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A Conserved Dedicated Olfactory Circuit for Detecting Harmful Microbes in *Drosophila*

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SUMMARY

Flies, like all animals, need to find suitable and safe food. Because the principal food source for *Drosophila melanogaster* is yeast growing on fermenting fruit, flies need to distinguish fruit with safe yeast from yeast covered with toxic microbes. We identify a functionally segregated olfactory circuit in flies that is activated exclusively by geosmin. This microbial odorant constitutes an ecologically relevant stimulus that alerts flies to the presence of harmful microbes. Geosmin activates only a single class of sensory neurons expressing the olfactory receptor Or56a. These neurons target the DA2 glomerulus and connect to projection neurons that respond exclusively to geosmin. Activation of DA2 is sufficient and necessary for aversion, overrides input from other olfactory pathways, and inhibits positive chemotaxis, oviposition, and feeding. The geosmin detection system is a conserved feature in the genus *Drosophila* that provides flies with a sensitive, specific means of identifying unsuitable feeding and breeding sites.

ab4B

INTRODUCTION

Animals respond with innate behaviors to certain stimuli in their environment. Innate behaviors, in contrast to learned behaviors, are hardwired; i.e., confronted with a specific stimulus, the animal will respond with a stereotyped behavior (Tinbergen, 1951). Many innate behaviors are triggered by odors. Prime examples are pheromones (Karlson and Lüscher, 1959), which have been particularly well studied in insects. In the vinegar fly *Drosophila melanogaster*, the male-produced pheromone *cis*-vaccenyl acetate (cVA) activates a single class of olfactory sensory neurons (OSN), which provides input to a single glomerulus (Kurtovic et al., 2007; van der Goes van Naters and Carlson, 2007) and a sexually dimorphic and functionally segregated circuit within the olfactory system (Datta et al., 2008; Ruta et al., 2010). In insects, odors associated with food or oviposition substrates can also elicit innate behaviors. The smell of vinegar confers obligate attraction in flies (Stökl et al., 2010). Although the vinegar odor activates a number of OSN classes, only a single glomerulus is sufficient and necessary for positive chemotaxis (Semmelhack and Wang, 2009). Pathways underlying hardwired attraction have thus been well characterized. Olfactory circuits mediating odorant-induced innate avoidance are, however, poorly understood. From an evolutionary perspective, being able to detect and respond quickly to harmful features in the environment should be an essential task for the olfactory system. In the fly, CO_2 elicits innate avoidance, which, like the attraction pathways, is mediated via a single glomerular circuit devoted exclusively to this stimulus (Suh et al., 2004). No dedicated avoidance circuit for an odorant sensu stricto (i.e., a volatile organic compound) has, however, been found in the fly or in any other insect. So far, all identified aversive odorants have activated multiple glomeruli (Knaden et al., 2012), and their identification depends on decoding of complex combinatorial glomerular activation patterns.

A volatile compound of interest in this context is geosmin (trans-1,10-dimethyl-trans-9-decalol) (Figure 1A). This substance is produced by a select number of fungi (Mattheis and Roberts, 1992), bacteria (Gerber and Lechevalier, 1965), and cyanobacteria (Jüttner and Watson, 2007) and to the human nose has a distinct and immediately recognizable earthy odor. A recent study found that addition of a small amount of geosmin reduced the attraction of flies to vinegar volatiles (Becher et al., 2010). Given its capacity to modulate innate attraction, this microbial volatile must be a very potent repellent and, as such, is possibly a candidate stimulus for a dedicated pathway for innate avoidance.

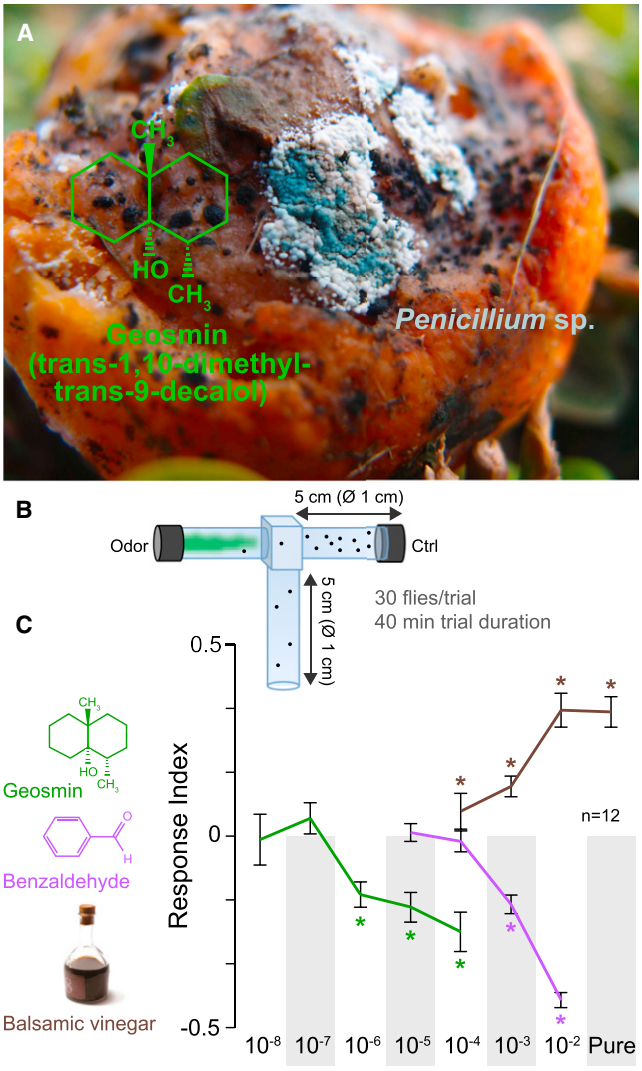

Figure 1. Geosmin—the Odor of Mold—Is Repellent to the Vinegar Fly

(A) Geosmin has a peculiar structure (left), which is distinct from odor ligands identified for *D. melanogaster*. Although a very common compound in nature, geosmin is produced only by a specific subset of microorganisms, including *Penicillium* sp. molds, shown here growing on an orange. Photo, MCS.

(B) Schematic drawing of the T-maze assay.

(C) Response indices of WT flies to geosmin, benzaldehyde, and balsamic vinegar in a T-maze assay. Deviation of the response index against zero was tested with a Student's t test ($p < 0.05$). Error bars represent SEM.

Here, we examine the functional significance of geosmin to the fly and show that geosmin activates only a single class of OSNs; these neurons express an odorant receptor that is exclusively tuned to this compound. Furthermore, we show that the geosmin-activated circuit constitutes a functionally segregated pathway, transferring the message arising from the periphery unaltered to central processing centers. We also demonstrate that this circuit alone is sufficient and necessary to trigger the avoidance behavior. Moreover, we show that, upon activation, the geosmin circuit overrides input from other circuits and inhibits positive chemotaxis. Additionally, we show that the peripheral part of the geosmin detection system is highly conserved across the genus *Drosophila*. Finally, we clearly demonstrate the ecological significance of this pathway, which is to detect toxic microbes.

RESULTS AND DISCUSSION

A Single Class of Olfactory Sensory Neurons Detects Geosmin

We first set out to determine the behavioral significance of geosmin by using a T-maze (Figure 1B). In this two-choice olfactory assay, geosmin on its own elicited avoidance at very low concentrations (10^{-6}) (Figure 1C). For comparison, benzaldehyde—a well-known repellant to flies—in the same assay required a 1,000-fold higher dose than geosmin to trigger repulsion (Figure 1C). The actual fold difference in flies' behavioral sensitivity toward these two compounds is greater once volatility is factored in. The vapor pressure of geosmin is 1,000-fold lower than for benzaldehyde (0.001 mmHg versus 1.27 mmHg at 25°C). Thus, at a given dose and temperature, the number of geosmin molecules in vapor phase is substantially lower than for benzaldehyde. Geosmin is accordingly not only repellent but is also repellent when present in exceedingly low amounts.

Flies are evidently equipped with a sensitive detection system for geosmin. To identify the population of OSNs that is activated by geosmin, we next turned to electrophysiology. Specifically, we performed single-sensillum recording (SSR) measurements, a method that allowed us to assess odor-induced OSN activity extracellularly. We aimed to obtain SSR measurements from all antennal olfactory sensillum types while stimulating the contacted OSNs with geosmin. The ~450 olfactory sensilla of the fly antennae (Shanbhag et al., 1999) can be divided into 17 functional types, which in total house 46 functionally distinct OSN classes (de Bruyne et al., 2001; Hallem et al., 2004; Couto et al., 2005; Yao et al., 2005; van der Goes van Naters and Carlson, 2007; Benton et al., 2009). In addition to these well-classified sensilla, morphological data indicate that the antennae also contain one more type, the so-called intermediate sensilla; these sensilla house an unknown number of functional OSN classes (Shanbhag et al., 1999). The second olfactory organ of the fly, the maxillary palp, houses an additional three types for a total of six distinct OSN classes (de Bruyne et al., 1999). By performing a considerable number of SSR measurements ($n > 1000$) using diagnostic odors and by comparing the response properties of contacted OSNs with previously published ligand affinities, we were able to locate and record from all sensillum types present on the antennae (including two types of intermediate sensilla), as well as from the three types found on the maxillary palps (Figure 2A).

Response to geosmin came from just a single class of antennal OSNs, namely, the ab4B OSNs (Figures 2B and 2C). These neurons express the odorant receptors (OR) *Or56a* and *Or33a* (Couto et al., 2005; Fishilevich and Vosshall, 2005), of which only the former is functional in the *Canton-S* strain we used here (Kreher et al., 2008). Although ab4B OSNs have been measured from previously (e.g., de Bruyne et al., 2001), geosmin is the first ligand reported for this neuron class. To confirm that

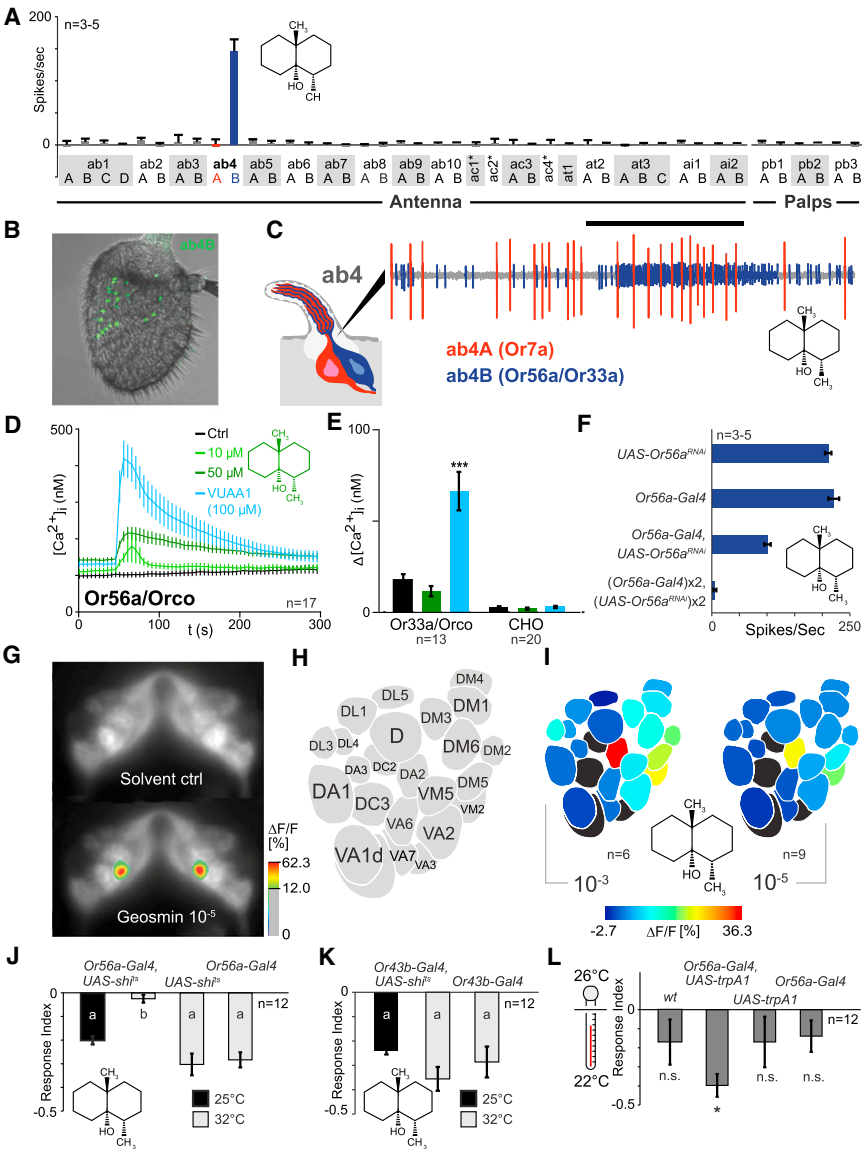

Figure 2. Geosmin Activates a Single Class of Antennal Olfactory Sensory Neurons

(A) SSR measurements from all olfactory sensilla with geosmin (10^{-3}) as a stimulus. ab, antennal basiconic sensilla (s.); ac, antennal coeloconic s.; at, antennal trichoid s.; ai, antennal intermediate s.; pb, palp basiconic s. Stars denote that activity from individual OSNs was not separated. Error bars represent SEM.

(B) Distribution of ab4B neurons on the antenna as visualized by the expression of GFP from the *Or56a* promoter.

(C) Representative SSR traces from an ab4 sensillum. The smaller amplitude spiking neuron, i.e., ab4B responds to geosmin (10^{-3}). The duration of the stimulus delivery (0.5 s) is marked by the black bar.

(D) The free intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ in CHO cells expressing Or56a and Orco increases after the application of geosmin and VUAA1 (100 µM), but not of saline (control). Error bars represent SEM.

(E) Mean increase in free intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ in CHO cells expressing Orco and Or33a or nontransfected CHO cells after the application of saline (control), geosmin (50 µM), and VUAA1 (100 µM). Star denotes response significantly different from control (Student's t test, $p < 0.05$). Color scale as in (D). Error bars represent SEM.

(F) Quantification of responses to geosmin (10^{-3}) from ab4B OSNs of flies expressing RNAi against Or56a in the ab4B OSNs and the corresponding parental lines. Error bars represent SEM.

(G) False color-coded images showing solvent-induced (top) and geosmin-induced (bottom) calcium-dependent fluorescence changes in the AL of a fly expressing the activity reporter GCaMP3.0 from the *Orco* promoter.

(H) Glomerular atlas of the AL.

(I) Odor-induced activity plotted on schematic ALs (average % $\Delta F/F$).

(J) RI to geosmin (10^{-5}) of flies expressing *Shibire^{ts}* from the *Or56a* promoter and corresponding parental lines in a T-maze assay. Significant differences are denoted by letters (analysis of variance [ANOVA] followed by Tukey's test; $p < 0.05$). Error bars represent SEM.

(K) RIs to geosmin (10^{-5}) of flies expressing *Shibire^{ts}* from the *Or43b* promoter and the corresponding parental lines in a T-maze assay. No significant differences (ANOVA followed by Tukey's test; $p > 0.05$). Error bars represent SEM.

(L) RIs of flies expressing *dTRPA1* from the *Or56a* promoter, the corresponding parental lines, and WT in a T-maze assay confronted with a choice between 22 and 26°C. Deviation of the RI against zero was tested with a Student's t test ($p < 0.05$). Error bars represent SEM.

See also Figure S1.

Or56a is indeed the geosmin receptor, we next expressed this protein in Chinese hamster ovary (CHO) cells that stably expressed the OR coreceptor *Orco* (Larsson et al., 2004). Because insect ORs are Ca^{2+}-permeable ionotropic receptors, OR activation can be monitored by measuring the free intracellular Ca^{2+} concentration $[Ca^{2+}]_i$. The application of geosmin transiently increased $[Ca^{2+}]_i$ in a concentration-dependent manner (Figure 2D). The cells responding to geosmin were seen to respond to the Orco agonist VUAA1 (Jones et al., 2011), although there was no response to control application of saline (Figure 2D and Figure S1A available online). We then expressed *Or33a* in the same CHO cell line. Although the cells responded to VUAA1, we found no responses to geosmin (Figure 2E). CHO cells not expressing *Orco* or either of the two tuning ORs produced no Ca^{2+} signals in response to the application of geosmin or VUAA1 (Figure 2E). Loss of function of *Or56a* should render ab4B OSNs insensitive to geosmin. We next used SSR to examine the function of ab4B OSNs expressing a *UAS*-RNA interference (RNAi) construct against *Or56a*. The expression of *UAS-Or56a^{RNAi}* reduced the response to geosmin in a dose-dependent manner (Figures 2F and S1B). In flies carrying one copy each of *Or56a-Gal4* and *UAS-Or56a^{RNAi}*, the response to

geosmin was reduced by ~50% compared to the response displayed by the parental lineages. With two copies of each, the response was essentially abolished (~98% reduction) (Figure 2F). Thus, we conclude that *Or56a* alone underlies the ability of the ab4B cells to detect geosmin.

To further verify that geosmin is detected only by a single class of OSNs, we next employed functional imaging to examine the activity pattern in the antennal lobe (AL) evoked by geosmin (Figures 2G and S1C). We used the *Gal4-UAS* system to express the Ca^{2+}-sensitive reporter gene GCaMP3.0 (Tian et al., 2009) from the *Orco* promoter, thereby labeling all OSNs except those relying on ionotropic receptors (Benton et al., 2009) for odorant detection. Activated glomeruli were then identified by comparing the activation pattern with the map of the fly AL (Couto et al., 2005; Fishilevich and Vosshall, 2005) (Figure 2H). We stimulated flies with diagnostic odors to assist glomerular identification (data not shown) and with geosmin at 10^{-3} and 10^{-5} dilutions (Figures 2G and 2I). At 10^{-5}, geosmin elicited repeatable signals from only a single locus in the AL—the DA2 glomerulus, which receives input from ab4B neurons (Couto et al., 2005; Fishilevich and Vosshall, 2005). We note that DA2 is also situated in the same lateral part of the AL that has previously been implicated in handling aversive odors (Knaden et al., 2012). In a number of recordings, we also noted activity from VM2; however, these signals were not consistently reproducible. In the SSR screen, we never observed any activity in response to geosmin from OSNs innervating VM2; these OSNs are housed in the ab8 sensillum (Figure 2A). Hence, the activity noted from VM2 most likely does not reflect actual peripheral input but, rather, may stem from intrinsic AL processes. We therefore conclude that geosmin is indeed detected by a single class of OSNs. It should be stressed that the level of specificity shown here toward a nonpheromonal odor is most unusual, if not unique, among the olfactory systems investigated to date.

Activation of the ab4B Neurons Is Necessary and Sufficient for the Aversive Behavior

If the behavior triggered by geosmin is solely derived from the activity of ab4B neurons, silencing this OSN subpopulation should also abolish the aversive behavior. To silence these neurons, we expressed the temperature-sensitive mutant dynamin *Shibire^{ts}* (Kitamoto, 2001) from the *Or56a* promoter. At the restrictive temperature (32°C), flies carrying this construct displayed no aversive behavior toward geosmin (Figure 2J). The same flies, tested at a permissive temperature (25°C), showed a strong aversion to geosmin. Parental lines tested at the nonpermissive temperature showed a somewhat increased repellency, which was likely caused by the increased volatility of geosmin at the higher temperature. Silencing the ab4B neurons had no effect on flies' behavior in response to benzaldehyde (Figure S1D). In line with the SSR experiments, silencing input to VM2—via the expression of *Shibire^{ts}* from the *Or43b* promoter—did not affect flies' behavior in response to geosmin (Figure 2K). The ab4B OSNs are evidently necessary for the aversive behavior.

We next asked whether selectively activating these neurons is sufficient to cause aversion. We expressed the temperature-sensitive cation channel *dTRPA1* in the ab4B neurons, a procedure that allowed us to conditionally activate these OSNs at temperatures >26°C (Hamada et al., 2008). As a control, we first examined the temperature preference (26°C versus 22°C) of wild-type (WT) flies in a T-maze assay. WT flies showed a tendency toward aversion against the higher temperature (Figure 2L). Having established baseline behavior in the assay, we next asked whether flies bearing the *Or56a-Gal4*, *UAS-dTRPA1* construct displayed a stronger aversion toward the higher temperature. In fact, flies expressing *dTRPA1* in ab4B OSNs showed significant avoidance toward the warm side, whereas parental control flies showed moderate (but insignificant) aversion (Figure 2L). Thus, specifically activating these neurons induces aversion in flies. In summary, these experiments demonstrate that the aversive behavior caused by geosmin is mediated solely through a single class of OSNs.

The ab4B Neurons Respond Exclusively to Geosmin

As seen, geosmin is detected by a single class of OSNs, ab4B. We next asked whether or not these neurons are exclusively tuned to geosmin. We again used SSR but now screened with 103 structurally diverse odorants (tested at 10^{-2} dilution) (Figure S2A). The larger spiking neuron in the ab4 sensillum responded to a range of compounds (Figure S2B). Interestingly, we note that the most potent ligands for these OSNs are all known repellants. The functional significance, if any, of having two neurons both responding to aversive odorants that are cocompartmentalized is unclear. The ab4B neurons, in contrast, displayed a striking degree of selectivity, as none of the screened odorants—apart from geosmin—elicited any increased spike firing (Figure 3A). Showing specificity in the context of the olfactory system is, however, difficult, as there are thousands of volatile chemicals in nature. Our tested set thus represents only a fraction of the volatile chemicals potentially present in the natural habitat of *D. melanogaster*.

To address this issue and to more firmly examine the specificity of these neurons, we next expanded our SSR investigation by using a gas chromatograph (GC) for stimulus delivery. GC-linked SSR enables the screening of headspace collections from complex odor sources and, consequently, enables the probing of large numbers of volatiles. We first sampled odors from a wide range of sources present in the natural habitat of *D. melanogaster* in native Africa as well as in the "Diaspora." We collected odors from 14 sources, including avoided ones, such as feces (from African mammals) and rotting meat, as well as attractive ones, such as fruits and vinegar. The total number of volatiles present in these samples is difficult to firmly establish, but the number of distinguishable flame ionization detection (FID) peaks amounts to ~2,900 in total. The actual number of compounds present is, however, likely considerably higher. The headspace of many fruits typically contains >400 volatiles (e.g., Petro-Turza, 1987); hence, in our samples, many more compounds were presumably present but only in amounts below the FID limit. These compounds were nevertheless effectively screened, as insects, including *Drosophila*, are capable of detecting compounds present well below the FID limit.

Having collected and verified the odor samples, we then proceeded to perform GC-SSR measurements from ab4B neurons.

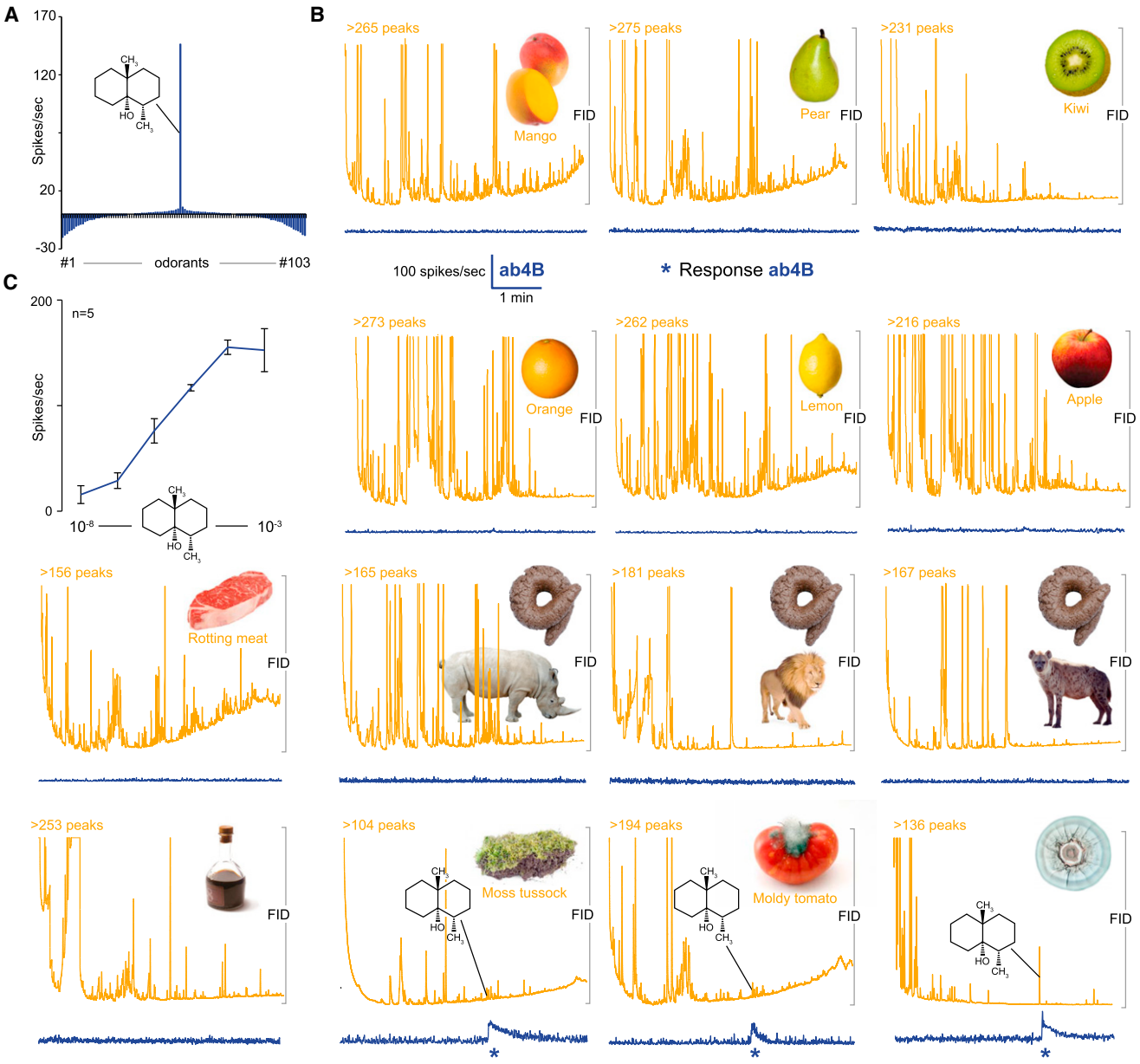

Figure 3. The ab4B Neurons Respond Exclusively to Geosmin

(A) Tuning curve for the ab4B neuron type based on a screen of 103 synthetic substances (10^{-2} dilution). Error bars represent SEM.

(B) Gas-chromatography-linked SSR measurements from ab4B neurons. The orange trace represents the FID, photos depict the screened odor sources, and the blue trace depicts the simultaneously recorded neural activity of ab4B neurons. Stars denote response. n = 1–3.

(C) Dose response curve from ab4B neurons toward geosmin. Error bars represent SEM.

See also Figure S2.

Out of the 14 odor samples we screened, only three evoked responses (Figure 3B), namely the headspace of a moldy tomato, a moss tussock, and isolated cultures of the common soil bacterium *Streptomyces coelicolor*. In each of the active samples, only a single FID peak elicited a response. We next used GC-linked mass spectroscopy (GC-MS) combined with synthetic standards to identify the functionally relevant peaks in these three samples; in all cases, these turned out to be geosmin. Thus, the ab4B neurons are indeed extremely specific, and it is reasonable to conclude that the sole function of these neurons is to detect geosmin.

How sensitive are the ab4B neurons toward geosmin? Our T-maze experiments (Figure 1C) had already shown that the flies respond behaviorally at very low concentrations. Indeed, the ab4B neurons respond to geosmin at 10^{-8} dilution (corresponding to 100 pg of substance in the stimulus pipette)

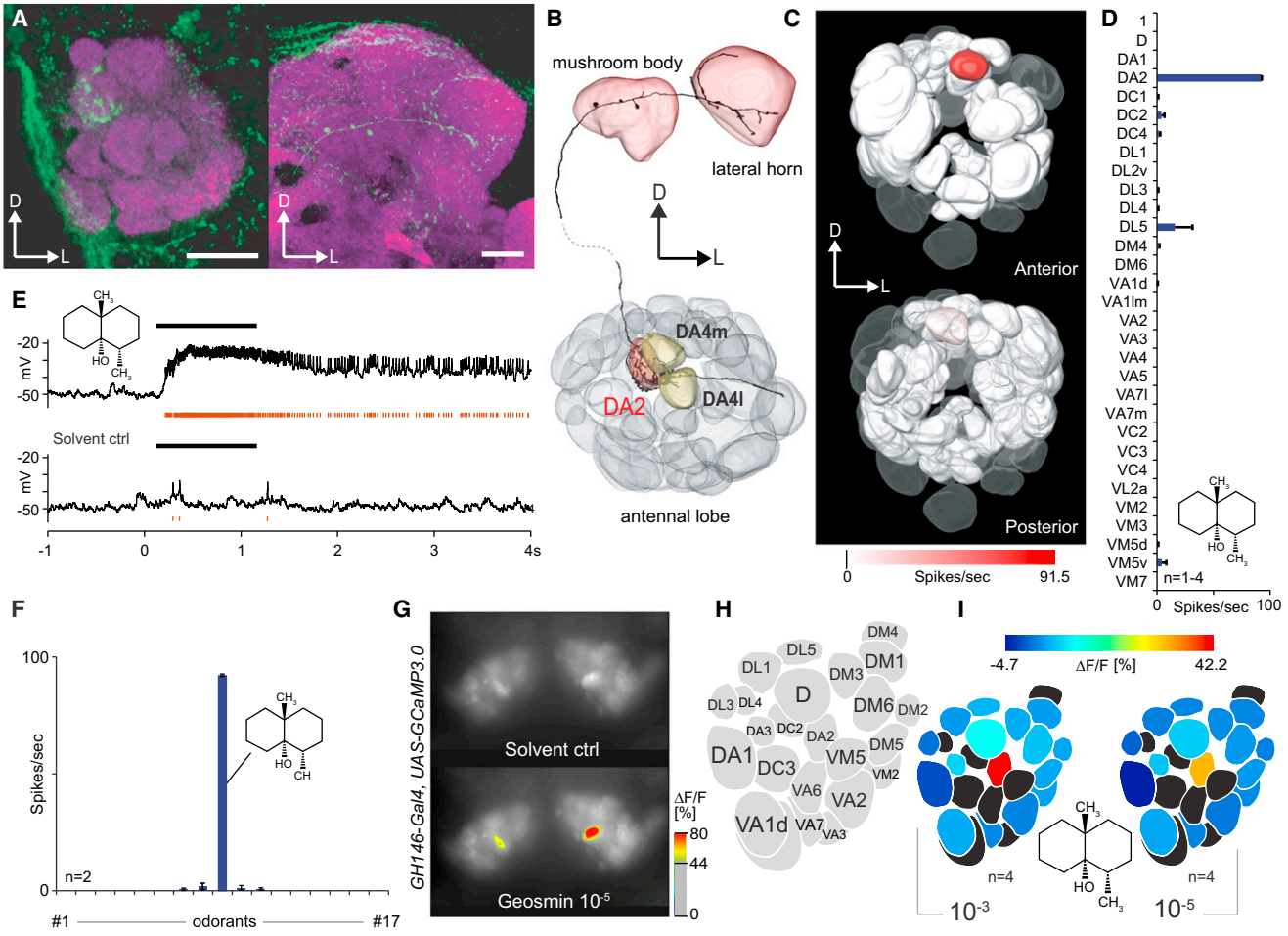

Figure 4. Geosmin Activates a Functionally Segregated Pathway

(A) A PN innervating the DA2 glomerulus (left) and sending its axon to the calyx of the mushroom body and terminating in the lateral horn (right). PN, green; nc82, magenta. D denotes dorsal, and L denotes lateral.

(B) Reconstruction of the neuron in (A).

(C) Glomeruli from which PN recordings were obtained (in solid), with the response to geosmin (10^{-3}) false color coded. Transparent glomeruli were not investigated.

(D) The net change in spike frequency in response to geosmin (10^{-3}) stimulation from PNs innervating 31 glomeruli. Error bars represent SEM.

(E) Example spike trace from a DA2 PN responding to geosmin (10^{-3}). Black bar marks the 1 s odor stimulus. Red trace represents extracted spikes.

(F) Tuning curve for DA2 PNs based on 17 synthetic substances (10^{-2} dilution, except geosmin, which was used at 10^{-3}). Error bars represent SEM.

(G) False color-coded images showing solvent-induced (top) and geosmin-induced (bottom) calcium-dependent fluorescence changes in AL PNs of a fly bearing the *GH146-Gal4*, *UAS-GCaMP3.0* constructs.

(H) Glomerular atlas of the AL.

(I) Odor-induced activity plotted on schematic ALs (average % $\Delta F/F$).

See also Figure S3.

(Figure 3C), which is in good agreement with the dilution of geosmin (1.74×10^{-7}) causing reduced upwind flight attraction to vinegar headspace when vaporized in the wind tunnel (Becher et al., 2010).

Geosmin Triggers a Segregated Pathway through the Antennal Lobe to Higher Brain Centers

How is the specific tuning in flies to geosmin seen in the peripheral sensory neurons transferred to higher brain centers? In *Drosophila*, the OSNs form synapses with projection neurons (PNs) and local interneurons within the AL. Most PNs innervate only a single glomerulus (Figures 4A and 4B), whereas local interneurons typically show broad innervation throughout the AL. The PNs send their axons to the mushroom body and lateral horn (Figures 4A and 4B) (Vosshall and Stocker, 2007). PNs tend to respond to a somewhat broader range of odors than do their corresponding OSNs (Wilson et al., 2004; Bhandawat et al., 2007). For instance, the PNs connected to OSNs that respond only to geranyl acetate respond to additional odors as well. However, PNs connected to OSNs that respond to the sex

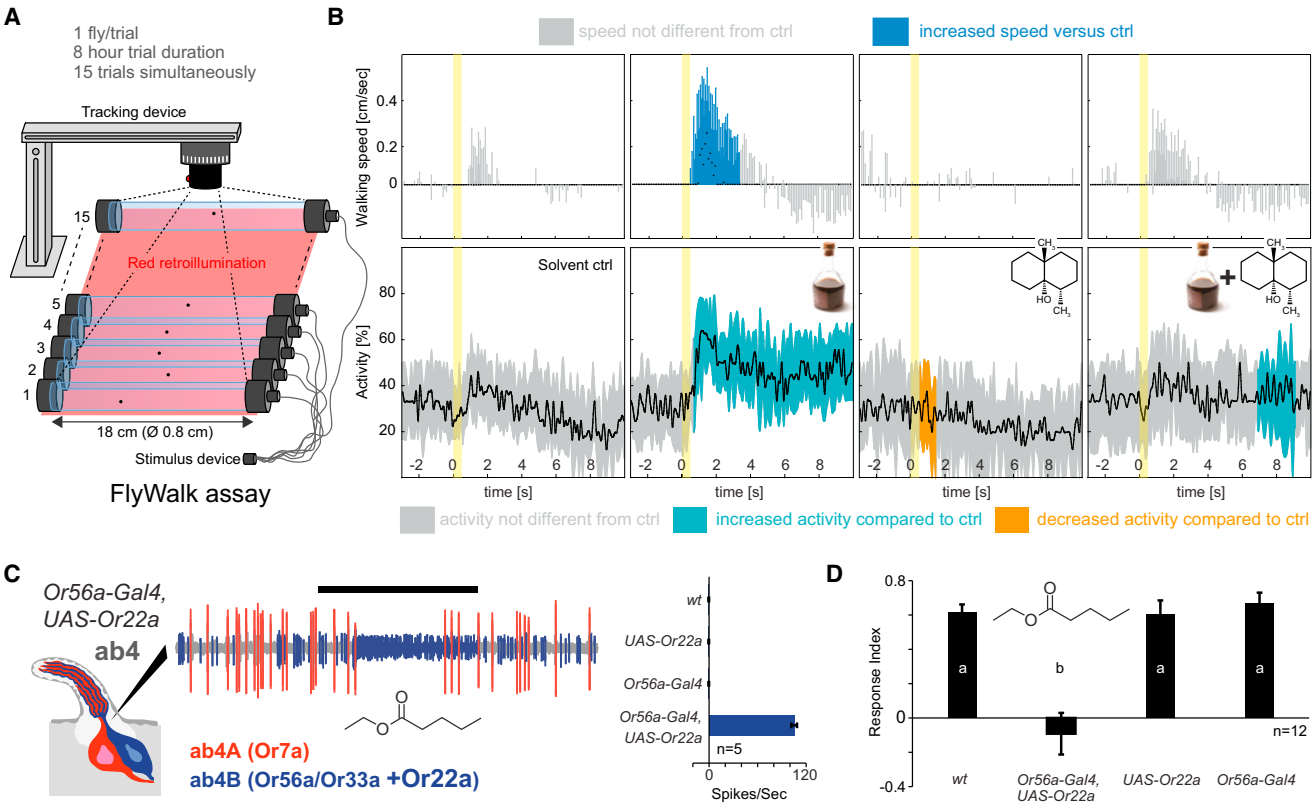

Figure 5. Activation of the Geosmin Pathway Reduces Attraction

(A) Schematic drawing of the Flywalk assay used in (B). For details, see Steck et al. (2012).

(B) Quantified behavior from individual flies stimulated with balsamic vinegar, geosmin (10^{-3}), and a mix of the two in the Flywalk assay. Top graphs, box plot representations of odor-induced changes in upwind speed of flies (n = 30); black line represents median upwind speed; box, interquartile range; whiskers, 90th and 10th percentiles. Lower graphs, undirected activity of flies (n = 30); black line, median activity; shaded area, interquartile range. Yellow area marks the 500 ms odor stimulus. Statistical analysis per Steck et al. (2012).

(C) Left, representative SSR trace from an ab4 sensillum, stimulated with ethyl butyrate (10^{-5}) in which the B neuron expresses Or22a. Right, quantification of mean responses to ethyl butyrate from control ab4B OSNs and ab4B OSNs misexpressing Or22a.

(D) Response indices of flies expressing Or22a in the ab4B OSNs, corresponding parental lines and WT flies to ethyl butyrate (10^{-5}) in a T-maze assay. Significant differences are denoted by letters (ANOVA followed by Tukey's test; $p < 0.05$). Error bars represent SEM.

See also Figure S4.

pheromone cVA do not show a broad response pattern and are just as specific as their cognate OSNs (Schlief and Wilson, 2007). We thus asked: how specific is the response of PNs that respond to geosmin?

We carried out whole-cell patch-clamp recordings from a large number of randomly selected uniglomerular PNs, stimulating with 17 chemicals, including geosmin (Figure S3). We obtained recordings and fills from 66 PNs (from 66 individual flies), which covered 31 different glomeruli. Geosmin elicited significant responses only from two PNs, both of which innervated the DA2 glomerulus (Figures 4A–4E). Although not all glomeruli were covered, this result strongly suggests that geosmin information does not diffuse broadly across the AL to other glomeruli. Moreover, DA2 PNs appear to be as selective as the input OSNs because these PNs responded exclusively to geosmin and not to any of the other screened compounds (Figures 4F and S3). To further examine the specificity of the AL output, we next imaged flies carrying the *GH146-Gal4* and *UAS-GCaMP3.0* constructs in which ~1/2 of the PNs express the GCaMP3.0 activity reporter (Stocker et al., 1997; Jefferis et al., 2001). Stimulation with geosmin again exclusively activated the DA2 glomerulus (Figures 4G–4I). Thus, we conclude that, like the labeled line pheromone pathway, the geosmin circuit forms a dedicated functionally segregated pathway, at least to the point of the calyx and lateral horn. The fate of the signal past this point remains to be elucidated.

The Geosmin Circuitry Can Modulate and Override Innate Attraction

As mentioned before, the addition of geosmin to vinegar significantly reduced positive chemotaxis in flies' response to this innately attractive odor. To verify that geosmin indeed has the capacity to reduce flies' attraction to vinegar, we next repeated the wind tunnel experiments with an alternative bioassay, the Flywalk (Steck et al., 2012) (Figure 5A). This assay enables high-resolution quantification of behavior from individual flies in

response to short pulses of an odor stimulus repeated during an extended period of time. Our Flywalk results parallel the findings from the wind tunnel (Figure 5B). Exposing flies to pulses of balsamic vinegar induced bursts of positive chemotaxis, which were significantly reduced when geosmin was added to the vinegar volatiles. Geosmin alone induced a "freezing" behavior, i.e., a decrease of the flies' activity, which, in this assay, reflects aversion (Steck et al., 2012). The ability of geosmin to reduce the attractiveness of vinegar is robust and can be repeated with both the trap assay (Larsson et al., 2004) (Figures S4A and S4B) and the T-maze (Figure S4C).

In light of the physiology findings, the cause of the reduced attractiveness of the geosmin-vinegar mix should stem from activation of the DA2 pathway. This circuit should consequently have the capacity to override and modulate an innate behavior. To test this notion, we used the *Or56a-Gal4* line to drive the expression of an additional odorant receptor (*Or22a* targeting glomerulus DM2) in ab4B OSNs (Figure 5C), enabling us to manipulate the activity of the DA2 circuit in the absence of geosmin and thereby to separate the chemical from the actual effect. In flies expressing Or22a under the *Or56a* promoter, stimulation with ethyl butyrate, a potent ligand for Or22a that is highly attractive to flies (Figure 5D), should result in the activation of both DM2 and DA2, in turn reducing the flies' attraction to ethyl butyrate. Through SSR, we first verified that the misexpression of Or22a conferred sensitivity toward ethyl butyrate in ab4B neurons (Figure 5C). Having established physiological function, we then tested the flies' behavioral response toward ethyl butyrate by using a T-maze. The parental control lines showed the expected strong positive response of WT flies toward this fruit ester. On the other hand, flies additionally expressing Or22a in the ab4B OSNs showed no attraction toward ethyl butyrate (Figure 5D). Thus, activating DA2 and the associated pathway can modulate and override innate attractive behavior.

Geosmin Is Used by the Fly to Detect Toxic Molds and Bacteria

We next asked what the possible evolutionary and ecological reason might be for the strong and hard-wired chemosensory avoidance of geosmin. Because geosmin itself is nontoxic to invertebrates as well as mammals (Young et al., 1996), the function of the circuit is not just to alert *D. melanogaster* to the presence of this compound. With some exceptions, the majority of volatiles flies detect are widely produced in nature and, thus, are difficult to firmly associate with a specific source. Geosmin—although very abundant in nature—is solely produced by a narrow range of microbes, in particular *Penicillium* fungal molds (Mattheis and Roberts, 1992) and *Streptomyces* soil bacteria (Gerber and Lechevalier, 1965). Has the system for detecting geosmin evolved to identify these specific microorganisms? We first examined whether flies could survive on these types of microbes. We transferred newly eclosed flies to vials with a yeast-containing medium or to vials additionally containing cultures of either *Streptomyces coelicolor* or *Penicillium expansum*. Flies were unable to survive in the presence of either of these microbes (Figure 6A), presumably due to the accumulation of toxins. Many fungal molds, including *P. expansum*, produce a range of toxic secondary metabolites, several of which have been shown to have strong insecticidal activity (Castillo et al., 1999). Many geosmin-producing microbes are not only toxic but are also known to outcompete or even kill the yeasts flies graze on (Arndt et al., 1999). Thus, for the fly, being able to detect and avoid fruit colonized by harmful molds and bacteria should be an essential skill.

Because many geosmin-producing microbes are detrimental to flies, we suspected that substrates colonized by this type of microbe are avoided for oviposition. Thus, we next looked for an olfactory-based oviposition preference in flies by using a two-choice assay (Figure 6B) in which flies were given the option of laying eggs on plates containing either standard *Drosophila* yeast medium or on plates additionally inoculated with *S. coelicolor*. Indeed, flies avoided laying eggs on plates containing *S. coelicolor* (Figure 6C). Is the avoidance of the bacterial plates mediated via geosmin? To address this question, we subsequently repeated the oviposition experiments. We inoculated one of the plates with a gene-targeted *S. coelicolor* strain (J3001), which carries a deletion in a key gene involved in the geosmin synthesis pathway (Gust et al., 2003). The J3001 strain is thus identical to WT *S. coelicolor* except for its inability to produce geosmin, the lack of which we also confirmed via GC-MS and GC-SSR (Figure 6D). Abolishing the production of geosmin completely eliminated the avoidance in response to *S. coelicolor* (Figure 6C). In the absence of geosmin, flies readily oviposited on the harmful media. Eggs deposited onto *S. coelicolor* did not develop into adult flies (data not shown), and survival on the J3001 strain did not differ from survival on WT *S. coelicolor* (log rank test; $p = 0.22$). In a pure olfactory choice assay, the trap assay (Figure S4A), flies also discriminated between the two strains, preferring J3001 over WT (Figure S5).

We next wondered whether the reluctance to oviposit in the presence of (WT) *S. coelicolor* is dependent on the DA2 circuit. To address this question, we examined the oviposition preference of flies carrying the previously used *Or56a-Gal4*, *UAS-Shibire^{ts}* construct. At permissive temperatures, these flies strongly avoided plates containing *S. coelicolor*, whereas at restrictive temperatures, there was no avoidance, and the flies even showed a slight preference for the bacterial substrate (Figure 6E). In line with our hypothesis, the presence of geosmin alone should also prevent egg laying, which it did. Plates containing geosmin (10^{-3}) were avoided as an oviposition substrate (Figure 6F). One could speculate that the presence of any strongly repellent odor would also prevent oviposition from occurring. However, benzaldehyde did not inhibit oviposition from occurring at 10^{-4} and 10^{-2} dilutions and barely did so even when tested as a pure substance (Figure 6F).

Are flies also hesitant to consume food contaminated with this type of microbe? We next examined feeding preference by using a capillary feeder assay (Figure 6G) (Ja et al., 2007); here, flies could choose between two 5% sucrose solutions, one of which was based on a wash from WT *S. coelicolor* colonies. Indeed, flies clearly preferred the pure sucrose solution (Figure 6H). We then repeated these experiments, replacing the WT *S. coelicolor* with the J3001 strain. The solution

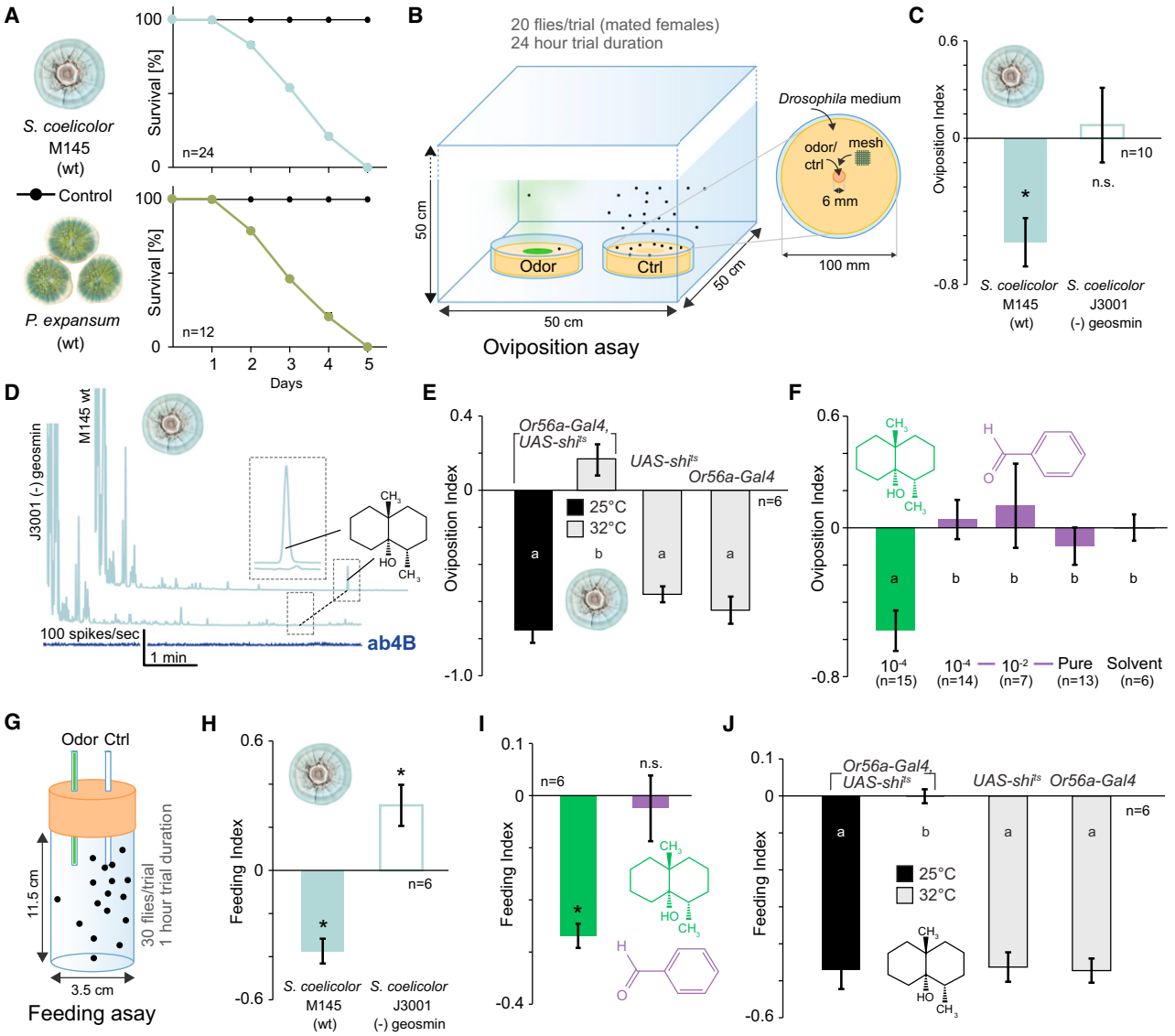

Figure 6. Geosmin Is Used by Flies to Detect Toxic Molds and Bacteria

(A) Survival rate of newly eclosed flies transferred to vials containing pure agar medium or medium with 1-week-old cultures of either of two geosmin-producing microbes.

(B) Schematic drawing of the oviposition choice assay used in (C), (E), and (F).

(C) Oviposition indices (OI) to WT (M145) and J3001 *S. coelicolor* of WT flies. The J3001 only differs from WT by its inability to produce geosmin. Deviation of the oviposition index against zero was tested with a Student's t test ($p < 0.05$). Error bars represent SEM.

(D) GC-MS and GC-SSR analysis of headspace from J3001 and M145. Pale blue represents flame ionization detection traces. The dark blue trace shows activity from an ab4B OSN being stimulated with J3001 headspace (no response).

(E) OIs to WT *S. coelicolor* of flies expressing *Shibire*^{ts} in the ab4B OSNs and corresponding parental lines at permissive (25°C) and restrictive (32°C) temperatures. Significant differences are denoted by letters (ANOVA followed by Tukey's test; $p < 0.05$). Error bars represent SEM.

(F) OIs to geosmin and benzaldehyde of WT flies. Significant differences are denoted by letters (ANOVA followed by Tukey's test; $p < 0.05$). Error bars represent SEM.

(G) Schematic drawing of the capillary feeding assay (modified from Ja et al. [2007]) used in (H)–(J).

(H) Feeding indices (FI) to 5% sucrose solutions containing traces of WT (M145) or J3001 *S. coelicolor* of WT flies. Deviation of the feeding index against zero was tested with a Student's t test ($p < 0.05$). Error bars represent SEM.

(I) FIs to 5% sucrose solutions containing geosmin (0.1%) or benzaldehyde (0.1%) of WT flies. Deviation of the feeding index against zero was tested with a Student's t test ($p < 0.05$). Error bars represent SEM.

(J) FIs to 5% sucrose solutions containing traces of WT (M145) *S. coelicolor* of flies expressing *Shibire*^{ts} from the *Or56a* promoter and corresponding parental lines at permissive (25°C) and restrictive (32°C) temperatures. Significant differences are denoted by letters (ANOVA followed by Tukey's test; $p < 0.05$). Error bars represent SEM.

See also Figure S5.

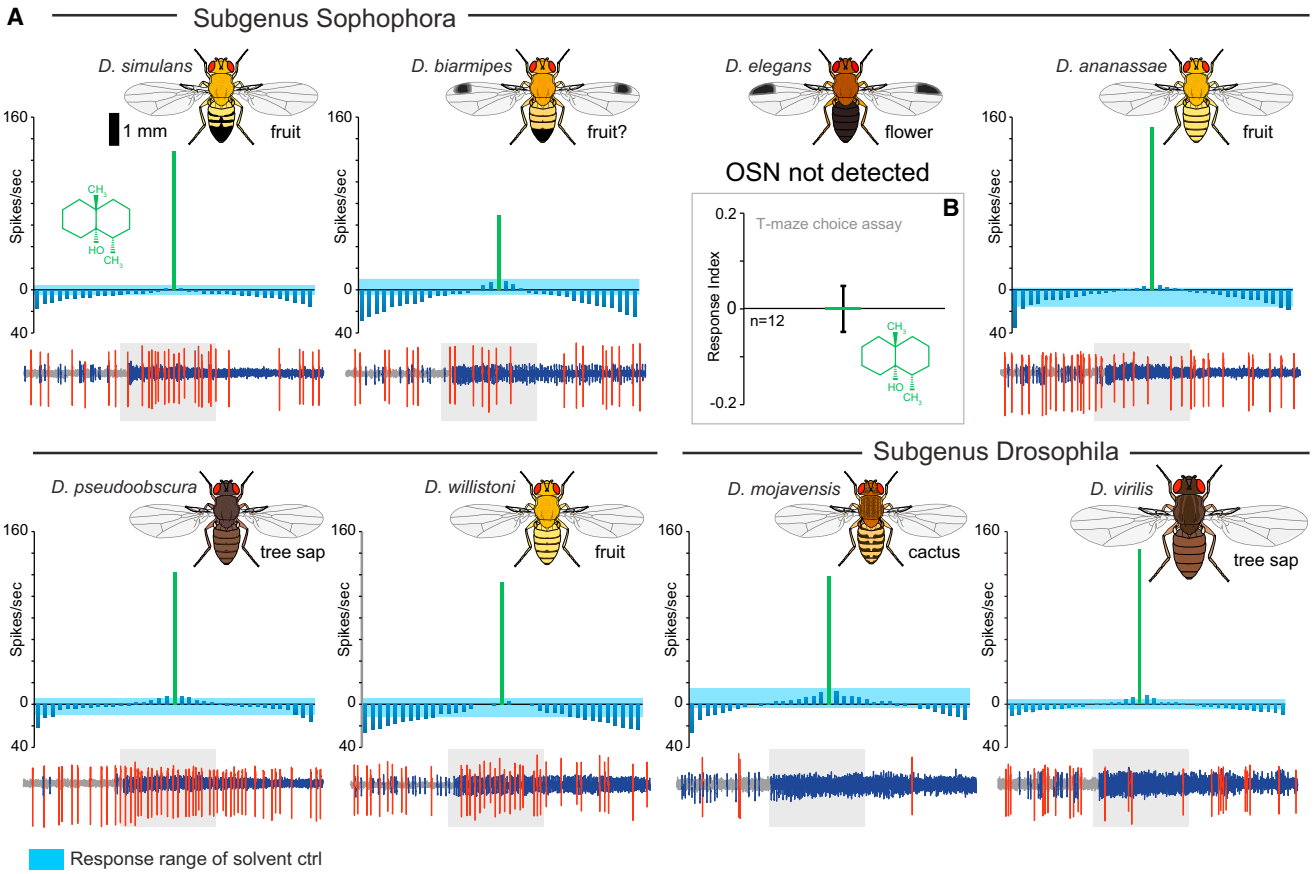

Figure 7. Responses to Geosmin in Drosophilids Are Deeply Conserved

(A) Tuning curves for neurons with similar response properties to the ab4B neurons of *D. melanogaster* from select members of the genus *Drosophila* (n = 3 for all species). The tuning curves are based on a screen with 37 compounds, tested at 10^{-2}. Below curves are representative SSR traces showing responses to geosmin (10^{-3}), with the gray box indicating the 0.5 s stimulus delivery period. The natural breeding substrates are indicated underneath the schematic drawings of the species. Error bars represent SEM.

(B) Response index to geosmin (10^{-5}) of *D. elegans* in a T-maze assay. Deviation of the response index against zero was tested with a Student's t test (not significant). Error bars represent SEM.

See also Figure S6.

containing J3001 did not reduce feeding but was slightly preferred over the sucrose-only solution (Figure 6H), suggesting that the aversion is due to the presence of geosmin. In line with this observation, adding geosmin (0.1%) also reduced feeding (Figure 6I). The addition of another aversive odor, benzaldehyde (0.1%), had no effect on feeding (Figure 6I). We next wondered whether the feeding aversion is due to olfactory input to the DA2 pathway. Indeed, the reduced feeding stems not from geosmin having an aversive taste but from the activation of ab4B OSNs because silencing input to this pathway—via *Shibire^{ts}*—also fully abolished the geosmin-induced feeding aversion (Figure 6J). Thus, geosmin also functions as an antifeedant, operating via the olfactory system.

Taken together, these findings strongly suggest that the ecological significance of geosmin is to alert flies to the presence of toxic molds and bacteria. The geosmin circuit performs a critical task, providing flies with a reliable and sensitive means of identifying unsuitable hosts.

The Geosmin Detection System Is Conserved across the Genus *Drosophila*

To shed light on the origin and evolution of the geosmin detection system circuit, we next turned to a comparative approach. We tested eight drosophilid species—chosen based on genome availability and phylogenetic and ecological considerations—for their capacity to detect geosmin (Figure S6A). We set out to identify neurons able to detect geosmin via SSR, stimulating with a set of 37 chemically diverse odorants (at 10^{-2} dilution) (Figure S3D). We located OSNs tuned to geosmin in all the screened species except *D. elegans* (Figure 7A). Electroantennogram recordings from this species also showed no response to geosmin (data not shown) and neither does this species respond behaviorally to geosmin in a T-maze assay (Figure 7B). As in *D. melanogaster*, in each of the species responding to geosmin, detection was noted only from a single class of OSNs, which also responded exclusively to geosmin (Figure 7A). The geosmin OSNs we found in the other species may well

serve the same function that they serve in *D. melanogaster*. The lack of a geosmin detection system in *D. elegans* may be a consequence of the low susceptibility to mold growth of this species' breeding substrate, namely, fresh flowers (Yoshida et al., 2000). Putatively functional orthologs of *Or56a* are also present across the species in which we have complete OR repertoires (Guo and Kim, 2007). We also located intact orthologs of *Or56a* in draft genome assemblies from an additional eight drosophilids (Figure S6B), including *D. biarmipes* and *D. elegans*. The function (if any) of the *Or56a* ortholog in the latter remains unknown. Analysis of selection pressure also showed that the *Or56a* genes are under overall purifying selection (Figure S6C). The response properties of the second neuron residing in these sensilla are much less conserved (Figure S6D). These neurons also do not express orthologous receptors across the examined species. In *D. melanogaster*, the ab4A neurons express Or7a (Hallem et al., 2004), orthologs of which are, however, found only in the subgenus *Sophophora* (Guo and Kim, 2007). Yet, also in species in which we can assume that Or7a underlies the response property, we did note variation in ligand affinity. The function of the ab4A OSNs hence likely reflects species-specific requirements. The striking specificity toward geosmin seen in the olfactory system of *D. melanogaster* is accordingly a basal feature of the genus *Drosophila*, conserved for at least ~40 million years (Russo et al., 1995).

Conclusions

The manner in which flies decode and rely upon geosmin has few, if any, direct parallels. Comparable circuits are essentially found only within the subset of the olfactory nervous system that relays pheromone information. However, also within this context, it is exceedingly rare for animals to rely on just a single chemical to identify a critical resource. Almost all pheromones characterized to date have been complex blends processed by multiple neuronal pathways. Moreover, the specificity toward geosmin shown here surpasses many pheromone-tuned neurons; if presented with enough odorants or with odorants in sufficient concentration, these neurons will also display responses to other substances (Hansson and Stensmyr, 2011).

The closest match to the geosmin pathway is found outside of the regular olfactory system, namely in the detection and processing machinery for the atmospheric trace gas CO_2. Although CO_2 is a fundamentally different chemical from geosmin, the similarity in which these two stimuli are decoded is striking. In flies, the CO_2 circuit forms a functionally segregated pathway that mediates innate avoidance. Input to the CO_2 circuit is likewise fed by sensory neurons exclusively tuned to a single stimulus (Suh et al., 2004). Although organized similarly, the ecological significance of these two circuits seems to differ. Geosmin is used by flies as a universal warning sign for the presence of toxic compounds that are comorbid with geosmin. The evolutionary significance of this circuit is clear: it provides flies with a sensitive and specific means to identify unsuitable hosts. The ecological meaning of CO_2 for *D. melanogaster* is, however, unclear. In fact, it is puzzling why flies would be repelled by CO_2 at all. *D. melanogaster* is highly adapted toward breeding (and feeding) on substrates with high ethanol content. Because CO_2 is a ubiquitous byproduct of alcoholic fermentation, it would make an ideal cue for flies to follow when searching for suitable hosts. Elucidating the role of CO_2 from the point of view of flies and using assays that better reflect the natural setting should be a focus of future studies.

Circuits analogous to the geosmin pathway are a likely feature in the olfactory systems of most, if not all, insects. Although these circuits are probably similar mechanistically and functionally (i.e., selective with regards to input, mediating innate aversion, and abolishing attraction), the identity of the eliciting stimulus will differ, reflecting the demands raised by the taxon-specific ecology.

EXPERIMENTAL PROCEDURES

Fly Stocks

All experiments with WT *D. melanogaster* were carried out with the Canton-S strain. Species other than *D. melanogaster* were obtained from the *Drosophila* species stock center (https://stockcenter.ucsd.edu/info/welcome.php). Transgenic lines were obtained from the Bloomington *Drosophila* stock center (http://flystocks.bio.indiana.edu/), except for *UAS-Or22a*, which was donated by L. Vosshall (The Rockefeller University, New York) and *UAS-Or56-a^{RNAi}*, which was obtained from the Vienna RNAi stock center (http://www.vdrc.at).

Stimuli and Chemical Analysis

All synthetic odorants tested were acquired from commercial sources (Sigma, http://www.sigma-aldrich.com and Bedoukian, http://www.bedoukian.com) and were of the highest purity available. (±)-Geosmin (of >97% purity) was obtained from Sigma. Stimuli preparation and delivery followed Stökl et al. (2010). The headspace collection of volatiles was carried out according to standard procedures. *S. coelicolor* M145 and J3001 strains were gifts from K. Flärdh (Lund University, Sweden) and K. Chater (John Innes Centre, UK), respectively. *P. expansum* was obtained from Centraalbureau voor Schimmelcultures (http://www.cbs.knaw.nl). Microorganisms were kept on strain-specific media (HiMedia, http://www.himedialabs.com), following standard protocols. Mammalian fecal samples were provided by the Leipzig Zoo. For GC stimulation, 1 μl of the odor sample was injected onto a DB5 column (Agilent Technologies, http://www.agilent.com), fitted in an Agilent 6890 GC, equipped with a four-arm effluent splitter (Gerstel, www.gerstel.com), and operated as previously described (Stökl et al., 2010) except for the temperature increase, which was set at 15°C min^{-1}. GC-separated components were introduced into a humidified airstream (200 ml min^{-1}) directed toward the antennae of a mounted fly. Signals from OSNs and FID were recorded simultaneously. GC-MS analysis was performed as previously described (Stökl et al., 2010).

Behavioral Assays

T-maze experiments were conducted as shown in Figure 1B, with flies starved for 4 hr prior to experiments with water provided ad libitum. The response index (RI) was calculated as (O-C)/T, where O is the number of flies in the baited arm, C is the number of flies in the control arm, and T is the total number of flies used in the trial. The resulting index ranges from −1 (complete avoidance) to 1 (complete attraction). Trap assay experiments (Figure S4A) were performed as described in Stökl et al. (2010) with RI calculated as above. The Flywalk experiments followed protocols outlined in Steck et al. (2012) (Figure 5A). Survival was measured for individual flies (males and females, except for tests with J3001, in which only females were examined), which were kept for 5 days (at 23°C) in glass tubes (16 × 100 mm) with metal caps containing 1-week-old cultures of *S. coelicolor* or *P. expansum* grown on yeast-containing media (HiMedia). Oviposition experiments were carried out as shown in Figure 6B. Oviposition index was calculated as (O-C)/(O+C), where O is the number of eggs on a baited plate, and C is the number of

eggs on a control plate. Feeding experiments were conducted as described in Figure 6G. A feeding index was calculated as (O-C)/(O+C), where O is the amount of food consumed from odorous solutions, and C is the amount from control sucrose-only solutions.

Physiology and Morphology

Electroantennogram (EAG) recordings were performed following standard procedures (e.g., Stökl et al., 2010). For SSR measurements, the recording electrode and the reference electrode (inserted into the eye) were positioned under a microscope (Olympus BX51W1; http://www.olympus.com). The recording electrode was positioned by using a motorized, piezo-translator-equipped micromanipulator (Märzhauser DC-3K/PM-10; http://www.marzhauser.com/de/). The signal was amplified (Syntech UN-06, http://www.syntech.nl), digitally converted (Syntech IDAC-4), and finally visualized and analyzed by using Syntech AutoSpike v3.2. CHO cells stably expressing dOrco (Trenzyme, http://www.trenzyme.com) were transiently transfected with dOr56a/pcDNA3.1(−) or dOr33a/pcDNA3.1(−) by using a Roti-Fect transfection kit (Carl Roth, http://www.carlroth.com) as described (Sargsyan et al., 2011). Ca^{2+} imaging of CHO cells was performed as described (Wicher et al., 2008). The functional imaging of odor-induced glomerular activity was conducted as outlined in Stökl et al. (2010). Patch-clamp recording was performed as previously described (Seki et al., 2010), except that in vivo preparation was used, and odor stimuli were given. Preparation followed Stökl et al. (2010), with the exception that the neurolemma was removed to allow the recording electrode access to the cell bodies of the PNs. Spike analysis, immunohistochemistry, laser scanning microscopy, and 3D reconstructions were performed as previously described (Seki et al., 2010).

Statistics and Bioinformatics

Estimates of the selection pressure were done by maximum likelihood as implemented in PAML (Yang, 1997). Additional orthologs of *Or56a* were identified via TBLASTN searches of draft genomes (courtesy of modENCODE/Baylor College of Medicine), downloaded from http://www.ncbi.nlm.nih.gov/bioproject/63477.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2012.09.046.

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REFERENCES

Arndt, C., Cruz, M.C., Cardenas, M.E., and Heitman, J. (1999). Secretion of FK506/FK520 and rapamycin by *Streptomyces* inhibits the growth of competing *Saccharomyces cerevisiae* and *Cryptococcus neoformans*. Microbiology *145*, 1989–2000.

Becher, P.G., Bengtsson, M., Hansson, B.S., and Witzgall, P. (2010). Flying the fly: long-range flight behavior of *Drosophila melanogaster* to attractive odors. J. Chem. Ecol. *36*, 599–607.

Benton, R., Vannice, K.S., Gomez-Diaz, C., and Vosshall, L.B. (2009). Variant ionotropic glutamate receptors as chemosensory receptors in *Drosophila*. Cell *136*, 149–162.

Bhandawat, V., Olsen, S.R., Gouwens, N.W., Schlief, M.L., and Wilson, R.I. (2007). Sensory processing in the *Drosophila* antennal lobe increases reliability and separability of ensemble odor representations. Nat. Neurosci. *10*, 1474–1482.

Castillo, M.-A., Moya, P., Cantín, A., Miranda, M.A., Primo, J., Hernández, E., and Primo-Yúfera, E. (1999). Insecticidal, anti-juvenile hormone, and fungicidal activities of organic extracts from different Penicillium species and their isolated active components. J. Agric. Food Chem. *47*, 2120–2124.

Couto, A., Alenius, M., and Dickson, B.J. (2005). Molecular, anatomical, and functional organization of the *Drosophila* olfactory system. Curr. Biol. *15*, 1535–1547.

Datta, S.R., Vasconcelos, M.L., Ruta, V., Luo, S., Wong, A., Demir, E., Flores, J., Balonze, K., Dickson, B.J., and Axel, R. (2008). The *Drosophila* pheromone cVA activates a sexually dimorphic neural circuit. Nature *452*, 473–477.

de Bruyne, M., Clyne, P.J., and Carlson, J.R. (1999). Odor coding in a model olfactory organ: the Drosophila maxillary palp. J. Neurosci. *19*, 4520–4532.

de Bruyne, M., Foster, K., and Carlson, J.R. (2001). Odor coding in the Drosophila antenna. Neuron *30*, 537–552.

Fishilevich, E., and Vosshall, L.B. (2005). Genetic and functional subdivision of the *Drosophila* antennal lobe. Curr. Biol. *15*, 1548–1553.

Gerber, N.N., and Lechevalier, H.A. (1965). Geosmin, an earthly-smelling substance isolated from actinomycetes. Appl. Microbiol. *13*, 935–938.

Guo, S., and Kim, J. (2007). Molecular evolution of *Drosophila* odorant receptor genes. Mol. Biol. Evol. *24*, 1198–1207.

Gust, B., Challis, G.L., Fowler, K., Kieser, T., and Chater, K.F. (2003). PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc. Natl. Acad. Sci. USA *100*, 1541–1546.

Hallem, E.A., Ho, M.G., and Carlson, J.R. (2004). The molecular basis of odor coding in the *Drosophila* antenna. Cell *117*, 965–979.

Hamada, F.N., Rosenzweig, M., Kang, K., Pulver, S.R., Ghezzi, A., Jegla, T.J., and Garrity, P.A. (2008). An internal thermal sensor controlling temperature preference in Drosophila. Nature *454*, 217–220.

Hansson, B.S., and Stensmyr, M.C. (2011). Evolution of insect olfaction. Neuron *72*, 698–711.

Ja, W.W., Carvalho, G.B., Mak, E.M., de la Rosa, N.N., Fang, A.Y., Liong, J.C., Brummel, T., and Benzer, S. (2007). Prandiology of Drosophila and the CAFE assay. Proc. Natl. Acad. Sci. USA *104*, 8253–8256.

Jefferis, G.S.X.E., Marin, E.C., Stocker, R.F., and Luo, L. (2001). Target neuron prespecification in the olfactory map of *Drosophila*. Nature *414*, 204–208.

Jones, P.L., Pask, G.M., Rinker, D.C., and Zwiebel, L.J. (2011). Functional agonism of insect odorant receptor ion channels. Proc. Natl. Acad. Sci. USA *108*, 8821–8825.

Jüttner, F., and Watson, S.B. (2007). Biochemical and ecological control of geosmin and 2-methylisoborneol in source waters. Appl. Environ. Microbiol. *73*, 4395–4406.

Karlson, P., and Lüscher, M. (1959). Pheromones': a new term for a class of biologically active substances. Nature *183*, 55–56.

Kitamoto, T. (2001). Conditional modification of behavior in *Drosophila* by targeted expression of a temperature-sensitive shibire allele in defined neurons. J. Neurobiol. *47*, 81–92.

Knaden, M., Strutz, A., Ahsan, J., Sachse, S., and Hansson, B.S. (2012). Spatial representation of odorant valence in an insect brain. Cell Rep. *1*, 392–399.

Kreher, S.A., Mathew, D., Kim, J., and Carlson, J.R. (2008). Translation of sensory input into behavioral output via an olfactory system. Neuron *59*, 110–124.

Kurtovic, A., Widmer, A., and Dickson, B.J. (2007). A single class of olfactory neurons mediates behavioural responses to a *Drosophila* sex pheromone. Nature *446*, 542–546.

Larsson, M.C., Domingos, A.I., Jones, W.D., Chiappe, M.E., Amrein, H., and Vosshall, L.B. (2004). Or83b encodes a broadly expressed odorant receptor essential for *Drosophila* olfaction. Neuron *43*, 703–714.

Mattheis, J.P., and Roberts, R.G. (1992). Identification of geosmin as a volatile metabolite of *Penicillium expansum*. Appl. Environ. Microbiol. *58*, 3170–3172.

Petro-Turza, M. (1987). Flavor of tomato and tomato products. Food Rev. Int. *2*, 309–351.

Russo, C.A.M., Takezaki, N., and Nei, M. (1995). Molecular phylogeny and divergence times of drosophilid species. Mol. Biol. Evol. *12*, 391–404.

Ruta, V., Datta, S.R., Vasconcelos, M.L., Freeland, J., Looger, L.L., and Axel, R. (2010). A dimorphic pheromone circuit in *Drosophila* from sensory input to descending output. Nature *468*, 686–690.

Sargsyan, V., Getahun, M.N., Lavista Llanos, S., Olsson, S., Hansson, B., and Wicher, D. (2011). Phosphorylation via PKC regulates the function of the Drosophila odorant co-receptor. Front. Cell. Neurosci. Published online June 16, 2011. http://dx.doi.org/10.3389/fncel.2011.00005.

Schlief, M.L., and Wilson, R.I. (2007). Olfactory processing and behavior downstream from highly selective receptor neurons. Nat. Neurosci. *10*, 623–630.

Seki, Y., Rybak, J., Wicher, D., Sachse, S., and Hansson, B.S. (2010). Physiological and morphological characterization of local interneurons in the *Drosophila* antennal lobe. J. Neurophysiol. *104*, 1007–1019.

Semmelhack, J.L., and Wang, J.W. (2009). Select *Drosophila* glomeruli mediate innate olfactory attraction and aversion. Nature *459*, 218–223.

Shanbhag, S., Mueller, B., and Steinbrecht, R. (1999). Atlas of olfactory organ of *Drosophila melanogaster* 1. Types, external organization, innervation and distribution of olfactory sensilla. Int. J. Insect Morphol. Embryol. *28*, 377–397.

Steck, K., Veit, D., Grandy, R., Badia, S.B., Mathews, Z., Verschure, P., Hansson, B.S., and Knaden, M. (2012). A high-throughput behavioral paradigm for *Drosophila* olfaction - The Flywalk. Sci. Rep. *2*, 361.

Stocker, R.F., Heimbeck, G., Gendre, N., and de Belle, J.S. (1997). Neuroblast ablation in *Drosophila* P[GAL4] lines reveals origins of olfactory interneurons. J. Neurobiol. *32*, 443–456.

Stökl, J., Strutz, A., Dafni, A., Svatos, A., Doubsky, J., Knaden, M., Sachse, S., Hansson, B.S., and Stensmyr, M.C. (2010). A deceptive pollination system targeting drosophilids through olfactory mimicry of yeast. Curr. Biol. *20*, 1846–1852.

Suh, G.S., Wong, A.M., Hergarden, A.C., Wang, J.W., Simon, A.F., Benzer, S., Axel, R., and Anderson, D.J. (2004). A single population of olfactory sensory neurons mediates an innate avoidance behaviour in *Drosophila*. Nature *431*, 854–859.

Tian, L., Hires, S.A., Mao, T., Huber, D., Chiappe, M.E., Chalasani, S.H., Petreanu, L., Akerboom, J., McKinney, S.A., Schreiter, E.R., et al. (2009). Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. Nat. Methods *6*, 875–881.

Tinbergen, N. (1951). The Study of Instinct (Oxford: Clarendon Press).

van der Goes van Naters, W., and Carlson, J.R. (2007). Receptors and neurons for fly odors in *Drosophila*. Curr. Biol. *17*, 606–612.

Vosshall, L.B., and Stocker, R.F. (2007). Molecular architecture of smell and taste in *Drosophila*. Annu. Rev. Neurosci. *30*, 505–533.

Wicher, D., Schäfer, R., Bauernfeind, R., Stensmyr, M.C., Heller, R., Heinemann, S.H., and Hansson, B.S. (2008). *Drosophila* odorant receptors are both ligand-gated and cyclic-nucleotide-activated cation channels. Nature *452*, 1007–1011.

Wilson, R.I., Turner, G.C., and Laurent, G. (2004). Transformation of olfactory representations in the *Drosophila* antennal lobe. Science *303*, 366–370.

Yang, Z.H. (1997). PAML: a program package for phylogenetic analysis by maximum likelihood. Comput. Appl. Biosci. *13*, 555–556.

Yao, C.A., Ignell, R., and Carlson, J.R. (2005). Chemosensory coding by neurons in the coeloconic sensilla of the *Drosophila* antenna. J. Neurosci. *25*, 8359–8367.

Yoshida, T., Chen, H.W., Toda, M.J., Kimura, M.T., and Davis, A.J. (2000). New host plants and host plant use for *Drosophila elegans* Bock and Wheeler, 1972. Drosoph. Inf. Serv. *83*, 18–21.

Young, W.F., Horth, H., Crane, R., Ogden, T., and Arnott, M. (1996). Taste and odour threshold concentrations of potential potable water contaminants. Water Res. *30*, 331–340.