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Olfactory processing and behavior downstream from highly selective receptor neurons

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In both the vertebrate nose and the insect antenna, most olfactory receptor neurons (ORNs) respond to multiple odors. However, some ORNs respond to just a single odor, or at most to a few highly related odors. It has been hypothesized that narrowly tuned ORNs project to narrowly tuned neurons in the brain, and that these dedicated circuits mediate innate behavioral responses to a particular ligand. Here we have investigated neural activity and behavior downstream from two narrowly tuned ORN types in *Drosophila melanogaster*. We found that genetically ablating either of these ORN types impairs innate behavioral attraction to their cognate ligand. Neurons in the antennal lobe postsynaptic to one of these ORN types are, like their presynaptic ORNs, narrowly tuned to a pheromone. However, neurons postsynaptic to the second ORN type are broadly tuned. These results demonstrate that some narrowly tuned ORNs project to dedicated central circuits, ensuring a tight connection between stimulus and behavior, whereas others project to central neurons that participate in the ensemble representations of many odors.

The brain monitors odors in the environment via ORNs. In most species, the majority of ORNs can be excited by multiple kinds of odor molecules[1–3]. However, a minority of ORNs appear to be narrowly tuned to a single chemical compound, or at most to a small number of highly related compounds. These neurons can be highly selective for pheromones, nonpheromonal social cues or important environmental odors[4,5]. Historically, narrowly and broadly tuned ORNs have been viewed as fundamentally different. In particular, 'specialist' ORNs have been hypothesized to serve an important role in triggering specific innate behavioral responses to their cognate ligands[6–8]. The 'specialist/generalist' dichotomy originates from classic studies of pheromone responses in insects[6,7], but there is also evidence that narrowly tuned ORNs exist in vertebrates[4,5,9].

A fundamental question in the field of olfaction is how inputs from narrowly tuned ORNs are processed by the brain. One obstacle to answering this question in vertebrates is the sheer number of unique olfactory processing channels. Each processing channel is defined by a receptor gene: all ORNs expressing the same odorant receptor gene have similar odor responses and project to the same glomerulus in the brain[10]. In rats and mice, there are ~1,000 different glomeruli, making it difficult to record from the pre- and postsynaptic neurons corresponding to the same glomerulus. In contrast, *Drosophila* has only ~50 types of ORNs, corresponding to ~50 identified glomeruli in the antennal lobe of the brain (analogous to the vertebrate olfactory bulb). Moreover, the odor tuning profiles of most *Drosophila* ORN types have been described in detail, the odorant receptor gene expressed by almost every ORN type has been identified, and the glomerular target of almost every ORN type has been mapped[3,11–19]. This makes it possible to compare directly the odor responses of neurons that are pre- and postsynaptic to the same glomerulus.

Experiments of this type show that most odors evoke distributed patterns of activity in the *Drosophila* antennal lobe and that most second-order neurons respond to multiple odors[20–22]. In fact, most second-order neurons in the *Drosophila* antennal lobe are more broadly tuned than their corresponding ORNs[22,23] (but see refs. 20,21). We wondered whether second-order neurons that are postsynaptic to very narrowly tuned ORNs might prove an exception to this rule. If these second-order neurons retain the high selectivity of their inputs, this might be a useful way to ensure a tight connection between an important olfactory cue and an innate behavioral response. Consistent with this idea, narrowly tuned second-order neurons have been observed (in both vertebrates and invertebrates) to respond specifically to a pheromone or another important ligand[24–30]. However, it is not known whether these narrowly tuned second-order neurons are typically postsynaptic to narrowly tuned ORNs. Also, some of these previous studies have used only a small number of test odors, or only pheromonal odors, and so the true tuning breadth of these second-order neurons is uncertain[27,28,30].

An alternative possibility is that some or all narrowly tuned ORNs project to broadly tuned second-order neurons that participate in the distributed representations of multiple odors. In this scenario, narrowly tuned ORNs might simply guarantee a high sensitivity for their cognate ligand, rather than segregating these signals into a distinct processing pathway. Recent reviews have questioned the idea that pheromones and general odors are processed differently by the brain[31,32]. Furthermore, the fundamental distinction between specialist and generalist ORNs has been challenged by a recent comprehensive survey of *Drosophila*

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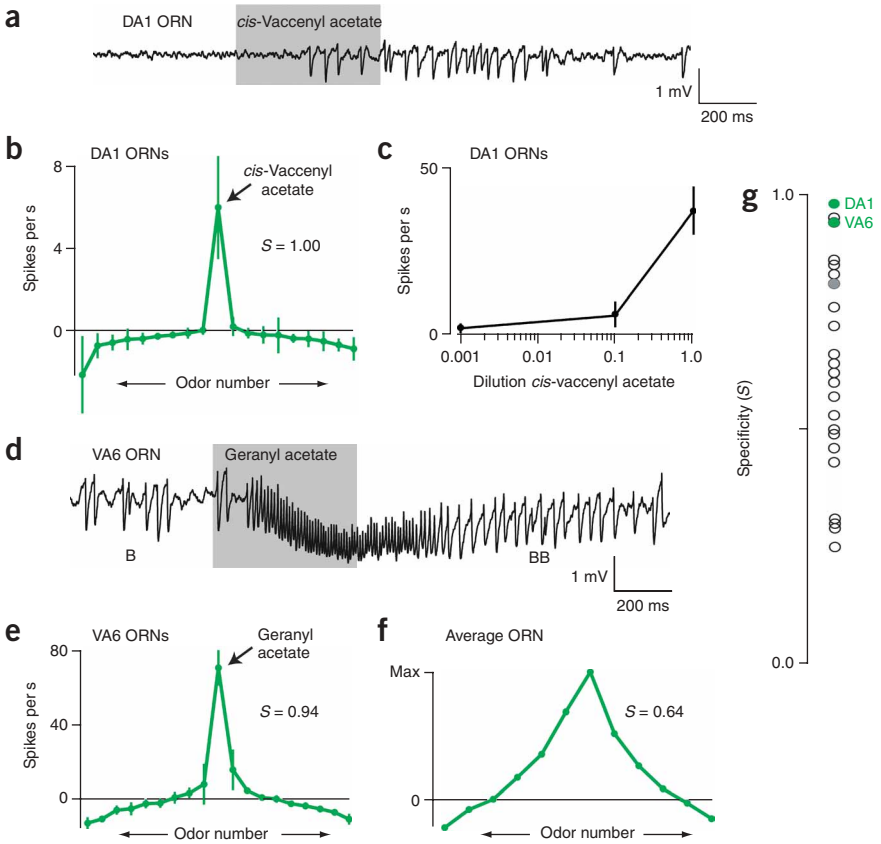

Figure 1 Two narrowly tuned ORN types. (**a**) Single-sensillum recording from a t1-type trichoid sensillum showing spikes from the DA1 ORNs. (**b**) A tuning curve for DA1 ORNs. Odors are arranged on the *x*-axis so that the strongest responses are in the center (see **Supplementary Table 1** for odor order). Negative values mean that the odor suppressed firing rates below spontaneous levels. The specificity (measured as lifetime sparseness, *S*) of this cell type is 1.00. Error bars in all panels are s.e.m. (**c**) A *cis*-vaccenyl acetate dose-response curve for DA1 ORNs. All other data in this study are collected at the 0.1 dilution (see Methods). (**d**) Single-sensillum recordings from an ab5 sensillum; large spikes originate from the VA6 ORNs; a few spikes (B symbols, smaller spikes) originate from the other ORN in the ab5 sensillum. (**e**) A tuning curve for VA6 ORNs. Note that odor order is different from **a**. (**f**) An average ORN tuning curve. Data from ref. 28 was used to construct a normalized tuning curve for each of 24 odorant receptors in the *Drosophila* antennae, and these normalized tuning curves were averaged together to produce a composite picture of the ORN cohort. (**g**) Distribution of specificity (*S*) values for DA1 and VA6 ORNs, as compared to all 24 odorant receptors in ref. 35. Gray symbol indicates the *S* value stated in ref. 28 for Or82a; this slightly lower *S* value may reflect experimental differences between the two studies. Among the open symbols (from ref. 28), the lowest *S* value corresponds to Or85f and the highest *S* value to Or47b.

odorant receptors that found a continuum of ORN tuning widths, rather than a bimodal distribution of narrowly and broadly tuned receptors[19]. This suggests that there may be nothing fundamentally different about narrowly versus broadly tuned ORNs. We have addressed this question in *Drosophila* by examining the odor tuning of second-order neurons downstream from two highly selective ORN types that are required for behavioral responses to their cognate ligands.

RESULTS

Narrowly tuned olfactory receptor neurons

We have measured the odor response profiles of two narrowly tuned ORN types using a panel of 19 structurally diverse odors. One of these ORN types reportedly responds to *cis*-vaccenyl acetate, a *Drosophila* pheromone[11,33,34]. However, its responses to other odors have not been characterized in any detail. These ORNs express the odorant receptor Or67d and project to glomerulus DA1 (refs. 15,16 and **Supplementary Fig. 1** online). Using single-unit recordings from individual sensory hairs (sensilla) on the surface of the antenna, we found that DA1 ORNs are excited exclusively by *cis*-vaccenyl acetate and do not respond to any other odor stimuli in our test set (**Fig. 1a–c**). We also characterized the odor tuning of a second ORN type using the same odor stimulus set. These ORNs are reported to be highly selective for geranyl acetate, a green-leaf volatile that also functions as a pheromone in some dipterans[3,19,35]. These ORNs express the odorant receptor Or82a and project to glomerulus VA6 (refs. 14–16). We have confirmed that these cells are indeed narrowly tuned to geranyl acetate (**Fig. 1d,e**) and that no highly similar odors elicit a larger response (**Supplementary Fig. 2** online).

We quantified the selectivity of these neurons by computing their lifetime sparseness (*S*), a measure that ranges from 0 (unselective) to 1 (maximally selective)[22,36]. Both of these ORN types showed high lifetime sparseness ($S = 1.00$ for DA1 ORNs, 0.94 for VA6 ORNs), meaning that they are very selective. Both are substantially more selective than most ORNs, judging from a recent study that reported the odor tuning of 24 *Drosophila* odorant receptors[19]. We re-analyzed the responses of these 24 odorant receptors to the 12 odors used in that study that overlapped with our stimulus set. We computed a tuning curve for each receptor, normalized the magnitude of each tuning curve to its peak and averaged together all 24 tuning curves. This average tuning curve was substantially less selective (**Fig. 1f**, $S = 0.64$, computed from data in ref. 19) than the tuning curves of the DA1 and VA6 ORNs. We also computed sparseness values individually for each of the 24 receptors in that study and found that the DA1 and VA6 ORNs fell near the extreme end of this distribution (**Fig. 1g**).

Receptor neurons required for innate behavioral responses

Some narrowly tuned ORNs are thought to trigger innate behavioral responses to their cognate ligand. Therefore, we next asked whether these two ORN types are required for innate responses to *cis*-vaccenyl acetate and geranyl acetate. We used a modified Y-maze assay to measure flies' innate odor preferences (**Supplementary Methods** online). Flies demonstrate attractive, neutral and aversive responses in this maze, depending on the odors present (**Fig. 2a**).

Drosophila are not attracted to pure *cis*-vaccenyl acetate in an acute navigation assay that lasts for several minutes[37] (although attraction has been shown in an assay measuring population aggregation over 48 h[33]). However, investigators using an acute navigation assay have shown that when *cis*-vaccenyl acetate is blended with another odor, the

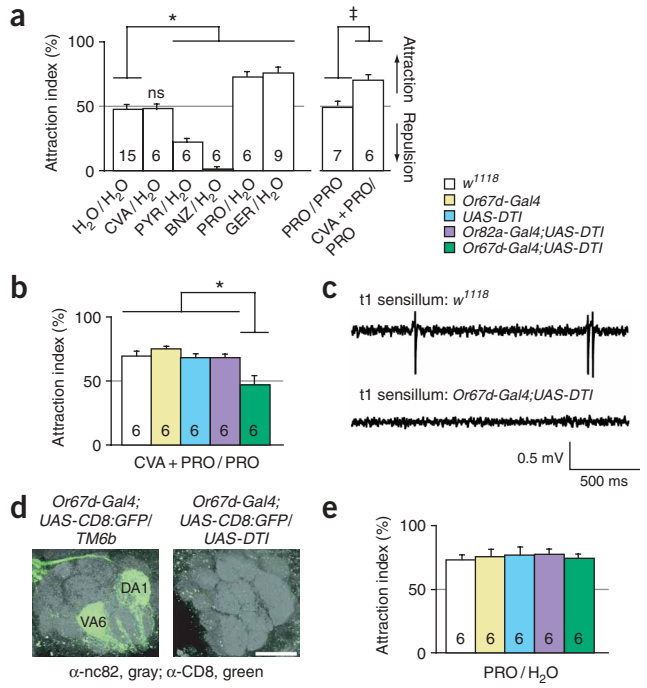

Figure 2 DA1 ORNs are required for behavioral attraction to *cis*-vaccenyl acetate. (**a**) Response index of control (w^{1118}) flies in a Y-maze (see **Supplementary Methods**). Flies demonstrate attractive, repulsive and neutral responses. All odors were diluted 1:250 in water. H_2O, water control; CVA, *cis*-vaccenyl acetate; PYR, pyrrolidine; BNZ, benzaldehyde; PRO, propionic acid; GER, geranyl acetate. The numbers inside each bar indicate the number of trials run for the given condition. Error bars on all bar graphs are s.e.m. Symbols indicate significance (*ANOVA, $P = 10^{-18}$, *post hoc* Tukey HSD, $P < 0.01$; ‡ *t*-test, $P = 0.02$). (**b**) Control (w^{1118}), Gal4-only (*Or67d-Gal4*) and UAS-only (*UAS-DTI*) flies are attracted to *cis*-vaccenyl acetate plus propionic acid, as compared with propionic acid alone. This synergistic attraction is absent in *Or67d-Gal4*;*UAS-DTI* flies (*ANOVA, $P = 0.0007$, *post hoc* Tukey HSD, $P < 0.05$). As an additional control, we tested *Or82a-Gal4*;*UAS-DTI* flies, which showed normal attraction. (**c**) Extracellular recordings from t1-type trichoid sensilla control flies, and in flies where *Or67d-Gal4* drives expression of diphtheria toxin. (**d**) Projections of confocal stacks through the antennal lobe. Dual immunofluorescence uses an antibody specific for CD8 (green) to visualize ORN axons, and nc82 antibody (gray) to visualize glomeruli. One fly (left) carries the *Or67d-Gal4* transgene plus a *UAS-CD8:GFP* transgene; the other fly (right) also carries a *UAS-DTI* transgene. In the latter fly, the Gal4-expressing axons are ablated. Scale bar, 20 μm. (**e**) Flies lacking DA1 ORNs show normal attraction to propionic acid, an attractive odor that does not activate DA1 ORNs.

blend is more attractive than the second odor in isolation[37]. Consistent with this, we found that *cis*-vaccenyl acetate alone was not an attractant in our Y-maze (**Fig. 2a**, see **Supplementary Methods**). As expected, we found that when *cis*-vaccenyl acetate was blended with an attractive second odor, the blend was significantly more attractive than the second odor alone (**Fig. 2b**). Next, we genetically ablated ORNs projecting to glomerulus DA1 by expressing diphtheria toxin under the control of the Or67d promoter (*Or67d-Gal4*;*UAS-DTI*). To confirm that the intended neurons were killed, we performed single-sensillum recordings (**Fig. 2c**). The DA1 ORNs are housed individually in 't1-type' trichoid sensilla; this is the only sensillum type that contains exactly one ORN[15,16]. As expected, when we recorded from trichoid sensilla in control (w^{1118}) flies, we found 31 sensilla that contained exactly one ORN; all other trichoid sensilla contained multiple ORNs. When we recorded from trichoid sensilla in flies where the Or67d promoter drives diphtheria toxin expression, we found 11 sensilla that contained zero ORNs; all other trichoid sensilla contained multiple ORNs. This argues that most or all DA1 ORNs had been killed. We also coexpressed diphtheria toxin with green fluorescent protein (GFP), and verified that all GFP-expressing axons were absent from the antennal lobes (**Fig. 2d**).

In these flies lacking DA1 ORNs, the synergistic behavioral effect of *cis*-vaccenyl acetate was abolished (**Fig. 2b**). This suggests that these neurons are important in triggering this behavior. Innate attraction to another odor was unaffected (**Fig. 2e**), arguing that this effect is specific to *cis*-vaccenyl acetate. Because this Or67-Gal4 line ectopically expresses Gal4 in the VA6 ORNs (**Supplementary Fig. 1**), it is important to check that this behavioral phenotype reflects the loss of DA1 ORNs, and not VA6 ORNs. Therefore, we repeated these assays in

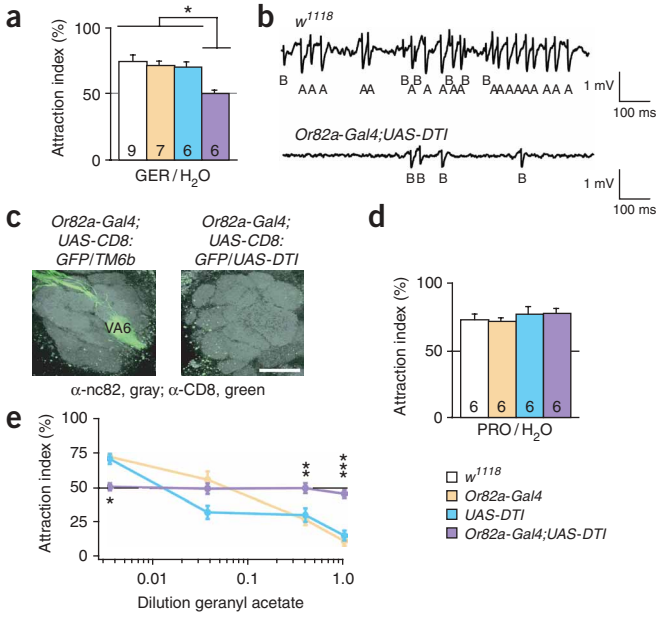

Figure 3 VA6 ORNs are required for behavioral attraction to geranyl acetate. (**a**) Control (w^{1118}), Gal4-only (*O82a-Gal4*) and UAS-only (*UAS-DTI*) flies are attracted to geranyl acetate. This attraction is absent in *Or82a-Gal4*;*UAS-DTI* flies (*ANOVA, $P = 0.0003$; *post hoc* Tukey HSD, $P < 0.01$). The numbers inside each bar indicate the number of trials run for the given condition. Error bars on all bar graphs are s.e.m. (**b**) Extracellular recordings from ab5 sensilla in control flies, and in flies where Or82a-Gal4 drives expression of diphtheria toxin. In the top trace, the larger spikes (A symbols) arise from the VA6 ORNs (refs. 31,32,41). The A spikes are absent in the bottom trace. (**c**) Projections of confocal stacks through the antennal lobe. Dual immunofluorescence uses an antibody specific for CD8 (green) to visualize ORN axons, and nc82 antibody (gray) to visualize glomeruli. One fly (left) carries the *Or82a-Gal4* transgene plus a *UAS-CD8:GFP* transgene; the other fly (right) also carries a *UAS-DTI* transgene. In the latter fly, the Gal4-expressing axons are ablated. Scale bar, 20 μm. (**d**) Flies lacking VA6 ORNs show normal attraction to propionic acid. (**e**) A dose-response curve for geranyl acetate. Flies lacking VA6 ORNs are not attracted to geranyl acetate at any concentration tested. Six trials were run for each condition, except as indicated in **a**. Symbols indicate a significant difference between the *Or82a-Gal4*;*UAS-DTI* genotype and both controls (*ANOVA, $P = 10^{-4}$; *post hoc* Tukey HSD, $P < 0.01$; **ANOVA, $P < 0.005$, *post hoc* Tukey HSD, $P < 0.05$; ***ANOVA $P = 10^{-6}$, *post hoc* Tukey HSD, $P < 0.01$). Error bars are s.e.m.

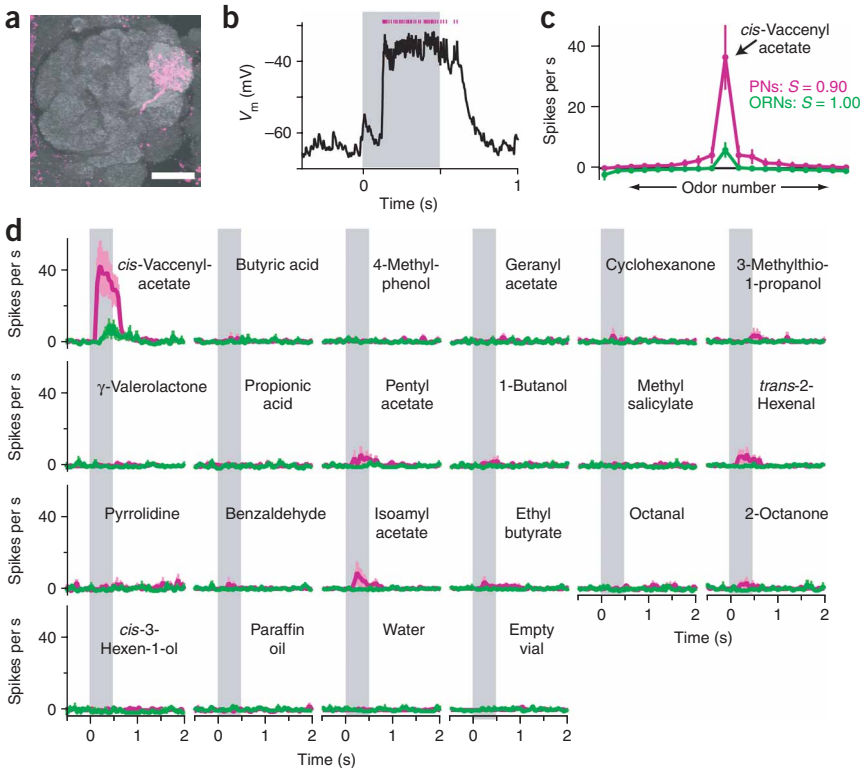

Figure 4 DA1 projection neurons are narrowly tuned to *cis*-vaccenyl acetate. (**a**) Projection of a confocal stack through the antennal lobe. A portion of a biocytin-filled projection neuron is shown (magenta) extending a dendritic tuft into glomerulus DA1. Glomeruli are outlined with nc82 antibody (gray). Scale bar, 20 μm. (**b**) A raw trace illustrating the response of a DA1 projection neuron to *cis*-vaccenyl acetate. Magenta symbols indicate the timing of action potentials and the gray bar shows timing of odor stimulation. (**c**) Tuning curves for DA1 projection neurons and ORNs. Odors are arranged so that the strongest responses are in the center, meaning that the odor order for the ORN and projection neuron graphs is not the same (**Supplementary Table 1**). Error bars are s.e.m. (**d**) PSTHs showing the responses of DA1 ORNs (green) and projection neurons (magenta) to 19 odor stimuli and 3 controls (paraffin oil, water and empty vial). Error bars (in lighter colors) are s.e.m.

flies where only the VA6 ORNs were ablated (*Or82a-Gal4*;*UAS-DTI*). In these flies, the behavioral response to *cis*-vaccenyl acetate was normal (**Fig. 2b**). Together, these results demonstrate that DA1 ORNs are required for the synergistic attractive effect of *cis*-vaccenyl acetate in the context of our assay.

We also tested the role of VA6 ORNs in triggering innate responses to geranyl acetate. This odor was attractive to control flies (**Fig. 3a**). We killed VA6 ORNs by expressing diphtheria toxin under the control of the Or82a promoter (*Or82a-Gal4*;*UAS-DTI*). Again, single-sensillum recordings confirmed that the toxin ablated the intended neurons (**Fig. 3b**). Each VA6 ORN is housed together with another ORN in an 'ab5-type' sensillum; of this pair, the VA6 ORN is always the neuron producing larger action potentials[3,14–16] (the 'A' neuron, **Fig. 3b**). We recorded from 31 ab5 sensilla in control flies and found that both ORNs were always present. As expected, all 10 ab5 sensilla tested in *Or82a-Gal4*;*UAS-DTI* flies lacked the A neuron. We also coexpressed diphtheria toxin with GFP and verified that all GFP-expressing axons were absent (**Fig. 3c**). In these flies, the attractive response to geranyl acetate was abolished (**Fig. 3a**). However, these flies responded normally to an attractive odor that does not excite the VA6 ORNs (**Fig. 3d**).

Previous studies have shown that VA6 ORNs are not the only receptor neurons that respond to geranyl acetate[3,19]. Therefore, it seemed possible that killing the VA6 ORNs simply shifts the behavioral dose-response curve to the right, rather than qualitatively altering the behavioral response to this odor. However, we found that higher concentrations of geranyl acetate did not rescue the attractive behavioral response in these flies. Even at high concentrations that are aversive to control flies, geranyl acetate elicited no attraction, and minimal repulsion, in flies lacking VA6 ORNs (**Fig. 3e**). These results imply that VA6 ORNs are important in mediating innate responses to their cognate ligand, despite the fact that this odor also activates other ORN types.

Second-order neurons highly selective for a pheromone

Taken together, these electrophysiological and behavioral results demonstrate that the DA1 and VA6 ORNs clearly meet the traditional definition of specialist neurons. Both ORN types are very narrowly tuned and both are important for an innate behavioral response to their cognate ligands. Having established this, we then asked whether second-order olfactory neurons that are postsynaptic to these two glomeruli are narrowly or broadly tuned to odors. We made whole-cell patch-clamp recordings *in vivo* from projection neurons directly postsynaptic to glomerulus DA1. Projection neurons are the only output neurons of the antennal lobe, analogous to olfactory bulb mitral cells. To record selectively from DA1 projection neurons, we used an enhancer trap line to label these cells with GFP. In *Mz19-Gal4*,*UAS-CD8:GFP* flies, all six GFP-positive projection neurons with somata lateral to the antennal lobe are known to innervate glomerulus DA1 (ref. 38). We also used biocytin-streptavidin histochemistry to confirm that the recorded projection neurons innervated this glomerulus (**Fig. 4a**). It should be noted that this enhancer trap line does not label all DA1 projection neurons[38,39] and we cannot exclude the possibility that unlabeled cells respond differently to our stimuli.

We measured the odor responses of these projection neurons while stimulating the antennae with the same odors we used for the ORN recordings. We found that, like their presynaptic ORNs, projection neurons in glomerulus DA1 are highly selective for *cis*-vaccenyl acetate (**Fig. 4b–d**). Other odors in our test set elicited either no response or a comparatively small response. The lifetime sparseness of the average DA1 projection neuron tuning curve was 0.90, indicating high selectivity (**Fig. 4c**). This stands in contrast to most antennal lobe projection neurons, which are broadly tuned to odors (ref. 22, also V. Bhandawat, S.R. Olsen, M.L. Schlief, N.W. Gouwens and R.I. Wilson, unpublished data). In addition, the DA1 projection neurons showed a marked degree of sensitivity. A concentration of *cis*-vaccenyl acetate that elicited only a small response in their presynaptic ORNs evoked a robust response in these projection neurons (**Fig. 4b–d**). These results show that at least some narrowly tuned ORNs project to downstream neurons with high sensitivity and high selectivity for one ligand.

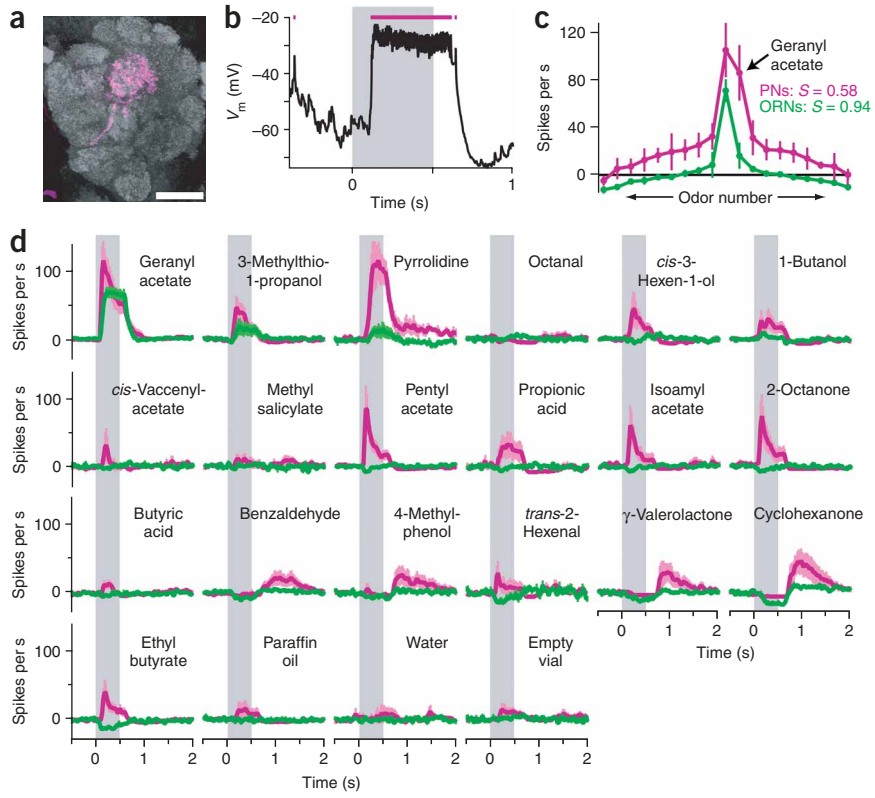

Figure 5 VA6 projection neurons are more broadly tuned to odors than their presynaptic ORNs. (**a**) Projection of a confocal stack through the antennal lobe. A portion of a biocytin-filled projection neuron is shown (magenta) extending a dendritic tuft into glomerulus VA6. Glomeruli are outlined with nc82 antibody (gray). Scale bar = 20 μm. (**b**) A raw trace illustrating the response of a VA6 projection neuron to geranyl acetate. Magenta symbols indicate the timing of action potentials and the gray bar shows timing of odor stimulation. (**c**) Tuning curves for VA6 projection neurons and ORNs. Odors are arranged so that the strongest responses are in the center, meaning that the odor order for the ORN and projection neuron graphs is not the same (**Supplementary Table 1**). Error bars are s.e.m. (**d**) PSTHs showing the responses of VA6 ORNs (green) and projection neurons (magenta) to 19 odor stimuli and 3 controls (paraffin oil, water and empty vial). Error bars (in lighter colors) are s.e.m.

Broadly tuned second-order neurons

Next, we characterized the odor tuning of projection neurons postsynaptic to glomerulus VA6. To target these projection neurons, we recorded from *Mz612-Gal4,UAS-CD8:GFP* flies. In this genotype, all GFP-positive projection neurons innervate glomerulus VA6 (ref. 40). We also filled each recorded neuron with biocytin to confirm the identity of these cells (**Fig. 5a**). We note that this enhancer trap line may not label all VA6 projection neurons.

In contrast to the DA1 projection neurons, projection neurons in glomerulus VA6 show broad odor tuning (**Fig. 5b–d**). These projection neurons were excited by geranyl acetate, but also by several other acetates. Several odors dissimilar to geranyl acetate (pyrrolidine and 2-octanone) also evoked a robust response. The lifetime sparseness of the VA6 projection neuron tuning curve was 0.58, meaning that these neurons were considerably less selective than their presynaptic ORNs (**Fig. 5c**). We also tested the VA6 projection neurons with a panel of odors highly similar to geranyl acetate, and again the VA6 projection neurons showed broader tuning than their presynaptic ORNs (**Supplementary Fig. 2**).

Thus, neurons downstream from glomerulus VA6 are not dedicated to a specific odor, but instead participate in the ensemble code for multiple odors. The transformation in odor representations that occurs in glomerulus VA6 is therefore different from the transformation that occurs in DA1. To quantify this difference, we asked how many odors elicited a response that was significantly different in projection neurons versus their presynaptic ORNs (**Fig. 6**, tuning curves from **Figs. 4c** and **5c**). Because we were interested in tuning curve shape, and not the absolute value of the tuning curve peak, we first normalized each odor response to the peak of the corresponding average tuning curve (for example, every DA1 ORN odor response was normalized to the average DA1 ORN response to *cis*-vaccenyl acetate). Then we compared ORN and projection neuron responses for each odor using an unpaired two-tailed Student's *t*-test. At the confidence level corresponding to $P < 0.05$, only 1 of the 19 test odors elicited DA1 projection neuron responses that were significantly different from DA1 ORN responses, and none of these were significant after a Bonferroni correction for multiple comparisons (**Fig. 6a**). In contrast, 9 of the 19 test odors for glomerulus VA6 elicited responses that were significantly different at $P < 0.05$, and 3 of these

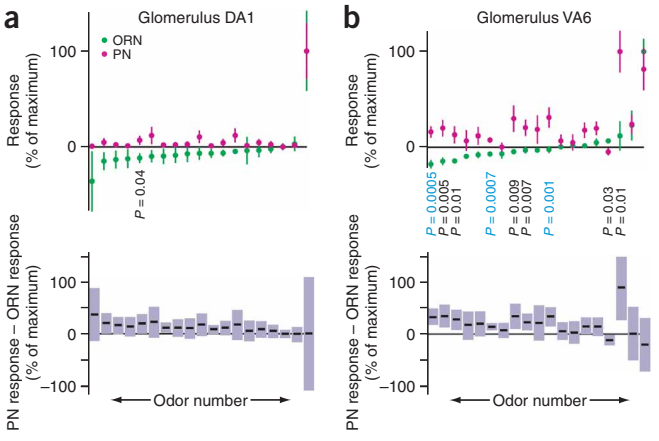

Figure 6 There is a significant transformation of odor tuning in glomerulus VA6, but not in DA1. Tuning curves for (**a**) DA1 and (**b**) VA6. ORN responses are in green, projection neuron responses in magenta. Error bars are s.e.m. Odors are arranged so that the strongest ORN responses are at right. Odor order is the same for ORN and projection neuron graphs. All values are normalized to the maximum average response for that cell type. The *P* value is indicated below the odor for ORN–projection neuron comparisons that are significant at the criterion $P < 0.05$ (unpaired two-tailed *t*-tests). Only *P* values in blue remain significant after a Bonferroni correction for multiple comparisons ($P < 0.0026$). Lower panels plot the mean normalized projection neuron response minus the mean normalized ORN response for each odor (black symbols). Violet bars indicate the confidence interval corresponding to $P < 0.05$ for each test, showing a similar level of statistical power for the DA1 dataset as compared to the VA6 dataset.

odors were still significant after the stringent Bonferroni correction ($P < 0.05 \div 19 = 0.0026$; **Fig. 6b**). For each glomerulus we also computed the confidence interval corresponding to $P < 0.05$ for each of the 19 odors. We obtained a similar range of confidence intervals for DA1 as compared to VA6, meaning that our DA1 dataset had statistical power comparable to that of our VA6 dataset (**Fig. 6**).

DISCUSSION

A neural circuit dedicated to a *Drosophila* pheromone

The *Drosophila* pheromone *cis*-vaccenyl acetate has been reported to elicit an excitatory response in the ORNs that project to glomerulus DA1 (the t1-type ORNs)[11,33,34]. Because previous studies used a limited odor set or described only qualitative responses, it was not possible to assess how narrowly these neurons are tuned. Here, we show that DA1 ORNs were strictly selective for *cis*-vaccenyl acetate, whereas all of the other odors in our diverse test set elicited no response. Ablating these ORNs abolished the synergistic behavioral attraction to *cis*-vaccenyl acetate, implying that these neurons are necessary to trigger behavioral responses to this chemical cue.

Following this circuit into the brain, we have found that projection neurons postsynaptic to glomerulus DA1 retain the extreme selectivity of their ORN inputs. These projection neurons responded robustly to *cis*-vaccenyl acetate, whereas all of the other odors elicited little or no response. In this respect, these projection neurons are unlike most projection neurons in the antennal lobe, which participate in the ensemble representations of multiple odors. The narrow tuning of this central olfactory processing channel could be useful in preventing a nonpheromonal odor from triggering a pheromone-cued behavior. Segregating a pheromone signal into a separate parallel pathway wastes coding capacity, but should help ensure an appropriate connection between stimulus and behavior. In this respect, it may be relevant that the DA1 ORNs express *fruitless*, a transcription factor linked to sexual behavior[41,42]. This suggests that *cis*-vaccenyl acetate might have a role in courtship or mating, behaviors that have been previously linked with the notion of dedicated sensory channels[43].

Although DA1 projection neurons are dedicated to *cis*-vaccenyl acetate, neural signals elicited by this odor must converge at some point downstream with information from other olfactory channels. This is because *cis*-vaccenyl acetate is not attractive by itself in our assay, but rather increases the attractiveness of other odors in a blend. In our experiments, for example, *cis*-vaccenyl acetate increased the attractiveness of propionic acid. We observed that DA1 projection neurons were insensitive to propionic acid even at a high concentration (10% saturated vapor, data not shown). This implies that the behavioral response to the blend requires integration across glomeruli.

Notably, DA1 projection neurons are much more sensitive to *cis*-vaccenyl acetate than their presynaptic ORNs are. This may reflect the fact that 110–120 ORNs project to each DA1 glomerulus[44]. This convergence could produce a robust response in the DA1 projection neurons even if the stimulus intensity were only sufficient to elicit scattered spikes in a few DA1 ORNs. Additionally, if each ORN spike has a large impact on its postsynaptic projection neurons, this would also tend to amplify responses in the projection neuron layer. Future experiments should determine exactly how many ORNs make synapses with each projection neuron, and how much postsynaptic depolarization is elicited by each presynaptic spike.

An ensemble code for most odors

Most ORNs respond to multiple odors, and most odors elicit responses in multiple ORN types. This constitutes an ensemble (or 'combinatorial') code for odors in the sensory periphery. Similarly, most second-order neurons in the *Drosophila* antennal lobe respond to multiple different odors, and most odors elicit responses in multiple types of projection neurons. Moreover, when the responses of ORNs and projection neurons corresponding to the same glomerulus are compared directly, most projection neurons are more broadly tuned than their presynaptic ORNs[22,23]. It should be noted that some functional imaging experiments in the *Drosophila* antennal lobe paint a somewhat different picture, suggesting that the selectivity of most projection neurons closely matches that of their presynaptic ORNs[20,21]. These results may reflect the low sensitivity and limited dynamic range of the fluorescent probes used in these experiments[45,46].

In this sense, VA6 projection neurons are typical: they are more broadly tuned than their presynaptic ORNs, and even respond to odors from different chemical classes. Thus, these projection neurons participate in the ensemble representations of multiple odors. The mechanism by which most projection neurons acquire broad tuning is currently unknown (either for VA6, or for any other glomerulus). One possibility is that most projection neurons receive indirect input from several ORN types via excitatory local interneurons[23]. This would account for why the VA6 projection neurons respond differently to two odors (for example, 3-methylthio-1-propanol and pyrrolidine) that elicit similar activity in the VA6 ORNs (**Fig. 5d**). If interglomerular excitatory connections contribute to broad tuning in VA6 projection neurons, then DA1 projection neurons may receive less interglomerular excitatory input than VA6 projection neurons. Consistent with this idea, we have previously found that some local networks in the antennal lobe selectively exclude glomerulus DA1 (ref. 47).

Another possibility is that feedforward nonlinearities contribute to broad tuning curves in projection neurons. If ORN–to–projection neuron synapses are powerful, then a robust projection neuron response might be triggered by only a few spikes in the neuron's presynaptic ORNs. This synapse might saturate at high ORN firing rates, producing a broad projection neuron tuning curve. If broad projection neuron tuning reflects primarily feedforward nonlinearities, then there must be something different about the VA6 and DA1 glomeruli with respect to feedforward excitation. Consistent with this, there are more weak responses in VA6 ORNs than in DA1 ORNs; these weak responses could interact with feedforward nonlinearities to produce a broader tuning curve in VA6 projection neurons. However, there are also more inhibitory responses in VA6 ORNs as compared with DA1 ORNs, and it is difficult to see how feedforward mechanisms alone could excite a VA6 projection neuron in response to an odor that inhibits its presynaptic ORNs (for example, pentyl acetate or 2-octanone, **Fig. 5d**).

Manipulating ensemble representations of odor quality

Killing VA6 ORNs abolishes the innate behavioral attraction to geranyl acetate in the conditions of our assay. Yet these are not the only ORNs that respond to this odor[3,19], and these ORNs do not project to a central circuit dedicated to geranyl acetate. Why does killing these ORNs eliminate attraction to this odor?

Drosophila show different innate responses to different odors (**Fig. 2a**). Thus, attraction requires not only odor detection, but also qualitative perception. Geranyl acetate elicits activity in several glomeruli, which together signal the quality of this stimulus. We hypothesize that killing ORNs projecting to VA6 alters this ensemble code such that it no longer triggers the same innate associations in higher brain regions. Indeed, there is behavioral genetic evidence that activity in most glomeruli is not decoded in isolation but rather in the context of other glomeruli[48].

Our results do not exclude the possibility that output from glomerulus VA6 is somehow intrinsically privileged in triggering an innate attractive response. However, we have found that one of the odors eliciting a robust response in VA6 projection neurons (pyrrolidine, **Fig. 5d**) is innately aversive in the Y-maze assay (**Fig. 2a**). This argues that output from this glomerulus is decoded in the context of output from other glomeruli, and robust VA6 projection neuron activity is not sufficient to elicit attraction.

Revisiting the notion of olfactory specialists

These results caution against a simple view of narrowly tuned ORNs. We have shown that the narrowly tuned ORNs project to downstream neurons that are dedicated to signaling the presence of a pheromone. Conversely, although the VA6 ORNs are also narrowly tuned to a particular odor, these neurons project to projection neurons that respond to multiple odors. Unlike *cis*-vaccenyl acetate, geranyl acetate is not a pheromone and has no known special significance for *Drosophila*. It may be that the Or82a receptor is useful primarily because it confers increased sensitivity to volatile terpenoids, and the narrow tuning of this receptor is merely the byproduct of other biophysical constraints. Another possibility is that the VA6 ORNs simply reflect the evolutionary history of this species. This is consistent with the observation that odorant receptor genes do not appear to evolve rapidly during speciation[49].

We would argue that the specialist/generalist classification of ORNs, as currently defined, may not correspond to a meaningful functional division. Our results show that some narrowly tuned ORNs project to central neurons that are dedicated to their cognate ligand, whereas other narrowly tuned ORNs project to central neurons that respond to multiple odors. Another challenge to the specialist/generalist classification is the recent finding that there is not a bimodal distribution of narrowly and broadly tuned receptors in the *Drosophila* antenna; instead, there is a continuum of receptor tuning widths[19]. Our results extend this idea, showing that even ORNs at the narrowly tuned end of this continuum can project to projection neurons that participate in the ensemble code for multiple odors. Thus, in order to understand the role of any single odorant receptor in odor coding, it is necessary to follow the ORNs expressing that receptor into the brain, and to observe whether and where these signals are integrated with information from other ORN types.

METHODS

Flies. All flies were females aged 3–8 d, raised on a 12-h light/12-h dark cycle at 25 °C, 50–60% relative humidity, on standard cornmeal-agar medium. Except where otherwise noted, control flies were w^{1118}. The Or67d-Gal4$_{II}$ and Or82a-Gal4$_{II}$ lines were obtained from L. Vosshall (Rockefeller Univ.). For the experiments in **Supplementary Figure 1**, we tested both the Vosshall Or67d-Gal4$_{II}$ line and a Or67d-Gal4$_{II}$ line obtained from B. Dickson (IMP Vienna); the same result was obtained with both lines ($n = 3$ for each line). Flies expressing diphtheria toxin light chain under UAS control (*UAS-DTl*) were obtained from L. Stevens (Univ. Texas, Austin), *Mz19-Gal4* and *Mz612-Gal4* flies from L. Luo (Stanford Univ.), and *UAS-CD8:GFP* flies from the Bloomington Stock Center.

Olfactory stimulation. During ORN and projection neuron recordings, a constant stream of charcoal-filtered air (2.2 l min^{-1}) was directed at the fly. Odors were diluted 1:100 vol/vol in paraffin oil (J.T. Baker, VWR #JTS894), except for 3-methylthio-1-propanol and propionic acid, which were diluted 1:100 vol/vol in water, and 4-methylphenol, which was diluted 1:100 wt/vol in water. We did not dilute *cis*-vaccenyl acetate in the odor vial (except in **Fig. 1c**). After a trigger, a three-way solenoid valve redirected 10% of the airstream (0.22 liter min^{-1}) through the headspace of the odor vial for 500 ms. Thus, all odors (including *cis*-vaccenyl acetate) were diluted tenfold in air just before reaching the fly. The odor stream rejoined the non-odor stream 16 cm from the end of the end of the delivery tube, which was 3 mm in diameter. The end of the delivery tube was 8 mm from the fly. Odor presentations (typically six trials per odor) were spaced 40–60 s apart. *cis*-Vaccenyl acetate was obtained from Pherobank, geranyl formate and geranyl propioniate from Advanced Biotech, and all other odors from Sigma. Item numbers and purities for all odors are listed at http://wilson.med.harvard.edu/odors.html. Odor dilutions in paraffin oil were kept at 23 ± 1 °C, and replaced with fresh dilutions every 10 d. In **Figure 1b**, 0.001 means a 1:100 dilution in paraffin oil followed by a 1:10 dilution in air, 0.1 means a 1:10 dilution in air, and 1 corresponds to a concentration near saturated vapor pressure (100% of a 1 liter min^{-1} airstream was driven through the odor vial).

Olfactory receptor neuron recordings. Flies were immobilized in the trimmed end of a plastic pipette tip, and a pair of pulled glass pipettes was used to stabilize one antenna. A saline-filled glass capillary was inserted into the eye as a reference electrode, and a sharp saline-filled glass capillary (tip <1 μm) was inserted into a sensillum. Recordings from t1-type trichoid sensilla (DA1 ORNs) were performed using sharp saline-filled quartz capillaries made on a laser puller (Sutter P-2000). Signals were recorded on an A-M Systems Model 2400 amplifier with a 10-MΩ headstage, low-pass filtered at 2 kHz and digitized at 10 kHz. DA1 ORNs were identifiable because these neurons are housed in t1-type trichoid sensilla in the proximal region of the trichoid zone (antennal zone V)[33]. Also, these are the only sensilla containing a single ORN; in single-sensillum recordings, this corresponds to a single spike waveform and the absence of interspike intervals <2 ms. VA6 ORNs were identifiable as the neuron generating larger spikes (the A cell) in type ab5 sensilla. These sensilla were always in zone IV or V, and were uniquely identifiable on the basis of the strong response of the B cell (>200 Hz) to the odors 3-methylthio-1-propanol and pentyl acetate[3]. If a sudden increase in spontaneous spiking was observed, the recording was terminated.

Projection neuron recordings. *In vivo* whole-cell patch-clamp recordings from projection neurons were performed as described previously[47]. Extracellular saline contained 103 mM NaCl, 3 mM KCl, 5 *N*-tris(hydroxymethyl)-methyl-2-aminoethane-sulfonic acid, 8 mM trehalose, 10 mM glucose, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 1.5 mM $CaCl_2$ and 4 mM $MgCl_2$. Saline osmolarity was adjusted to 270–275 mOsm, and the pH equilibrated near 7.3 when bubbled with 95% O_2/5% CO_2. Signals were recorded on an A-M Systems Model 2400 amplifier with a 10-MΩ headstage, low-pass filtered at 5 kHz and digitized at 10 kHz. Recordings were obtained from GFP-positive projection neuron somata in *Mz19-Gal4,UAS-CD8:GFP* or *Mz612-Gal4,UAS-CD8:GFP* flies using an Olympus BX51F with IR-DIC optics, a 40× water-immersion objective, and a fluorescence attachment with a GFP filtercube. One projection neuron was recorded per fly. We confirmed the identity of the recorded projection neurons by visualizing their biocytin fills *post hoc,* using nc82 antibody to outline glomerular boundaries. Histochemistry with biocytin-streptavidin, α-CD8 antibody and nc82 antibody was performed as described previously[47], except that in the secondary incubation we used 1:250 goat anti-mouse–Alexa-Fluor633 and 1:1,000 streptavidin-AlexaFluor568. The nc82 antibody was obtained from the Developmental Studies Hybridoma Bank (Univ. Iowa).

Electrophysiological data analysis. Spike times were extracted from raw ORN and projection neuron recordings using custom software written in Igor Pro (Wavemetrics). Each cell was tested with multiple odors, with each odor presented six times at intervals of 40–60 s (a block of trials). Each trial was converted into a peristimulus time histogram (PSTH) by counting the number of spikes in sliding 50-ms bins that overlapped by 25 ms. These single-trial PSTHs were then averaged together to generate a PSTH describing that block of trials. The average spontaneous spiking rate during the 7 s preceding stimulus onset was then subtracted from each of these block PSTHs. Each cell type (DA1 ORN, DA1 projection neuron, VA6 ORN, VA6 projection neuron) was tested with a given odor in multiple experiments, each with a different fly. The *n* for each odor/cell-type combination is listed in **Supplementary Table 1** online. The PSTHs represent the mean ± s.e.m. computed across experiments.

Tuning curves (**Figs. 1, 4** and **5**) were constructed by computing the overall spike rate (in spikes s^{-1}) during the 500-ms period beginning 100 ms after odor valve opening, and ending 100 ms after odor valve closing. This count was

averaged across six trials to yield the response to that block of trials, and the mean baseline firing rate for that block of trials was subtracted from this value. These responses were then averaged across experiments to generate a mean ± s.e.m. for each odor (the order of odors on the *x*-axis of each tuning curve in **Figs. 1**, **4**, **5** is different; see **Supplementary Methods** for odor orders). We quantified tuning curve shape by computing S[22,36]:

$$S = \{1 - [(\sum N_{j=1} r_j/N)^2 / \sum N_{j=1}(r_j^2/N)]\}/[1 - (1/N)]$$

where N = number of odors and r_j is the analog response intensity of the neuron to odor j. Any negative values of r_j were set to zero before computing S; otherwise, $S > 1$ can result.

Behavior. See **Supplementary Methods**.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

This study was jointly designed by M.L.S. and R.I.W. The experiments and data analysis were performed by M.L.S., and M.L.S. and R.I.W. jointly wrote the paper.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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