Supplementary Methods and Results

Supplementary Methods

Surgical procedures and animal care. In the initial surgical procedure, we implanted a 22 ga. hollow guide cannula (C313G, Plastics One, Roanoke, VA) for intranasal pressure measurements and a stainless steel head-bolt (10-32 x 24 mm) for restraint. Rats were anesthetized with isoflurane (1.5 -3%) and body temperature maintained at 37 °C throughout the procedure. The intranasal cannula extended 2.4 mm from the base of its threading and was lowered into the dorsal recess (frontal-nasal fissure: 0 mm AP, ML: 0.9 mm, angle toward midline: 4°) and secured to the skull with ethyl 2-cyanoacrylate glue (Advanced Formula Krazy Glue, Elmer's Products, Inc.), 4 small skull screws (#000 x 3/32”) and dental cement. The restraint head-bolt was positioned on top of the skull and secured with dental cement anchored by six skull screws (0-80 x 1/8”). Wound margins were treated with local anesthetic (1 mg/ml bupivicaine, Sigma-Aldrich, St. Louis, MO). Buprenex (10 µg/kg IM, Reckitt Benckiser Healthcare, Richmond, VA) was also provided as an analgesic. Following surgery, animals were supplied with ad libitum water containing Ibuprofen (15 mg/kg) for 3 days, and given antibiotic (Baytril, 3 mg/kg SC). Animals were carefully monitored throughout their surgical recovery. Animals were singly-housed and kept on a 12:12 hr (light:dark) schedule, with all behavioral training and testing occurring during the light phase of the cycle. Food was available ad libitum except during testing. Rats began water deprivation at least 1 week post surgery, and 4 days prior to behavioral training. For water deprivation, rats were maintained at 10-15% below baseline bodyweight. During testing and training sessions (see below), rats received approximately 3 – 5 ml water; following which they were given 30 mins ad libitum water access in their home cage.
Rat ORNs were loaded with calcium-sensitive dye 3 – 4 days prior to imaging using a procedure modified slightly from that already described in mice\(^1\). Rats were anesthetized with ketamine/xyloazine (56mg/kg, resp. 6 mg/kg, i.p.) and placed on their back with the dorsum of the nose angled at 60° from horizontal, and an infusion canula was inserted 17 mm into one naris. 25 – 40 µl of a solution containing the dye (3% w/v) and 0.2% Triton X-100 (ICN Biomedicals Inc., Aurora, OH) was then infused into the naris and left for 10 min. The animal was then placed on its front for five minutes before repeating the procedure for the other naris.

One day prior to imaging, the chronic optical window was installed over the dorsal surface of each olfactory bulb under isoflurane anesthesia by thinning the overlying bone to ~100 µm thickness and coating the thinned bone with ethyl 2-cyanoacrylate glue to preserve optical clarity. This procedure required only thinning of previously-exposed bone, and so was considered noninvasive. Care was taken not to pierce the bone or underlying dura, and hemorrhaging was prevented by applying minimal pressure, positioning the head above the trunk, and injecting dexamethasone (2 mg/kg i.p.).

**Behavior.** The behavioral apparatus (Supplementary Fig. 1) consisted of a clear acrylic restraint chamber (9” long x 3.25” wide x 2.5” tall)\(^2\) placed inside a larger isolation chamber made of black ABS (18” long x 12” wide x 13.5” tall). A steel tab with a through hole extended horizontally from the roof of the restraint chamber, allowing quick restraint of the animal by its head bolt. A cross-shaped profile on top of the head-cap fitted tightly with its inverse profile in the tab, preventing head movement and allowing repeatable positioning of the animal in different sessions. A lick-spout for water delivery was attached to a post
extending from the top of the restraint box and was positioned in front of the animal’s mouth at the start of each session. The outer isolation chamber was designed to reduce extraneous light and noise. An exhaust fan (12V, 3” diameter) on the wall of the chamber directly behind the animal aided in venting odorants away from the animal after presentation. Small holes in the roof, side and rear walls allowed access for the odorant delivery tube (side wall, facing animal), a video camera (rear wall, to animal’s left), and the microscope objective (roof). Both the inner and outer chambers were cleaned with 95% ethanol between each behavioral session. Licking was recorded with a contact lickometer circuit (env-250, Med Associates Inc., St. Albans, VT) connected to a thin steel plate on the floor of the restraint chamber for paw contact, and to a small stainless steel tube contained in the lick spout assembly. The lick tube was encased in Teflon so as to only record contact due to licking.

After the initial habituation to head fixation, rats were restrained daily for a single session lasting 30– 60 mins (51 – 142 trials). During behavioral training, rats were rewarded with approximately 40 µl water for correct licks to the CS+ . Rats were rewarded when they licked during or up to 2 sec after the presentation of the CS+ (hit). False alarms (licks to CS-) initiated a punishment of 7 sec added to the next ITI. Rewards were not given for not licking to the CS-. Odorants were presented in pseudo-random fashion (50/50 CS+/CS-, less than 4 consecutive trials of same valence). The total number of correct responses (CS+ lick or CS-no lick) was compared to the total trials for an index of behavior performance (Supplementary Table 1). Odorants were presented in pseudo-random fashion (50/50 CS+/CS-, less than 4 consecutive trials of same valence); therefore, a performance index of 40-60% was considered chance performance and a score of 100% was perfect discriminatory behavior. Signs of distress (vocalizations, defecation, failure to
perform the task) resulted in cessation of the session and release of the animal from restraint.

**Olfactometer.** The olfactometer was made of glass odorant vials, stainless steel manifolds, and Teflon tubing and connectors. Odorants were diluted from saturated vapor of pure liquid odorant using mass flow controllers (1-10 ml/min; Aalborg). Nitrogen was used as the saturated vapor carrier to prevent odorant oxidation and diluted in a stream of filtered medical grade air flowing at 300 – 500 ml/min. Linearity and stability of the olfactometer were verified with a photoionization detector (MiniRae 2000, RAE Systems Inc., San Jose, CA). A dual, concentric odorant delivery tube (inner tube 20 mm shorter than outer tube), positioned ~ 6 mm from the animal’s nose, assured rapid onset and offset of the odorant. Odorant duration was 4 or 5.5 sec.

**Sniff measurements.** Sniffing was continuously recorded throughout all behavioral sessions by connecting the guide cannula implanted in the dorsal recess to a pressure sensor (24PCAFA6G, Honeywell, Morristown, NJ) via PE tubing (0.45 m x 0.1 mm ID x 0.15 mm OD). The response time-constant of the pressure sensor was < 1 msec; sensor output was amplified 100x, low-pass filtered at 100 Hz, then digitized at 500 Hz and saved to disk. Sniff signals obtained during imaged trials were digitized at 100 Hz, synchronized with the optical signals and saved to disk using Neuroplex software (RedShirtImaging). In two rats, a thermocouple (emtss-010g-12; Omega Engineering Inc., Stamford, CT) was inserted into the guide cannula to allow for simultaneous measurement of intranasal pressure and airflow (**Supplementary Results** and **Supplementary Fig. 2**).

**Data analysis.** Initial data handling. Offline movement correction was performed by
registering all frames in a trial with a cross-correlation based algorithm. A reference frame was constructed by averaging the first 5 frames. Each subsequent frame was high-pass filtered (subtracted with morphologically opened frame, disk radius = 10 pixels) and bilinearly interpolated to 1024 x 1024 pixels. If the peak of its spatial cross-correlation with the reference frame was different from the origin, the original frame was interpolated to 2048 x 2048 pixels and the peak of its cross-correlation with the reference was used to shift the unfiltered frame to match the reference, then down-sampled to 256 x 256 pixels.

Overall, movement artifacts were minimal (see Supplementary Results and Supplementary Movie). To reduce signal from widespread fluorescence changes – for example from intrinsic signals - high-pass spatial filtering was performed in frequency space using a cutoff frequency equal to the frame size/1.28 (corresponding to 2.6 mm⁻¹), at the cost of a ~20% reduction in focal response amplitudes (Supplementary Fig. 4).

Processed files were stored in their original file format (Neuroplex, RedShirtImaging) for subsequent analyses and data display.

Regions of interest (ROIs). ROIs to be used for subsequent analysis were selected by visual inspection of ‘sniff-triggered’ average response maps, constructed by extracting forty frames around the time of each sniff during odorant presentation and averaging over all sniffs and all trials for a given odorant. The minimum inter-sniff interval (ISI) was 280 msec. Sniffs were automatically detected by band-pass filtering the sniff signal (0.1 – 20 Hz) and detecting increases > 0.5 standard deviations above the mean.

Optical signals and sniff signals were averaged with no additional filtering and were then stored in Neuroplex file format. Optical signals averaged across each ROI were extracted from each data file and saved, along with the sniff trace, lick trace and additional relevant information, into a custom file format that allowed for easy visual inspection and automated
analysis of individual trials using custom software written in Matlab (Mathworks). ROIs used for analysis consisted of $72 \pm 35$ pixels (representative sample of 137 ROIs from 6 rats), having a mean area of $12359 \pm 6027 \ \text{m}^2$, which is equivalent to a circle with diameter $122 \pm 30 \ \text{m}$. Thus, ROI size was on average slightly smaller than the half-width of the signal foci (see Results).

Selecting ROIs from sniff-triggered average maps could yield ROIs that failed to show responses with an adequate signal-to-noise ratio (SNR) in single trials. We thus characterized the SNR for all ROIs on a trial-by-trial basis, then calculated the average SNR across all trials of a particular glomerulus-odorant pair. SNR for one trial was defined as the mean response amplitude across all odorant responses in that trial, divided by the standard deviation of sniff-evoked signals measured for all pre-odor sniffs and across all trials.

*Temporal deconvolution of calcium signals.* Temporal patterns of ORN action potential firing were estimated from presynaptic calcium signals by temporal deconvolution. Temporal deconvolution uses the calcium signal evoked by a known action potential pattern (typically a single action potential) to generate a filter that is then deconvolved with the calcium signal measured in response to an unknown pattern of action potentials. The result of this deconvolution (in the ideal case) is the original action potential pattern. We imaged responses to single, brief electrical shocks (0.1 msec; 400 – 600 A) delivered to the olfactory nerve layer by a small concentric bipolar electrode (OP: 125 m SS, IP: 25 m Pt/Ir; Frederick Haer &Co., Bowdoinham, ME) in three urethane-anesthetized rats. Responses were imaged at 50 or125 Hz and 128x128 pixel resolution. Electrical stimulation evoked signals in multiple glomeruli. The decay of the shock-evoked signal was well-fit by a single-exponential function with time-constant of $259 \pm 62$ msec (mean $\pm$ sd, n
= 15 glomeruli, 3 rats), a value identical, although more variable, to that seen in slices of the mouse OB (241 ± 21 msec). Signals evoked by a train of shocks (5 pulses, 100 Hz) showed higher amplitudes but similar decay rates (268 ± 60 msec; n = 3 glomeruli, 3 rats). The measurement of sniff-evoked transient half-width (see Results) was robust to changes in the decay time-constant used, ranging from 143 ± 19 ms to 154 ± 29 ms when deconvolved using a time constant of ± 1 s.d. from the mean). Because the deconvolution procedure is sensitive to high-frequency noise, calcium signals were low-pass filtered (4th-order Butterworth) at 15 Hz prior to deconvolution.

Comparison of response maps. For the test/background odor experiments, response maps to single odorants during low-frequency sniffing were made by averaging the maps evoked by the first sniff during odorant presentation in 4 – 5 trials. In these experiments the test odorant was ethyl butyrate or 2-hexanone; the background odorants were 2-butanone, 2-heptanone, 2-octanone, benzaldehyde, valeric acid, 3-methyl butyraldehyde, or butyraldehyde. Response maps to the binary mixture and the test/background response were constructed from single trials.

Display and averaging of optical signal traces. For some ROIs, fluctuations in baseline of the optical signal remained after initial image processing due to more localized intrinsic signals associated with blood vessels (see Supplementary Fig. 4 and Supplementary Results). For display in the Figures (Fig. 3b, Fig. 6 and Fig. 7a), we corrected for the local intrinsic signal by selecting a ROI from one or more non-responding areas adjacent to each glomerulus. For each trial, the ‘surround’ ROI trace was low-pass filtered at 3 Hz then subtracted from the trace of its corresponding glomerulus (Supplementary Fig. 4). Local signal correction was also performed for each trace used in the averaged-trace analysis shown in Fig. 5A and Supplementary Fig. 5.
**Sniff-evoked response measurements.** Peak sniff-evoked response amplitude was calculated automatically. First, all sniffs in a trial were automatically detected. Next, the fluorescence change or deconvolved signal evoked by each sniff was calculated as the difference between the mean signal in the three frames (120 msec) after and including the peak of the sniff and the maximum signal in the subsequent 7 frames (280 msec). Response maximum, rather than mean, was used because a large temporal window was necessary to capture response peaks due to latency differences across glomeruli. This method risked assigning amplitudes to the wrong sniff if the latency from sniff onset to response peak plus the ISI was less than 400 msec. Given an average latency of 277 msec (response latency plus time-to-peak; see Text), this corresponded to an inter-sniff interval of 123 msec (8.1 Hz). Such sniff frequencies were very rarely observed (Fig. 1c).

**Response amplitude vs. inter-sniff interval (ISI).** For analysis of response amplitude versus ISI (Fig. 5d), we conservatively omitted all measurements at ISI < 200 msec (5 Hz). Peak response amplitudes were normalized and placed in 50 msec time-bins according to the ISI. Fitting of response amplitude versus ISI was performed on the mean within each ISI time-bin rather than on unbinned data because of the nonuniform distribution of sniffs at different ISIs. The means of each bin were fit to a simple exponential using a modified maximum likelihood method that relied on s.e.m. rather than s.d. for error minimization.