

## **SUPPLEMENTARY METHODS**

### **Behavioral training and testing**

Rats were trained and tested on a two-alternative odor discrimination task. The behavioral apparatus consisted of a 30 cm cubic box containing three conical nose pokes (38 mm inner diameter, 38 mm depth). The odor port was located at the middle of one wall at a height of 32 mm from the floor to center. Two choice ports were located 57 mm left and right of the odor port (center-to-center). An infrared photodiode/phototransistor pair placed on either side of the nose poke at 15 mm depth from the surface was used to determine the timing of nose pokes.

The animals were maintained on a reverse 12 hr light/dark cycle and tested during their dark period. They were allowed free access to food but were restricted to water available during the behavioral session and for 30 minutes after the session. Animals were first habituated to the behavioral apparatus and experimenter (one week) and then trained to perform the two-alternative odor mixture discrimination task using the following sequence of steps:

- (1) Nose pokes at one of two choice ports were rewarded by water delivered at that port.
- (2) A nose poke at the odor sampling port (which triggered the delivery of one odor) was required before water was available at the choice port. The required duration for the nose poke was gradually increased up to 0.3 s.
- (3) The first choice port was covered and the second choice port and a second odor were introduced and reinforced as above.
- (4) Both ports were available and delivery of two odors were randomly interleaved and rats were rewarded only for correct choices.

Following training to criterion (90% accuracy) on a pure odor pair (total of 10-12 sessions over two weeks for the initial training including shaping, and  $4.00 \pm 0.87$  sessions when switched to new odor pairs), binary odor mixtures in six different proportions (80/20, 68/32, 56/44, 44/56, 32/68, 20/80) were gradually introduced (over five to seven sessions) and rats were rewarded for choosing the dominant component. The mixture ratios were produced by differential air flow dilution using a pair of mass flow controllers (see below). After reaching criterion (90% accuracy in pure odor discrimination) in a session with eight different odor mixture ratios, data were collected for the analysis.

For the main data set, each of four rats was tested on six odor pairs with eight mixture ratios (100/0, 80/20, 68/32, 56/44, 44/56, 32/68, 20/80, 0/100). Throughout testing, rats were always rewarded for correct choices as during the training procedure. Complementary mixture ratios (0/100 and 100/0, 80/20 and 20/80, etc.) were pooled together for the analysis. The main data set therefore consisted of 96 experimental conditions (4 rats x 6 odor pairs x 4 complementary mixture ratios). Each of four rats was tested in one session (~250 trials) per day and five to ten sessions per condition, yielding a total of 33,948 trials.

### **Odor delivery**

Odorants used and their abbreviations are as follows: butyric acid (C4-COOH), valeric acid (C5-COOH), caproic acid (C6-COOH), hexanol (C6-OH), heptanol (C7-OH), S(+)-2-octanol (S(+)-2-oct) and R(-)-2-octanol (R(-)-2-oct). Aliphatic acids were diluted in mineral oil in the proportions 1:100 (C4-COOH) and 1:10 (C5-COOH and C6-COOH).

A custom olfactometer was constructed of Teflon tubing (Cole-Parmer), fittings (Omnifit) and solenoid valves (Neptune Research) (**Supplementary Fig. 3**). Saturated odor vapor was produced by flowing clean (carbon-filtered) air through disposable syringe filters (glass microfiber, 2.7  $\mu\text{m}$  pore size, #6823-1327 Whatman) which were loaded with 30  $\mu\text{l}$  liquid odorant. Filters were inserted into a Teflon or polyetheretherketone (PEEK) manifold downstream of all valves and controllers. Odor streams were mixed at the manifold directly before the odor sampling port with a clean air stream (“carrier”) to produce a total flow rate of 1,000 ml/min. The flow rates of odorant and carrier streams were regulated using computer-controlled mass flow controllers (Aalborg). By varying the flow rate of odorant streams between 5 and 50 ml/min, variable air flow dilution from 0.5 to 5% was produced. Two independent odorant flow controllers allowed us produce various mixture ratios. Flow rates were set so that the combined flow rate of the two odorant streams was 50 ml/min. Filters were replaced and the manifold cleaned and sonicated daily.

The performance of the olfactometer was tested for vapor saturation, linearity of air flow dilution, and concentration precision using a gas chromatograph (HP 6890) fitted with a flame ionization detector (FID). Odor concentration achieved using the mass flow controller was linear with command voltage over the range of 10-100 ml/min and was repeatable with a coefficient of variation of <1%. We periodically verified that rats used the delivered odor cues by testing on pairs of identical odors. In all tests performance levels dropped to chance levels. Continued reinforcement over up to three consecutive sessions (~1000 trials) in no cases yielded discrimination.

To test that the air-flow dilution method did not contribute substantial variability in flow rates (which could compromise mixture ratios) or extraneous cues, we compared results using this method to a liquid mixture method in which a constant stream of air

through odorants mixed together as liquids at different ratios. To avoid potential problems with differential volatility of the two odorants in the mixture, this comparison was done using the stereoisomers S(+)- and R(-)-2-octanol. Accuracy and odor sampling times obtained using the two methods did not differ.

The arrival of stimuli at the nose was delayed due to latency in valve opening and flow of air from the valve to the sampling port. This latency was measured by recording the odor-evoked electroolfactogram (EOG) in four urethane-anesthetized, freely-breathing rats. To record the EOG, a tungsten microelectrode (0.5 M $\Omega$ ) was inserted in a dorsal hole 1-2 mm in anterior to the naso-frontal suture and 1-2 mm lateral from the midline. The EOG onset was detected as the peak of the second derivative of voltage, corresponding to the inflection on the rising phase. This procedure yielded a delay to onset of  $172 \pm 16$  ms ( $n = 4$ ). Because the EOG onset may be delayed relative to odor reaching the epithelium, we subtracted a latency of 32 ms (ref. <sup>1</sup>) to obtain a more conservative estimate, 140 ms, which was used for all calculations.

### **Behavioral data analysis**

Psychometric functions were fit using the logistic function:  $\ln(p/(1-p)) = b_0 + b_1x$ , where the parameters  $b_0$  and  $b_1$  give the bias and slope of the function. Fitting was performed using a maximum likelihood procedure (GLMFIT, MATLAB).

To compute the conditional accuracy function<sup>2</sup>, trials were partitioned into windows according to reaction time and the percentage of correct trials plotted as a function of the midpoint of each window. For this analysis we used a 100 ms sliding window. We quantified the rate of rise of the conditional accuracy in three ways. First, we

...calculated the time to reach asymptote at the midpoint of the first time window in

which accuracy exceeded 95% of the maximum accuracy in any time window ( $T_{95}$ ). This was done using a sliding window of 100 ms as described above. Second, we estimated the time constant,  $\tau$ , of the rising phase by fitting the equation  $A(t) = 1 - e^{-t/\tau}$ . This method did not require windowing or binning. Third, we estimated the time at which the positive correlation between odor sampling time and performance ceased by calculating Spearman's rank correlation coefficient over windows with different starting times. All three analyses again yielded similar results.

The correlation between odor sampling time and performance was calculated on the conditional accuracy function<sup>2</sup> for each of 96 conditions (4 rats x 6 odor pairs x 4 mixture ratios). The time range for this analysis was from 250 ms (after the apparent asymptote of the conditional accuracy function) to 650 ms (discarding longer trials near the tail of the distribution to avoid possible contamination by trials in which the behavior was interrupted). The results were not sensitive to the choice of time window.

### **Measurement of respiration**

Three rats were tested while measuring nasal respiration patterns. After initial training, a temperature sensor (0.005" Teflon-coated thermocouple, Omega) was implanted in one nostril through a hole made in the dorsal skull overlying the nostril and secured with dental cement. After recovery (one week), the rats were tested on the odor mixture discrimination task while voltage signals from a thermocouple were recorded through an amplifier. Timing of onset of inhalation and exhalation were determined as the local maxima (exhalation onset) and minima (inhalation onset) of the temperature signals. There is some ambiguity in how to count partial sniffs. We analyzed this in two ways. First, we considered the onset time of the inhalation and

counted partial inhalations including the onset as a full sniff. Second, we excluded all trials with partial inhalations. These two methods yielded similar results.

### **Intrinsic signal imaging**

Six Long Evans rats (200 – 250 g) were used for imaging experiments. Animals were deeply anesthetized with medetomidine (0.5 mg/kg, IP), fentanyl (0.01 mg/kg, IP) and diazepam (5 mg/kg, IM) and the bone and dura covering the dorsal surface of one olfactory bulb were removed. A well of dental cement was constructed, filled with agarose gel, and sealed with a glass cover slip to reduce brain movement. Anesthesia was maintained by periodic dosage with medetomidine (0.25 mg/kg/hr, IP). Light reflectance from the olfactory bulb (~630 nm wavelength illumination from light emitting diodes) was captured using an analog CCD camera (TELI), frame grabber (Matrox Pulsar) and custom acquisition software written using MATLAB (The Mathworks, Inc.).

Intrinsic signals evoked by odorants used in behavioral experiments were imaged using the same concentration and flow rate as those used in behavioral experiments. Odors were delivered using a custom-made olfactometer of the same design as for behavioral experiments (see below). Odorants were presented for 5 s duration and randomly interleaved at intervals of 60-90 s. Baseline ( $R_0$ ) and response ( $R_1$ ) images were obtained by averaging frames before (-5 to 0 s) and after (5 to 10 s) stimulation and relative change in reflectance,  $(R_1 - R_0)/R_0$ , was used to obtain an activation map.

High-pass spatial filtering was used to separate glomerular punctate signals from diffuse component<sup>3</sup> (**Supplementary Fig. 4**). We localized glomeruli by using a pixel-by-pixel statistical test (Kolmogorov-Smirnov) to identify areas of the bulb whose reflectance decreased significantly in response to at least one of the odors

tested compared to control (air) stimulation. The amplitude of glomerular signals was calculated by averaging the signal amplitude of pixels in circular regions of interest (50-150  $\mu\text{m}$  diameter) placed over the center of each spot of activation (**Supplementary Fig. 4b**).

Similarity of the glomerular activity patterns was quantified as follows using MATLAB Statistics Toolbox. An array of signal intensity values across identified glomeruli was extracted for each odor and treated as a vector. The similarity of a pair of odorants was calculated as  $1 - \cos(\alpha)$ , where  $\alpha$  is the angle between two glomerular activity vectors. We used this metric because it is insensitive to the overall intensity of glomerular signals and yields values normalized to between 0 (most similar) and 1 (least similar). Euclidian distance and standardized Euclidian distance produced similar results (**Supplementary Fig. 5**). The results were also insensitive to the way we selected the glomeruli and to the use of high-pass filtering. To visualize the relationships between multiple odors, a hierarchical clustering algorithm was applied and the results were visualized using a dendrogram. The clustering results were insensitive to linkage method.

#### **References:**

1. Mackay-Sim, A. & Kesteven, S. Topographic patterns of responsiveness to odorants in the rat olfactory epithelium. *J Neurophysiol* **71**, 150-60 (1994).
2. Luce, R. D. *Response Times: Their Role in Inferring Elementary Mental Organization* (Oxford Univ Press, New York, 1986).
3. Meister, M. & Bonhoeffer, T. Tuning and topography in an odor map on the rat olfactory bulb. *J Neurosci* **21**, 1351-60 (2001).