Supporting Online Material

Materials and Methods

Fly preparation. Flies were reared on standard cornmeal agar medium (1). Except where otherwise noted, flies were wild-type (Canton-S). *UAS-CD8-GFP* flies were a gift from Seymour Benzer. *GH146-Gal4* flies were a gift from L. Luo. *Or22a-Gal4, UAS-CD8-GFP/TM6* flies were a gift from L. Vosshall. *GH298-Gal4, UAS-CD8-GFP/TM6b* flies were a gift from R. Stocker. All experimental animals were females, 4-12 days post-eclosion. Flies were anesthetized in a glass vial on ice just until movement stopped (~20 sec.), and then were gently inserted into a hole in a piece of aluminum foil. Small drops of wax (55°C) were used to suspend the fly in the hole, with the edge of foil defining a horizontal plane around the head and thorax, from the first antennal segment anteriorly to the scutellum posteriorly. The dorsal side of the foil was bathed in saline, while the ventral side (including antennae and maxillary palps) remained dry and accessible to odors. A window was cut in the dorsal head cuticle between the eyes, extending from the ocelli to the first antennal segment. Fat and air sacs dorsal and anterior to the brain were removed, and the perineural sheath was gently picked away from the antennal lobes. In some preparations, brief exposure to a protease solution was used to weaken the sheath; activity in these brains was not noticeably different from brains desheathed mechanically. Spontaneous leg and proboscis movements were typically observed in this preparation for the duration of the recording (1.5-3 hours). The saline composition was (in mM): NaCl 103, KCl 3, TES 5, trehalose 10, glucose 10, sucrose 7, $NaHCO_3$ 26, NaH_2PO_4 1, $CaCl_2$ 1.5, $MgCl_2$ 4 (adjusted to 280 mOsm). The saline was bubbled with 95% O_2/5% CO_2 and continuously perfused over the preparation (2ml/min).

Whole-cell patch-clamp recording. Recordings were obtained from PN or LN somata under visual control using a Axioskop FS-1 with IR-DIC optics and a 40X water-immersion objective. One PN or LN was recorded per fly, except for paired recordings (Fig. 4F-G). Patch-clamp electrodes (5-7 MΩ) were filled with a solution of (in mM): KCH_3SO_3H 160, HEPES 10, MgATP 4, Na_3GTP 0.5, EGTA 1 (pH = 7.3, adjusted to 265mOsm). In most experiments, 0.5% neurobiotin was added to the internal solution. Neurobiotin-filled PNs in wild-type flies were electrophysiologically similar to PNs recorded without neurobiotin in *Gal4-GH146, UAS-CD8-GFP* flies. In paired recordings (Fig. 4F-G), one electrode contained Alexa Fluor 488 (1 μM) and the other contained Alexa Fluor 568 (1 μM). Hyperpolarizing voltage steps (2-5 mV) were used to continually measure intrinsic membrane properties. Cells were accepted for recording if R_{input}>500 MΩ and R_{access}<50 MΩ; R_{access} in many experiments was <30 MΩ. A small constant hyperpolarizing current was injected in LN and PN recordings, beginning immediately after break-in, in order to bring the membrane potential between –55 to –60 mV; under these conditions, spontaneous spiking rates in LNs matched the rate observed in cell-attached mode prior to break-in. Spikes were not observed in PN recordings in cell-attached mode, probably because spikes are not actively propagated to the soma in these neurons (Fig. 1B). Voltage was acquired in current-clamp mode on an Axopatch-1D amplifier at 10kHz and low-pass filtered at 5kHz.

OSN recording. Flies were immobilized in the trimmed end of a plastic pipette tip, and a pair of pulled glass pipettes were used to stabilize one antenna. GFP+ antennal sensilla were identified by fluorescence microscopy using a 40X air objective. A saline-filled reference electrode was inserted into the eye, and a saline-filled sharp glass electrode (tip <1 μm) was inserted into a sensillum. The signal was acquired on a Axoclamp-2A amplifier via a HS-2 headstage at 40kHz and low-pass filtered at 20kHz. Recordings were accepted only if the two spike sizes were clearly discriminable; sudden increases in spontaneous spiking were interpreted as indicating sensillum damage and recordings were terminated if this occurred (2). Genetic ablation experiments indicate that the *Or22a*+ OSN consistently corresponds to the larger spikes (the "A neuron") in the ab3 sensillum (3). Each odor was tested on 3-5 sensilla in different flies.

Odors. Odor concentrations and delivery conformed to conditions previously used to characterize OSN tuning (2-3). Briefly, each odor was diluted 100-fold v/v in paraffin oil in a glass vial fitted with a silicone stopper. 1,4-diaminobutane, 4-methylphenol, 2,3-butanedione, and 3-methylthio-1-propanol were diluted in water. A constant airstream was directed at the fly throughout the recording. Following a trigger, a solenoid redirected 9% of the airstream through the headspace of the odor vial for 500ms. The odor stream (3.7 ml/sec) rejoined the non-odor stream (37ml/sec) 16 cm. from the end of the end of the delivery tube, which measured 3mm in diameter. The end of the delivery tube was positioned 8mm from the fly. All odor concentrations, therefore, are ~1000X dilutions, not including the additional dilution factor between the end of the odor tube and the antenna. Odor presentations (typically 5-8 trials per odor) were spaced 45-60 sec apart.

The 32 odors and 2 control stimuli, as arranged along the x-axis of the tuning curves in Fig. 3F, are: 1, 1-heptanol; 2, empty vial; 3, paraffin oil; 4, 2-octanone; 5, hexanal; 6, phenyl acetaldehyde; 7, benzaldehyde; 8, 4-propyl phenol; 9, 1-octanol; 10, 3-octanol; 11, cis-3-hexen-1-ol; 12, trans-2-hexenal; 13, 1,4-diaminobutane, 14, linalool; 15, pentyl acetate; 16, 1-octen-3-ol; 17, isoamyl acetate; 18, ethyl butyrate; 19, 2-heptanone; 20, 1-hexanol; 21, cyclohexanone; 22, geranyl acetate; 23, γ-valerolactone; 24, 3-methylthio-1-propanol; 25, methyl salicylate; 26, cyclohexanol; 27, 4-methyl phenol; 28, octanal; 29, butyric acid; 30, 1-butanol; 31, pyrrolidine; 32, propionic acid; 33, L-carvone; 34, 2,3-butanedione. Heptanal was included in the stimlus set in most PN recordings (Fig. 1-2) for a total of 33 odors and 2 control stimuli.

Immunocytochemistry. Brains were fixed for 20 min. in 4% paraformaldehyde in PB, rinsed in PB, permeabilized in PBST (0.2% Triton-X in PBS) for 20 min, and blocked in 5% goat serum for 20 min. Brains were incubated in 1:10 mouse nc82 antibody (gift of E. Buchner and A. Hofbauer) for 24 hrs. at 4°C, and then washed 30 min. in several changes of PBST before incubation with 1:1000 goat anti-mouse:Alexa Fluor 568 and 1:1000 streptavidin:Alexa Fluor 488 for 24 hrs. at 4°C. After washing 20 min. in PBST, brains were mounted in Vectashield on a slide flanked by two #1 coverslips to prevent flattening. Confocal fluorescence microscopy was performed on a LSM 510 using a 63X oil-immersion objective to acquire 0.7-μm slices through the antennal lobes. Wider-field images (Fig. 1A) were acquired using a 40X oil-immersion objective in 1-μm slices. In

triple-labeling experiments (Fig. 3), the first incubation contained 1:10 nc82 and 1:25 rat anti-mouse CD8a, and the second incubation contained 1:250 goat anti-mouse:Alexa Fluor 633, 1:250 goat anti-rat:Alexa Fluor 488, and 1:1000 streptavidin:Alexa Fluor 568. Glomeruli were identified by comparing the nc82 signal to a published atlas (4).

Data analysis. All means are reported ± SEM. Spike times were extracted from raw voltage traces using custom software written in IgorPro, using an automated routine to detect minima in the second differential of the membrane potential. Spontanous spiking rate (mean: 5 ± 0.7 Hz) varied widely across PNs (range: 0-17 Hz), perhaps reflecting variations in the number of OSNs expressing each olfactory receptor type (2-50 OSNs per receptor per antenna, depending on receptor type, (5)), and the heterogenous distribution of OSN firing rates (1-32 Hz, again depending on receptor type, (2)). Therefore, the threshold for an excitatory response is best defined with reference to the spontaneous firing statistics for the PN in question. Each block of odor presentations consisted of 5-8 trials. We divided each trial into 100ms bins. The mean and SD of the baseline firing rate was calculated across all baseline bins for that block of trials (7 sec. of baseline × 10 bins/sec × 5-8 trials). A PN was defined as responding if, in any bin, the mean PSTH exceeded 2 SDs above the mean baseline. This threshold is conservative: visual inspection of rasters suggests that some responses may be missed by this definition, and only 3% of blocks contained a "false response" during the baseline period.

We have also described responses using a higher threshold, defined as 50Hz above the mean baseline rate (Fig. 1E). 50Hz was generally greater than 4 SDs above the mean baseline rate. A 100-ms bin size was also used to construct the PSTHs and tuning curves in Fig. 3. Because the shortest-latency OSN responses were detected about 130 ms after odor onset, we always shifted our 100ms bins so that a bin began at 30ms after the trigger, 130ms after the trigger, etc.

Inhibitory epochs were detected by converting spike times into interspike intervals, concatenating all the trials in a block, and re-sorting the ISIs by time relative to the stimulus. A custom IgorPro algorithm then searched for a window (0.5-4 sec wide) after stimulus onset when ISIs were significantly greater ($p < 0.01$, two-tailed t-test) than spontaneous ISIs during the baseline period. A "false" inhibitory response was detected during the baseline period in only 4% of odor blocks using this threshold.

Empty vial elicited an excitatory response above the 2 SDs threshold in 33% of PNs, suggesting that PNs receive mechanosensory input. Paraffin oil generally elicited the same response (or non-response) as empty vial (both controls tested sequentially in n=7 PNs). (We detected no response to empty vial or paraffin oil in the two OSNs we have characterized (Fig. 3E-F).) A PN or LN was counted as responding to an odor only if its response was substantially and reliably larger than the response to empty vial or paraffin oil.

We quantified tuning curve shape by quantifying the lifetime sparseness (S) of the DM2 PN and OSN tuning curves over the first 500ms of the response:

$$S = (1-\{[\Sigma^{N}_{j=1}\, r_j/N]^2 / \Sigma^{N}_{j=1}\, [r_j^2/N]\})/(1-1/N)$$

where N=number of stimuli, and r_j is the response of the neuron to stimulus j. Analog response intensities for a given cell-odor pair were computed taking the mean PSTH for that odor (using 100ms bins), subtracting the mean baseline rate, and setting all negative-

valued bins equal to zero. S varies between 0 and 1, where 0 describes a flat tuning curve and 1 corresponds to just one non-zero response.

EPSP analysis (Fig. 4K) was performed for the 5 PNs which had (1) spontaneous EPSP frequencies <40Hz, (2) at least one block of trials displaying a robust inhibitory epoch, and (3) good signal-to-noise ratio (R_{access}<30 MΩ).

1. E. B. Lewis, *Drosophila Information Service* **34**, 117 (1960).
2. M. de Bruyne, K. Foster, J. R. Carlson, *Neuron* **30**, 537 (2001).
3. A. A. Dobritsa, W. van der Goes van Naters, C. G. Warr, R. A. Steinbrecht, J. R. Carlson, *Neuron* **37**, 827 (2003).
4. P. P. Laissue *et al.*, *J Comp Neurol* **405**, 543 (1999).
5. L. B. Vosshall, A. M. Wong, R. Axel, *Cell* **102**, 147 (2000).

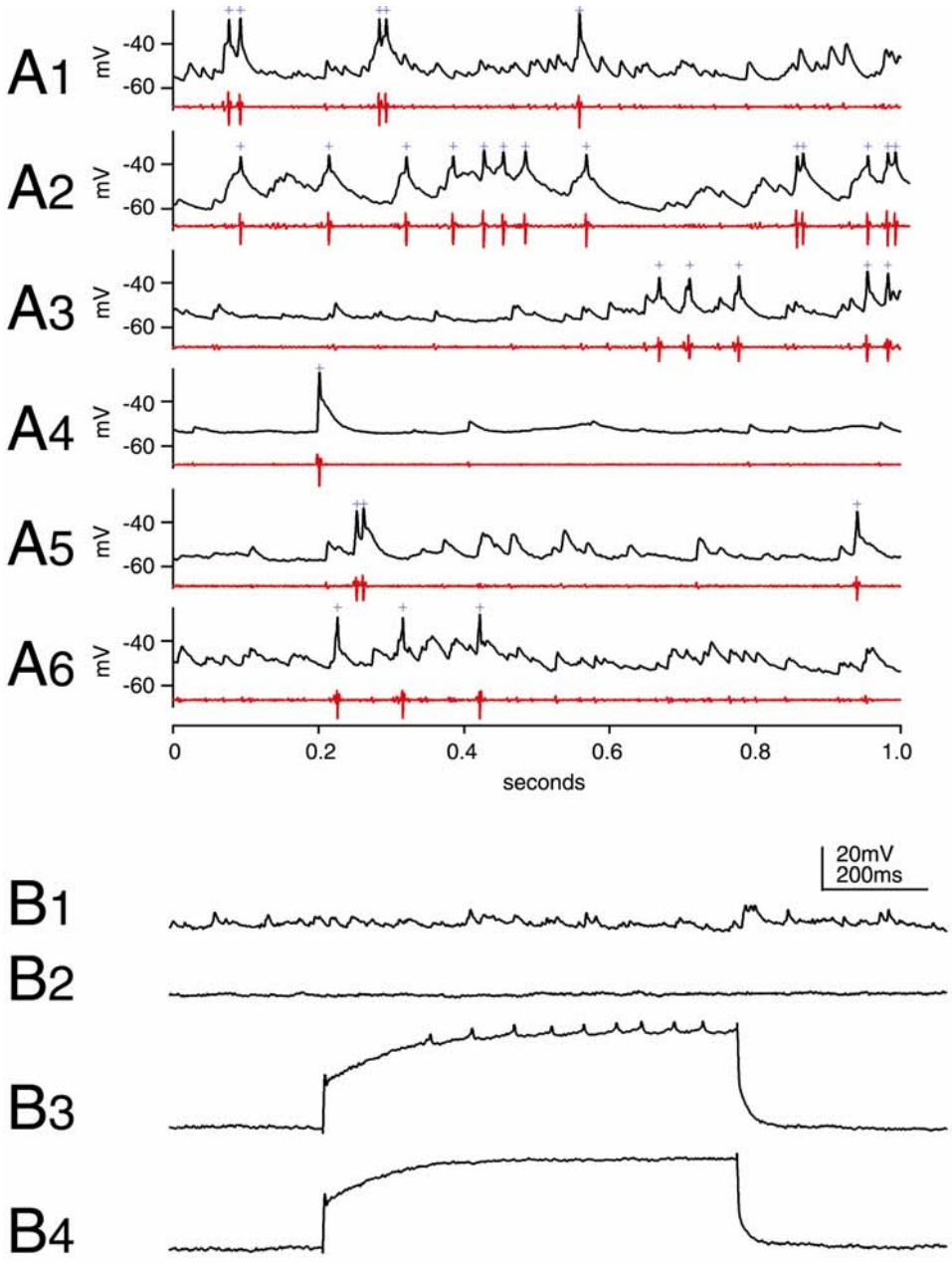

Fig. S1. (A1-A6) Spontaneous activity in six different PNs. Note fast EPSPs and small-amplitude spikes. Spikes (blue crosses) were detected by an automated program as minima in the second differential of the membrane potential (shown below each trace in red). Recordings were only accepted for analysis if the spikes were easily discriminable from EPSPs. (B1) Spontaneous EPSPs in another PN. (B2) A nicotinic acetylcholine receptor antagonist (250 μM mecamylamine) blocks EPSPs. Fast IPSPs were not observed in PNs in the presence of mecamylamine, suggesting that either PNs do not receive direct $GABA_A$-type inputs, or else these are too electrotonically distant to be detectable in somatic recordings. (B3) Depolarizing current injection to the soma can still elicit spikes in the presence of mecamylamine. (B4) TTX (1 μM) blocks depolarization-evoked spikes.

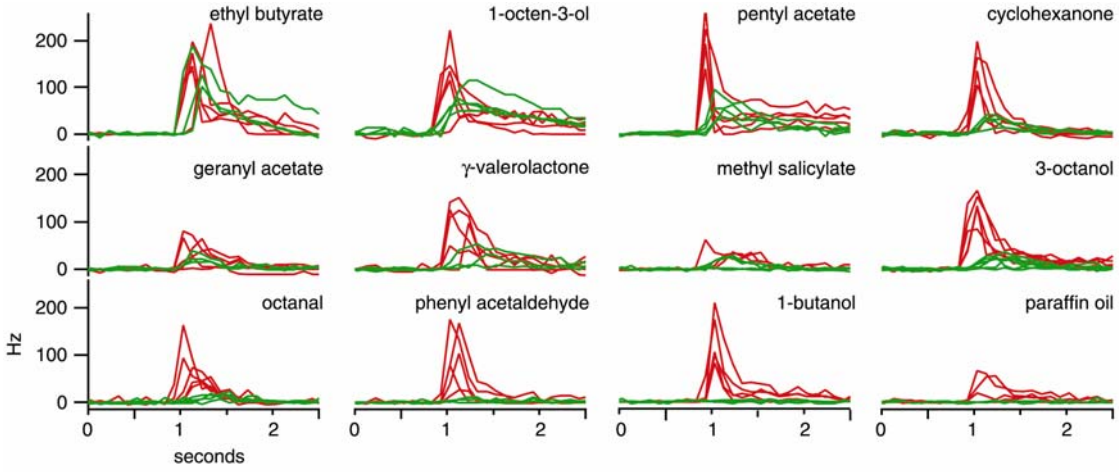

Fig. S2. Individual PSTHs show the responses of all *Or22a+* OSNs (3-5 OSNs per odor) and DM2 PNs (3-5 PNs per odor) tested with the twelve stimuli shown in Fig. 3E. All panels use the same axes. Odor: from 0.5 to 1.0 sec. In many cases, there was little variability in the PSTH among cells of the same type, compared to the difference in the means of the two cell types. This implies that the differences in the OSN and PN tuning curves cannot be explained by statistical noise.

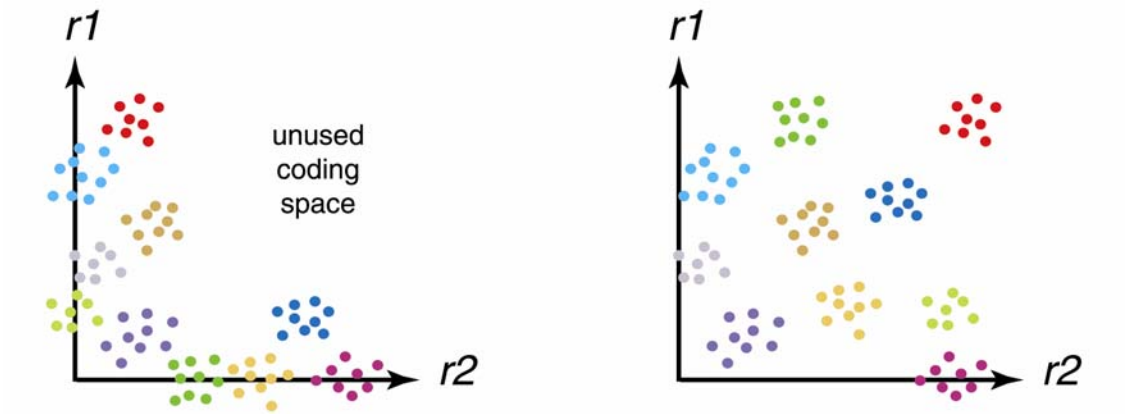

Fig. S3. Consider an olfactory system with only two glomeruli (or input channels) and two PNs. Imagine first that these two PNs have sharp (and different) tuning curves mirroring their respective OSN preferences. Represent the responses of these PNs along two axes (*r1* and *r2*). Because the tuning of each PN is sharp and because the two PNs are tuned to different odors, every time one PN responds vigorously to one odor, the other will likely be inactive (and vice versa): odor representations, plotted on the *r1r2* plane, will hover in the neighborhood of the two axes. In other words, most of the space available for coding will remain unused. If, by contrast, PN tuning curves become less sharp and reflect input from both channels, odor representations will move to occupy the entire plane or coding space. Odor stimuli and neurons are noisy, and so to be recognizable, each odor representation should be allowed to vary in a "sphere of noise" without risking interference with others. Under such conditions, it might be advantageous for the brain to separate odor representations in coding space by distributing representations across many broadly tuned PNs.