

Glomerular activation patterns and the perception of odor mixtures

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Abstract

Odor mixtures can produce several qualitatively different percepts; it is not known at which stage of processing these are determined. We asked if activity within the first stage of olfactory processing, the glomerular layer of the olfactory bulb, predicts odor mixture perception. We characterized how mice respond to components after training to five different mixture ratios of pentanal and hexanal, and found two types of responses: elemental perception and overshadowing. We then used intrinsic signal imaging to observe glomerular activity in response to the same mixtures and their components. As has been previously described, glomerular activity patterns produced by mixtures resemble the linear combination of responses to components. Mice trained to identify mixtures with more hexanal than pentanal recognized hexanal but not pentanal when the odorants were presented alone (overshadowing). Consistent with these behavioral responses, the imaged activity pattern in response to mixtures was similar to that produced to hexanal alone. Moreover, there was no significant effect of glomerular inhibition in the imaged response. In contrast, the glomerular activity patterns did not predict elemental perception: when trained to identify mixtures with more pentanal than hexanal, mice recognized both components equally well, even with highly overlapping activation patterns. This suggests that spatial activity patterns within the olfactory bulb are not always sufficient to specify component recognition in mixtures.

Introduction

It has been suggested that activity patterns within the mammalian olfactory bulb can explain similarities in the perception of some monomolecular odorants (Johnson *et al.*, 1999; Linster & Hasselmo, 1999; Rubin & Katz, 1999; Johnson & Leon, 2000a,b; Linster *et al.*, 2001). These glomerular activation patterns have been observed with a variety of imaging strategies and appear to be relatively stable across individuals. Behavioral studies show that odorants with similar chemical structures, and therefore overlapping glomerular activation patterns, smell more alike than do odorants of very different chemical structure (Laska & Teubner, 1999; Laska *et al.*, 1999; Linster & Hasselmo, 1999; Linster *et al.*, 2001; Cleland *et al.*, 2002).

It is not known, however, whether glomerular activity patterns also explain how odor mixtures are perceived. Previous studies suggest that mixtures of odorants that either smell alike or have the same functional group often produce configural or synthetic percepts, in which the mixture does not smell like the components. In contrast, mixtures of odorants that do not smell alike or that have very different chemical structures often produce elemental percepts, in which the mixture smells like the components (Kay *et al.*, 2003; Wiltrout *et al.*, 2003). These behavioral results suggest that mixtures in which the components activate overlapping glomeruli produce configural percepts, while mixtures in which the components activate separate populations

of glomeruli allow the individual components to be perceived (elemental; Bell *et al.*, 1987; Laing *et al.*, 1989; Kay *et al.*, 2003; Wiltrout *et al.*, 2003).

Despite the simplicity of the glomerular overlap hypothesis of mixture perception, its predictions have not been tested. We do not know what behavioral responses are produced by mixtures with little or large overlap in the glomerular representation of their components. We also do not yet know whether overshadowing, where the mixture smells like only one of the components, results from more intense representation by one component or through blocking or antagonism of the other component. We address these questions with parallel behavioral and imaging experiments in C57/BL6 mice using an odorant pair that at different concentration ratios produces different types of perception.

We use intrinsic signal imaging to follow the activity of individual glomeruli in response to pentanal, hexanal and five mixtures of the two odorants. We found that perceptual behavior followed glomerular activation patterns for several, but not all, mixtures. Surprisingly, mixtures of odorants that had substantial overlap in their glomerular activity patterns produced overshadowing but not configural perception. We also found that overshadowing occurs in the absence of antagonism between odorants, such that overshadowing mixtures produced glomerular activity patterns that appeared to be linear combinations of the patterns produced by the individual components. Finally, we found elemental perception even with substantial overlap of glomerular activity patterns, suggesting that input patterns are not sufficient to explain odor mixture perception.

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Materials and methods

Behavioral assay

Subjects

Twelve adult male C57/BL6 mice (obtained from NCI, Bethesda, MD, USA) were housed singly and maintained on a 12 : 12 h light : dark schedule (lights on at 08.00 h CST). They were dieted to 90% of their *ad libitum* weight by restricting food only. Testing was done in dim light in the morning to mid-afternoon. All procedures were performed with approval and oversight by the University of Chicago Institutional Animal Care and Use Committee, and conformed to AAALAC standards.

Odors

Odors were purchased from Sigma-Aldrich (valeraldehyde/pentanal 97%, hexanal 98%) and Fisher Scientific (amyl acetate 98%). All training odorants were binary mixtures of hexanal and pentanal diluted in mineral oil to five different ratios (Table 1; all concentrations are above threshold for identification in mice; unpublished results of L. Kay). The ratio set includes the value at which the binary mixture ratio is inversely proportional to the ratio of theoretical vapor pressures at 25 °C (approximately 3 : 1 hexanal to pentanal). Theoretical vapor pressures were estimated using the ACD/1-Laboratory Web service, ACD/Vapor Pressure 5.0 (Advanced Chemistry Development 2003).

Behavior

Mice were trained to dig in a round glass Petri dish filled 2/3 full of corncob bedding material scented with a drop of diluted odorant solution placed on top of the bedding in a random location and covered with a few of the bedding granules (1% methyl salicylate for initial behavioral training) for a reward of sweet cereal (~1/8 of a Froot Loop[®]), as we have reported previously for rats and mice (Nusser *et al.*, 2001; Kay *et al.*, 2003, 2005). A similar dish with only a drop of mineral oil served as a control on each trial. Both dishes were presented simultaneously (odor side randomized and balanced across trials) in a modified mouse housing cage (polycarbonate rectangular cage with a divider separating the cage into two chambers of 2/3 and 1/3 of the cage area lengthwise). The mouse was put in the smaller sub-chamber when odorant dishes were changed in between trials, and the opaque divider was lifted to initiate a trial. After training (approximately 3 weeks), the animals performed odor tests one-to-three times per week.

Each mouse was trained and tested on a particular mixture ratio on a single day, with each session consisting of 10 mixture training trials, followed immediately by test trials. On each training and test trial, both an odorant dish and a control dish (plain mineral oil) were present. The dish with mineral oil was used to ensure that the mice paid attention to the odorant and did not dig in any presented dish

(after training to the task mice rarely dig in the mineral oil control dish in this type of behavioral assay). During the 10 mixture training trials the reward was present (except for probe trials, one–two trials in which no reward was presented). In test trials, the mixture, each of the mixture components and the unrelated odorant were tested (four test trials with order balanced across individuals within each mixture ratio set). Between test trials, the trained odorant mixture was reinforced with a reward (one-to-three trials randomly) to avoid extinction to the trained mixture. During testing the experimenters were blind to the identity of the test odorants. Digging times were measured with a stopwatch and recorded at the end of each test trial, and digging duration was used as our measure of recognition. In a separate study we have shown that for rats, the digging task provides the same qualitative conclusions about mixture perception (e.g. elemental, overshadowing, configural) as a more controlled operant task using an air dilution olfactometer (Kay *et al.*, 2006). We have also verified that using the same unrelated test odorant (amyl acetate) for all tests has no effect on the generalization patterns (Kay *et al.*, 2005) and unpublished results of L. Kay. All mice dug in response to the training odor (five different ratios of pentanal and hexanal, 13.1 ± 0.9 s mean \pm standard error) longer than to the unrelated odorant (0.25% amyl acetate, 3.9 ± 0.8 s) across training sets, with average digging times within each of the five sets ranging from 10.9 ± 2.5 to 16.0 ± 2.1 s for the mixtures, and 2.0 ± 1.0 to 5.9 ± 2.2 s for amyl acetate (unrelated odorant). Digging in component odorants varied dependent upon the training mixture ratio. Each mixture ratio was tested on a different day for each mouse, and the order of ratio tests was balanced across mice. Tests reported here were interleaved with tests of other mixtures (data not reported here), so that the same set of odorants (at different ratios) was not tested in two successive tests.

Analysis

Data are digging times, in seconds, for each mouse for each test day, as reported in earlier studies (Nusser *et al.*, 2001; Kay *et al.*, 2003, 2005). Response times did not conform to a normal distribution, so we used the non-parametric Kruskal–Wallis test to test for variance across test odorants within each mixture ratio, followed by the Mann–Whitney test for pairwise comparisons.

Imaging the olfactory bulb

Subjects

Data from nine adult C57/BL6 male mice (Harlan, Indianapolis, IN) are reported. The animals were housed in groups of five in Plexiglas cages and maintained on a 12 h light/dark cycle. Food and water were provided *ad libitum*. Mice used for imaging were not the same that were trained and tested behaviorally.

Surgical procedure

Mice were initially anesthetized with a bolus of urethane (1500 mg/kg in saline, i.p.) followed by a urethane–ketamine mixture (40 mg/kg i.p.) after 5–15 min. Subsequent doses of the urethane–ketamine mixture (15 mg/kg i.p.) were given to maintain anesthetic plane. Dexamethasone (1.0–2.0 mg/kg, s.c.) was administered to reduce cerebral edema. Animals were placed in a stereotaxic apparatus and core temperature was clamped to 37.5 °C with a heating pad (Fine Science Tools, TR-100 temperature controller). Heart rate was monitored by electrocorticogram (ECG), and oxygen (1.5–2 L/min) was delivered within 3 cm of the nose. A midline incision was made in the scalp over the olfactory bulb, and the skull was thinned using a dental drill. The optical clarity of the thinned skull was preserved by a

TABLE 1. Olfactory stimuli: ratios and percentage (v/v dilution in mineral oil) of odorants and their mixtures

Ratio	Mixture (Pentanal : Hexanal)
20 : 1	1.25% : 0.0625%
10 : 1	1.25% : 0.125%
3 : 1*	0.25% : 0.75%
1 : 10	0.125% : 1.25%
1 : 20	0.0625% : 1.25%

*Partial pressures of components at this concentration ratio are nearly equal.

layer of 3% Type A agarose (Sigma-Aldrich) in saline and covered with a glass coverslip. At the end of the experiment, the animal was killed with an overdose of pentobarbital (150 mg/kg).

Odorants and dilutions

Because selected odorants had to activate the dorsal aspect of the olfactory bulb, glomerular responses to several odorants were screened. The aliphatic aldehydes pentanal and hexanal consistently elicited glomerular responses, represented by dark, punctate areas that were distributed across the dorsal bulbs. The aliphatic aldehydes in liquid phase were diluted in mineral oil to the same concentration ratios as used for behavioral studies (Table 1). In preliminary experiments amyl acetate only weakly activated a few glomeruli on the dorsal surface of the bulb, and was therefore not studied further. Odorants were prepared fresh daily in glass vials with screw caps. Vials remained capped except for 3 s prior to and during stimulus presentation.

Stimulus presentation and intrinsic signal imaging

Olfactory stimuli were presented in uncapped vials within 1 cm of the animal's nose in the oxygen stream for 10 s, with an interstimulus interval of 60 s. While the odor mixture was a similar distance from an animal's nose in both the imaging portions and the behavioral portions of the study (within 3 cm), the exact odor concentration that reaches the mouse's nose is not known for either case. However, the ratio between the odor components in the mixtures, which is the factor that determines perceptual quality for the same liquid phase concentration, is the same between the imaging and digging experiments. While the method of odor delivery and the state of the subjects (anesthetized vs waking) varied between our behavioral and imaging tests, imaged responses and mitral cell responses in anesthetized animals are reasonable predictors of monomolecular odor similarities in odor generalization tests (Linster & Hasselmo, 1999; Nusser *et al.*, 2001; Cleland *et al.*, 2002; Kay *et al.*, 2006; note that some predictions from monomolecular response patterns may break down for odor-discrimination tasks; Kay & Laurent, 1999; Cleland *et al.*, 2002; Beshel *et al.*, 2007; but the behavioral test used in these experiments is an odor generalization test). Stimuli were presented seven times in pseudo-randomly interleaved order.

Images were acquired using a CCD camera (Dalsa 1M30P) running at 30 frames/s. The camera has a 1024 × 1024 pixel chip, with a 100% fill factor, and images were spatially binned (2 × 2) by software to produce final images of 512 × 512 pixels. Two Nikkor lenses (50 mm and 135 mm) were mounted back-to-back to give a net magnification of 2.7. Green light (540 nm) was initially used to image the vascular pattern of the olfactory bulb. For intrinsic signal imaging, the camera was focused 0.3 mm below the vascular surface. The bulb was illuminated with red light (610 ± 15 nm, Thermo-Oriel Filter), and a matched filter was interposed between the lenses to reduce the impact of background light.

Responses to odors were averaged from 0.5 to 11.5 s after the beginning of odor presentation, and were normalized by the average response in the first 0.5 s of odor presentation. Images were spatially high-pass filtered; the high-pass filter was implemented by subtracting an image smoothed with a 35 × 35 pixel kernel (311 × 311 μm) from the unsmoothed image; this is the same general procedure used by other groups to process intrinsic signal images of olfactory bulb (Meister & Bonhoeffer, 2001; Belluscio & Katz, 2001). The images from the seven stimulus repetitions were averaged together. In final images (after magnification and software binning) each pixel is 8.9 μm along an edge.

Because there are spatial correlations in the noise from any method for imaging brain activity, we took several steps to minimize the effects of non-stimulus-dependent spatial correlations on the measurements of glomerular activity. (i) To reduce the effect of stimulus-independent correlations in neural activity, we averaged responses over multiple trials of the same stimulus. Spatially correlated activity that was only present during a single trial, or was out of phase with the randomly ordered stimuli, was therefore averaged out in response maps. (ii) To reduce vascular- and brain-motion artifacts we kept the skull intact (thinning it to improve the optical clarity); this substantially reduces the amount of motion of the surface of the olfactory bulb. (iii) We spatially high-passed images to remove long-range spatial correlations, leaving only modulations in activity that occur on the spatial scale of glomeruli. Glomeruli that appear active after high-pass filtering are well-correlated with histologically defined glomeruli (Meister & Bonhoeffer, 2001) and the pattern of activity varies with odor identity (Belluscio & Katz, 2001; Meister & Bonhoeffer, 2001; Belluscio *et al.*, 2002; Gurden *et al.*, 2006).

Analysis

We compared glomerular patterns in response to odorant mixtures and their components. All potential glomeruli were selected manually by outlining regions of activation of the appropriate size and shape from all mixture and component conditions. Then, some regions were excluded by an automated procedure that relied on two criteria. First, the average glomerular response had to exceed a threshold of 0.75 standard deviations above the mean pixel intensity in the mineral oil image. Second, the glomerulus could not be active in the mineral oil control image (its average intensity had to be less than threshold). The remaining regions were labeled as glomeruli and analysed using custom software written in IDL (RSI, Boulder, CO, USA).

The threshold for activity was set at 0.75 σ (standard deviations) to ensure that all the glomeruli that appear by eye to be active at the highest odorant concentrations are included in the activity pattern and that few inactive glomeruli are incorrectly classified as active (false positives). The threshold value chosen produced a linear relationship between the number of active glomeruli and odor concentration over the full range of concentrations used. We also considered lower and higher threshold levels. Substantially lower thresholds caused the count of active glomeruli to saturate at low odorant concentrations, so they did not allow discrimination among activity patterns at different concentrations. Substantially higher thresholds excluded many glomeruli that appeared active by eye. The use of a threshold is not meant to imply that glomerular activity is a binary event, rather the threshold allows us to compare activity patterns across conditions and to identify individual glomeruli that may behave differently in response to different odorants.

The threshold of 0.75 σ is high enough to exclude false positives. Because σ was measured in response to the mineral oil condition (blank condition), it represents the random fluctuations of individual pixels. When considering the average intensity across many pixels, the chance of randomly exceeding threshold therefore decreases with increasing numbers of pixels in the glomerulus. For individual pixels, 22.7% would exceed a 0.75 σ threshold by chance even when not stimulated (assuming a Gaussian distribution). However, in our experiments glomeruli encompassed many pixels (in the example images shown in Figs 4–6 the average number of pixels per glomerulus was 222). If each pixel was completely independent of its neighbor, the chances of an entire inactive glomerulus randomly exceeding threshold are negligible ($\ll 10^{-10}$). There are, however, likely to be correlations in the noise across pixels, so the intensities of

individual pixels are not independent of their neighbors'. To ensure that the threshold is high enough to exclude false positives, we recalculated the probability of a false positive by assuming that the number of independent pixels is less by a factor of 50. Reducing the number of independent pixels in the sample by a factor of 50 still sets the chance of an entire glomerulus randomly exceeding threshold at about 10^{-3} .

We also compared activity patterns by correlating the strength of responses in the set of glomeruli across conditions. This analysis does not rely on a threshold and allows comparison of activity levels in a group of glomeruli. However, it does not allow identification of individual glomeruli that were different between conditions. Assessment of non-linear responses was therefore made from the responses of individual glomeruli.

Results

Our goal was to determine if glomerular activity patterns predict component recognition in odor mixtures. We therefore assessed both how odor mixtures are perceived by mice and how the same odor mixtures activated glomeruli in the dorsal olfactory bulb.

The perception of odor mixtures

We used a digging odor generalization task to examine how mixtures of pentanal and hexanal are perceived by mice (Nusser *et al.*, 2001; Kay *et al.*, 2003). Mice were trained on a given test day to dig in a dish scented with one of five mixtures of pentanal and hexanal in a single drop on top of bedding material (10 training trials). When subsequently presented with a test odorant without reinforcement, the mice spent more time digging in response to the trained mixture than an unrelated odorant (amyl acetate). This confirms that the mice recognized the odors presented and were selective in digging to odors recognized from training sessions (Fig. 1; all mixture ratio tests showed significant variation across test odorants using the Kruskal–Wallis test, $P < 0.02$ for all test sets). Their ability to recognize the mixture components

presented individually varied depending on the concentration ratio of the components (summarized in Fig. 1).

We observed elemental perception, in which both the hexanal and pentanal components are recognized equally and responded to more than the unrelated odorant, when the airborne concentration of pentanal was much higher than that of hexanal in the trained mixture. For example, mice trained to respond to a mixture of 1.25% pentanal and 0.0625% hexanal dug significantly longer in each of the component odorants than in amyl acetate ($P = 0.04$ and 0.02 , respectively), and not differently between hexanal and pentanal ($P = 0.97$).

We observed overshadowing of pentanal by hexanal, in which mice generalize only to hexanal, when the estimated airborne concentration of hexanal was higher than or equal to that of pentanal. Mice trained to respond to a mixture of 0.0625% pentanal and 1.25% hexanal dug significantly longer in hexanal than in pentanal or the unrelated odorant ($P = 0.001$ and 0.002 , respectively). In contrast, they did not dig significantly longer in response to 0.0625% pentanal than to amyl acetate ($P = 0.18$). When the estimated airborne concentrations of hexanal and pentanal in the trained mixture were nearly equal (0.25% pentanal/0.75% hexanal), mice also dug longer in response to hexanal alone than they did to pentanal and the unrelated odorant ($P = 0.01$ and 0.0008 , respectively).

These five mixtures therefore produced two different behavioral responses: (i) hexanal overshadows the perception of pentanal when hexanal has a higher airborne concentration in mixture or when they are approximately equal; (ii) both components can be recognized (elemental perception) in a mixture composed of a high concentration of pentanal but a low concentration of hexanal. We did not observe configural (synthetic) perception, in which neither component is recognized, for any of the mixtures.

Olfactory bulb imaging

To determine how the odor mixtures activated the olfactory bulb, we imaged glomerular activity in response to the odor mixtures and the individual components (Table 1).

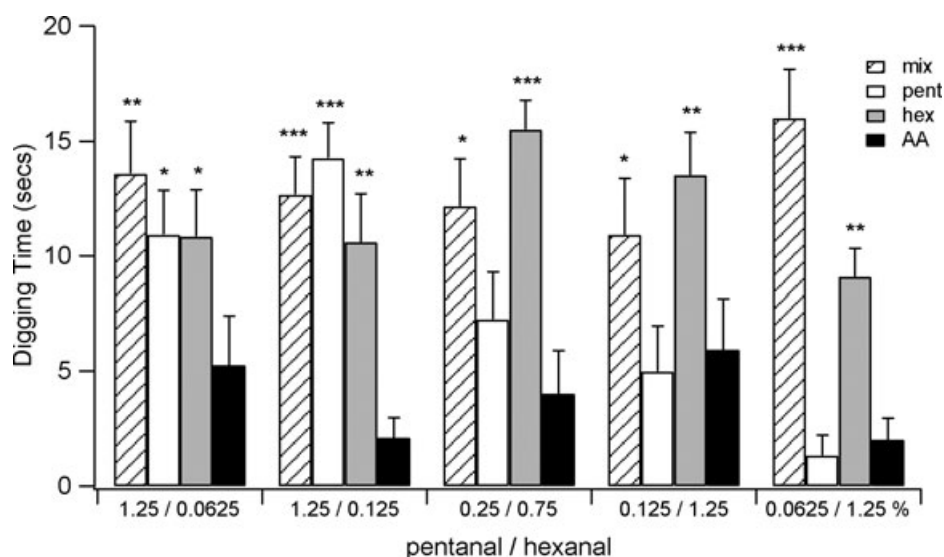


FIG. 1. Digging response to odor mixtures. Digging time (in seconds) in dishes scented with the mixture, pentanal and hexanal compared with the dish scented with an unrelated odorant, amyl acetate (AA). The horizontal axis labels indicate liquid phase concentrations. Error bars show SEM; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, all comparisons are to the unrelated odorant (amyl acetate).

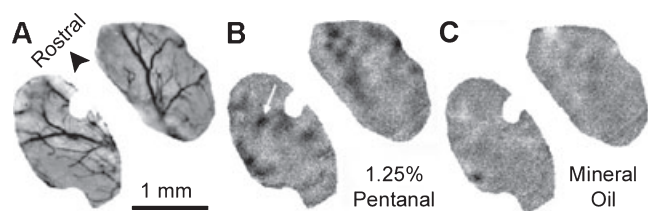


FIG. 2. Example of vascular pattern and intrinsic signal images. Glomeruli are active on the dorsal surface of both the right and left olfactory bulbs in response to odorant stimulus, but not the mineral oil control. (A) Vascular pattern generated by imaging green light reflected off the dorsal surface of the olfactory bulbs. A template is used to restrict the analysis region; portions of the image that are outside the template are not considered and are blank (white) in these images. (B) Dark, circular regions in intrinsic signal images represent active glomeruli in response to 1.25% pentanal; the white arrow points out one active glomerulus. (C) Few active glomeruli are observed in response to a mineral oil control.

General response patterns to individual odorants and mixtures

Optical responses to individual odorants were consistent with previous reports using intrinsic signal imaging (Rubin & Katz, 1999, 2001; Uchida *et al.*, 2000; Belluscio & Katz, 2001; Meister & Bonhoeffer, 2001; Takahashi *et al.*, 2004; Igarashi & Mori, 2005). Glomeruli appear as dark, punctate areas on the dorsal aspect of the bulb when activated by an odorant, but not when the animal is presented with a mineral oil control (Fig. 2). Each odorant activated a unique set of glomeruli, and the number of active glomeruli increased with odorant concentration (Fig. 3).

To determine if the overlap between mixture and component response patterns could predict the behavioral responses shown in Fig. 1, we looked at the glomerular response patterns to stimuli that produce both overshadowing and elemental perception. Regardless of the perceptual quality produced by a mixture, the pattern of activated glomeruli was always dominated by the component with the higher concentration. As Fig. 4 shows for one example experiment, most glomeruli activated by a mixture of 0.125% pentanal and 1.25% hexanal (red glomeruli in Fig. 4C) were also activated by 1.25% hexanal presented alone (red glomeruli in Fig. 4B). Similarly, most of the glomeruli activated by a mixture of 1.25% pentanal and 0.125% hexanal (red glomeruli in Fig. 5C) were also activated by 1.25% pentanal presented alone (red glomeruli in Fig. 5A). The overlap of glomeruli activated by all three conditions (the two components alone and the mixture) was greatest when the partial pressures of the components are nearly equal (the glomeruli coloured red in both Fig. 6A and B).

To quantify how much of the mixture response pattern was due to the individual components, we counted the number of glomeruli that were activated by both the mixture and one of its components. The overlap in mixture and component response patterns is plotted in Fig. 7 (average overlap of nine mice). For the mixtures in which the component concentrations were substantially different, approximately 80% of the glomeruli activated by the mixture were also activated by the higher concentration odorant. In contrast, less than 20% of the glomeruli were also activated by the lower concentration odorant. The measures of overlap fractions were similar whether pentanal or hexanal was at the higher concentration. Only when the component vapor pressures were equal was the overlap fraction similar for the two components. The gross patterns of activity therefore did not co-vary with how the odor mixtures were perceived.

It is possible that relative levels of glomerular activity, not just the spatial pattern of activated glomeruli, better predict perception. To test this possibility, we calculated the Pearson correlation coefficient for

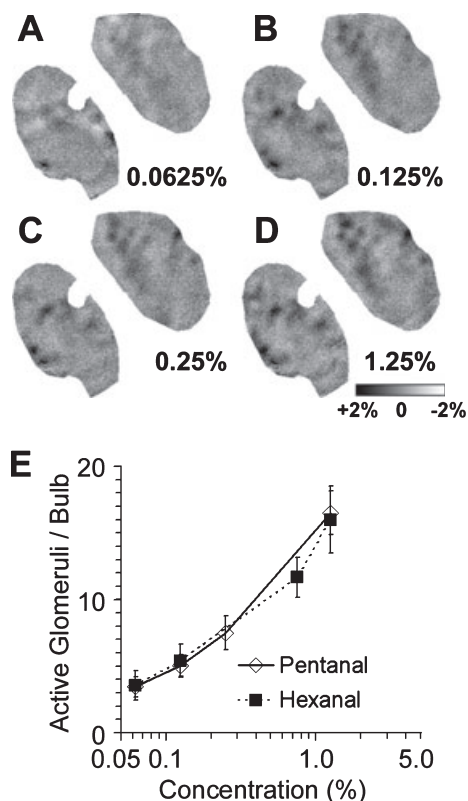


FIG. 3. The number of active glomeruli observed on the dorsal surface of the olfactory bulbs increases with odorant concentration. (A) Glomerular activity pattern in response to 0.0625% pentanal, (B) 0.125% pentanal, (C) 0.25% pentanal and (D) 1.25% pentanal. The scale bar represents 1 mm. The gray-scale represents image intensity as a percentage of the mean intensity in the image before stimulation (first-frame normalization); increased light absorbance (black) represents strong activation, and is therefore labeled positive. (E) The average number of active glomeruli per bulb increases monotonically as a function of odorant concentration. Error bars show SEM; measured in 15 bulbs from nine mice; $n = 9$.

glomerular activity levels in response to an individual odorant and the mixture (within-subject correlations were measured from patterns generated in each animal; statistical tests were then applied to the distribution of correlation values from all animals). If the relative levels of glomerular activity are a better predictor of perception, we would expect the correlations among activity patterns for different stimuli to co-vary more closely with odor perception than the glomerular overlap measures. This was not the case. The correlations among different activity patterns (Fig. 7B) were similar to the overlap measures shown in Fig. 7A, suggesting that relative activity levels are no better at predicting perceptual quality than are the overlap fractions.

Linear and non-linear responses

Because the overall pattern of glomerular activity did not predict perceptual differences, we asked if there were differences in glomerular responses at a finer scale. Specifically, we asked if there were non-linear glomerular responses when either pentanal or hexanal was at the higher concentration. Previous studies have suggested that a mixture's response pattern is the linear sum of the pattern generated by the components presented alone (Belluscio & Katz, 2001). In our data set the majority of active glomeruli obeyed this rule: 84% of glomeruli activated by a mixture were also activated by one or both of the

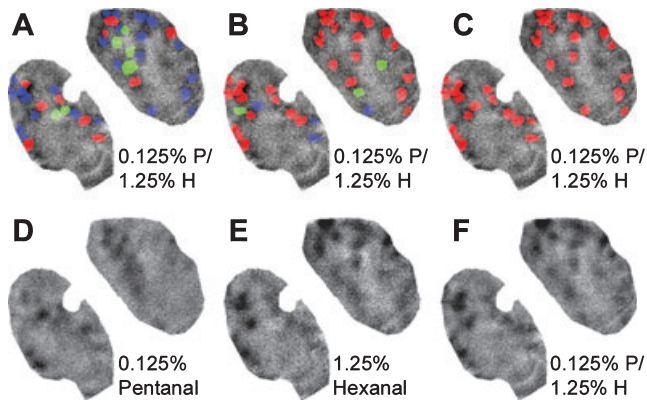


FIG. 4. Glomerular responses to a mixture of 0.125% pentanal and 1.25% hexanal and its components. The overlap of the mixture response pattern with that of the 0.125% pentanal component (A) and the 1.25% hexanal component (B) is highlighted in the top row. Glomeruli that were activated both by the mixture and the component are marked red, those activated by the mixture only are marked blue, and those activated by the component only are marked green. (C) All glomeruli that responded to the mixture are marked red. (D–F) Unmarked response images for the 0.125% pentanal condition (D), 1.25% hexanal (E), and the mixture composed of 0.125% pentanal and 1.25% hexanal (F).

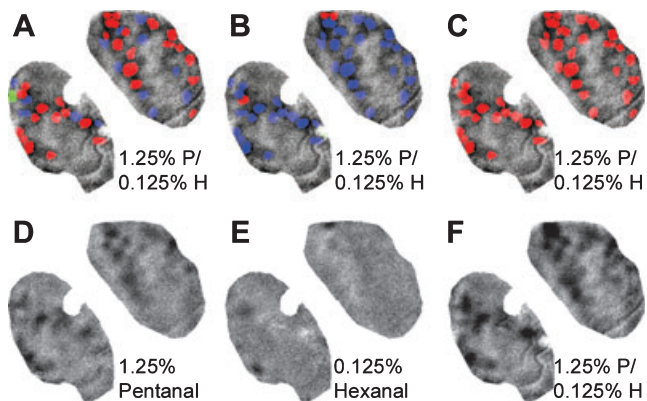


FIG. 5. Glomerular responses to a mixture of 1.25% pentanal and 0.125% hexanal and its components. The overlap of the mixture response pattern with that of the 1.25% pentanal component (A) and the 0.125% hexanal component (B) is highlighted in the top row. Glomeruli that were activated both by the mixture and the component are marked red, those activated by the mixture only are marked blue, and those activated by the component only are marked green. (C) All glomeruli that responded to the mixture are marked red. (D–F) Unmarked response images for the 1.25% pentanal condition (D), 0.125% hexanal (E), and the mixture composed of 1.25% pentanal and 0.125% hexanal (F).

components presented alone (1062 of 1267 glomerular responses measured in nine animals across five mixture ratios; Supplementary material, Table S1). The remaining 16% (205 of 1267) of glomeruli were activated only by the mixture. Conversely, 12% of glomeruli responded to individual odorants but not to the mixture (175 of 1442 glomerular activations). Together, these results suggest that an odorant mixture can generate a specific portion of the response that is independent of its components.

It is possible that a difference in the number or type of mixture-specific responses might underlie the difference in the perception of hexanal- or pentanal-dominated mixtures. We therefore characterized

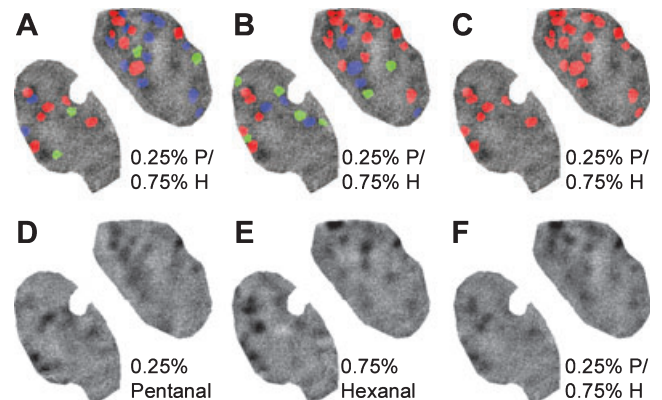


FIG. 6. Glomerular responses to a mixture of 0.25% pentanal and 0.75% hexanal and its components. The overlap of the mixture response pattern with that of the 0.25% pentanal component (A) and the 0.75% hexanal component (B) is highlighted in the top row. Glomeruli that were activated both by the mixture and the component are marked red, those activated by the mixture only are marked blue, and those activated by the component only are marked green. (C) All glomeruli that responded to the mixture are marked red. (D–F) Unmarked response images for the 0.25% pentanal condition (D), 0.75% hexanal (E), and the mixture composed of 0.25% pentanal and 0.75% hexanal (F).

the mixture-specific responses for pentanal-dominated and hexanal-dominated mixtures. Mixture-specific responses could be due to either a linear summation of responses to individual odorants or to non-linear processing of odor mixtures (e.g. inhibition or facilitation).

We first excluded mixture-specific glomeruli for which the response could be explained by a linear combination of the individual components' responses. If a glomerulus was weakly sensitive to both odorants, neither odorant alone would drive it to threshold (as defined for imaged responses), but together the odorants could strongly activate the glomerulus. We found that 72 of the 205 glomeruli that responded only to the mixture behaved in this way: the response to the mixture fell within 0.5 standard deviations of the sum of responses to the individual odorants (Fig. 8; supplemental Table S2, 'Linear'). This summation is linear and consistent with conclusions about mixture responses from previous studies (Belluscio & Katz, 2001).

The activity of the remaining 133 mixture-specific glomeruli (10.5% of all activated glomeruli) could not be characterized as the sum of responses to individual odorants (Fig. 8A; supplementary Table S2, A, 'Supralinear'). For these glomeruli, responses to the mixture were greater than the summed responses to the individual odorants. This supralinear response suggests that the combination of odorants activates specific glomeruli in a cooperative or facilitatory fashion. This non-linearity manifests at the first central stage of olfactory processing and could be due to interactions at the odorant receptor or processing within the olfactory bulb. However, there was no significant difference ($P > 0.05$, one-tailed t -test) between the numbers of supralinear glomeruli activated by pentanal-dominated and hexanal-dominated mixtures. This type of non-linearity is therefore not likely to produce the perceptual differences observed between the mixtures (Fig. 1).

To determine if suppression played a role in setting activity patterns, we considered glomeruli that were activated only by individual components and not by the mixture (Fig. 8B; supplementary Table S2, B, 'Suppression'). In the majority of these cases (115 of 175 glomeruli, 66%), glomerular activity in response to components exceeded threshold by less than 0.5 standard deviation. Because activity was near threshold, noise could easily affect whether

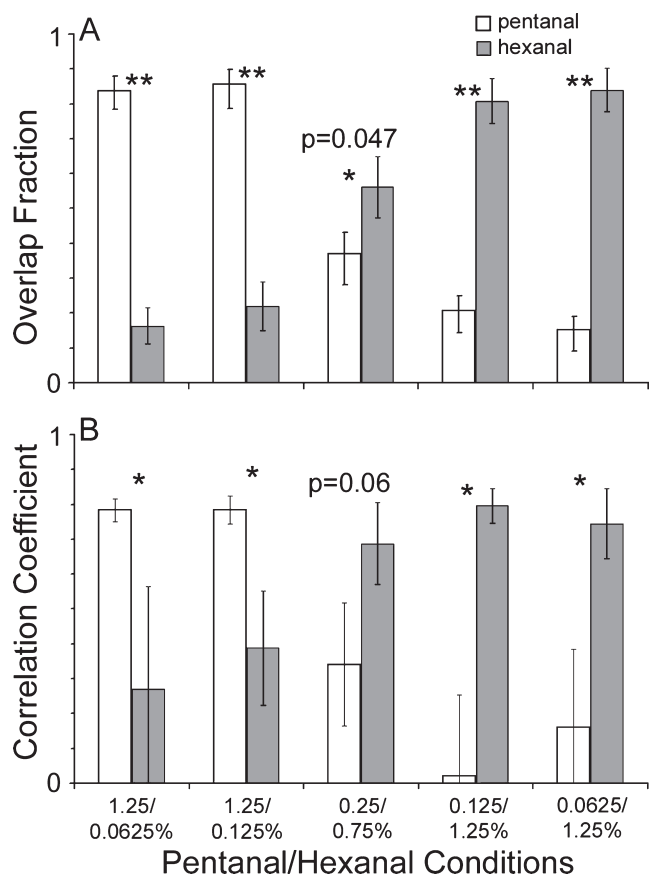


FIG. 7. Similarity between glomerular activity patterns produced by the mixture and by each component presented alone. (A) The fraction of mixture-responsive glomeruli that are also activated by one component presented alone is plotted against mixture composition. The amount of glomerular overlap between mixtures and their components varied depending on the concentration ratio of the components. For example, the glomerular pattern in response to a mixture of 0.0625% pentanal and 1.25% hexanal was dominated by the hexanal component (gray) compared with the pentanal component (white). (B) Correlation of mixture response pattern with the response pattern generated by each component. Pearson correlation coefficients were measured between sets of glomeruli activated by the mixture and components. For each mixture, the set of active glomeruli consisted of any glomerulus whose activity exceeded threshold in response to the mixture or either component. The correlation coefficient measures how similar the magnitudes of glomerular activity are between the mixture response and component responses. In both (A) and (B), * $P < 0.05$, ** $P < 0.001$, one-tailed t -test; error bars represent SEM, $n = 9$ mice.

threshold was reached, so activation would not always be detected for a stimulus of similar intensity (Fig. 8B; supplementary Table S2, B, 'Stochastic'). This suggests that only a small number of glomeruli within the field of imaging (approximately 4% of all glomerular activations observed; Fig. 8B; supplementary Table S2, B, 'Suppressive') are actively suppressed, perhaps through inhibition, when these two odorants are presented in a mixture. Again, the numbers of suppressed glomeruli were not significantly different ($P > 0.05$, one-tailed t -test) between the pentanal-dominated and hexanal-dominated mixtures. Neither facilitation nor suppression can therefore explain the difference between overshadowing or elemental mixture perception.

Glomerular representation of odorant impurities

The study is complicated somewhat by the fact that aldehydes oxidize into carboxylic acids, so the hexanal odorant is a mixture of unspecified

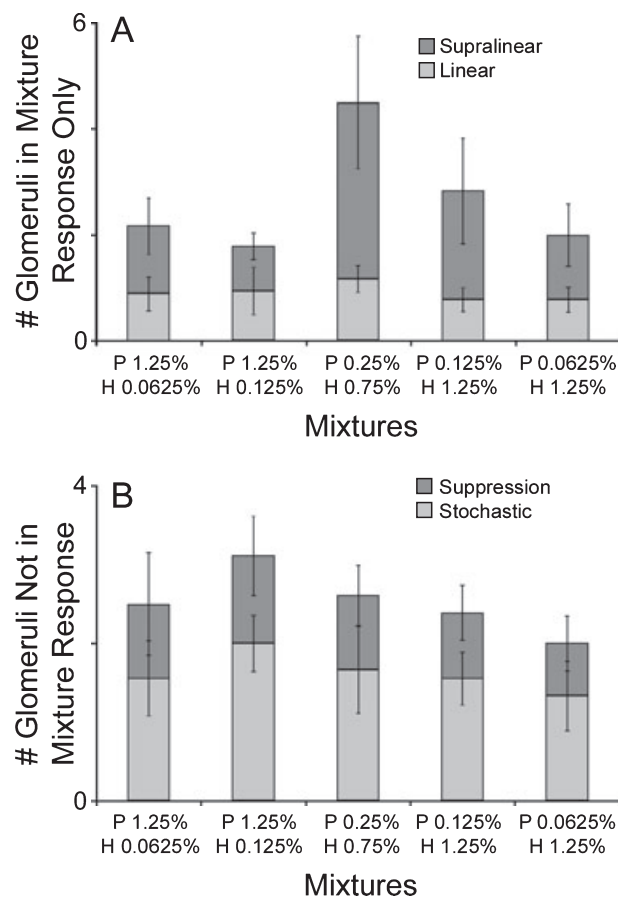


FIG. 8. Glomerular activity in response to mixtures that are not predicted from responses to individual odorants. (A) Average number of glomeruli in a bulb that are activated by a mixture, but not by either of the components ('blue glomeruli' in Figs 4–6). Most of these glomeruli responded more strongly to a mixture than to the summed responses to the two components presented alone (dark gray bars show the number of glomeruli per bulb that had supralinear responses). In a minority of glomeruli the magnitude of response to the mixture was similar to the sum of responses to the components presented alone (light gray bars show the number of glomeruli per bulb that had linear responses). (B) Average numbers of glomeruli activated by one or both components but not active in response to the mixture ('green glomeruli' in Figs 4–6). The majority of these glomeruli exceeded threshold by only a small amount, such that random fluctuations in optical responses could explain why they did not exceed threshold in response to the mixture (light gray, Stochastic). For a minority of glomeruli, activity appears to be inhibited by the mixture (dark gray, Suppressive). Error bars are SEM of number of glomeruli per bulb; counts from two bulbs imaged in the same animal were averaged together to give $n = 9$ from measurements in 15 bulbs.

ratios of hexanal and hexanoic acid, and the pentanal odorant is a mixture of pentanal and pentanoic acid (plus any impurities). Control experiments, however, suggest that aldehyde decomposition had little effect on glomerular activity patterns. Glomerular responses to high concentrations of hexanoic acid and pentanoic acid activate many of the same glomeruli as generated by hexanal and pentanal (supplementary Figs S1–S3). In addition, activity patterns generated by hexanal and pentanal were nearly the same whether the source of the odorant was old (first opened 1 year prior to test) or new (first opened on the day of test). Old pentanal activated 86% of the glomeruli that were activated by new pentanal, while old hexanal activated the same glomeruli (100%) that were activated by new hexanal. These similarities suggest that decomposition of the odorants did not substantially change glomerular responses.

Discussion

To determine if glomerular activity patterns predict mixture perception we assessed glomerular activity patterns in response to mixtures that produce qualitatively different perceptions as measured behaviorally in a generalization paradigm. Mixtures composed of hexanal and pentanal were perceived in one of two ways (Fig. 1). (i) When mice were trained on mixtures in which hexanal was the major component or when hexanal and pentanal were approximately equal in the vapor phase, they responded only to hexanal more than amyl acetate when components were presented alone (overshadowing). (ii) When pentanal was the major component in the trained mixture, they responded to both pentanal and hexanal more than to amyl acetate (elemental perception). The degree of overlap of glomerular activity between individual odorants and mixtures was then examined as a predictor of these two types of perceptual responses.

Perception and glomerular activity patterns

Our data were not consistent with a previous model of mixture perception derived from behavioral studies. According to the overlap model of mixture perception, configural (synthetic) perception should occur when odorant components produce overlapping glomerular activity (Kay *et al.*, 2003; Wiltrout *et al.*, 2003). Instead, we found either overshadowing or elemental perception with mixtures composed of chemically similar components that produced overlapping glomerular patterns.

Can the glomerular activity patterns explain why some mixtures produce elemental perception while others produce overshadowing? One possibility is that elemental or overshadowing perception is dictated simply by the size of the overlap of a component's pattern with the mixture pattern. This model, however, is only partially successful in predicting perception. The size of overlap in activity patterns did a good job of predicting how pentanal would be perceived in all mixtures: when the pentanal responses dominated the mixture response pattern pentanal was perceived, and conversely when the pentanal pattern accounted for only a small fraction of the mixture response pattern pentanal was not recognized in the mixture. Similarly, the large overlap of response patterns predicted that hexanal would be perceived when present at high concentrations. However, this reasoning failed to predict that hexanal would be perceived in mixtures in which it was present at very low concentration. It is questionable therefore that the spatial patterns of glomerular activity are the sole determinants of mixture perception.

The data also suggest that suppression of glomerular activity, produced by either competitive inhibition by odorants at the receptor (Araneda *et al.*, 2000) or inhibition within the glomerulus itself, is not needed to produce overshadowing. We would expect inhibition at the glomerulus, if present, to reduce the intrinsic optical signal. Gurden *et al.* (2006) found that direct application of dopamine or γ -aminobutyric acid (GABA)-B agonists reduced the intrinsic optical signal in the bulb, and that blockade of GABA-B receptors increased the signal, so inhibition modulates the optical signal in the opposite direction of excitation. While we did find that a small number of glomeruli were inhibited when the components were presented in a mixture, the number of inhibited glomeruli was nearly equal between conditions in which there was elemental perception and in which there was overshadowing. This suggests that overshadowing may not be produced by the suppression of responses of one odorant component by another, or if suppression does occur it is at a later stage of

processing, such as in the mitral or tufted cells or piriform cortex. The data are consistent with overshadowing resulting from the predominance of one component's pattern within the mixture response pattern.

One simple conclusion from these data is that individual odorants can be identified even when the mixture response includes many glomeruli that are not activated by the component odorant. This is most clearly seen in the response to the approximately equal-partial pressure mixture (1 : 3 pentanal : hexanal), in which 38% of the glomeruli (94 of 249) activated by the mixture were not activated by hexanal alone (Supplementary Table S1), yet hexanal was recognized by mice trained on the mixture. Consistent with this interpretation is the ability to recognize components even in the presence of a few non-linearly activated glomeruli. For all the mixtures studied, the fraction of non-linearly activated glomeruli was small, and one or both components of the same mixtures were recognized behaviorally. We speculate that if the fraction of non-linearly activated glomeruli was large, mixtures would be perceived as a novel odor (configural perception). These results suggest that a small number of active glomeruli that are not associated with the odorant component do not disrupt the ability to identify it within a mixture.

The ability to recognize components in the presence of active glomeruli that are not associated with the component seems to be limited. In the same equal-partial pressure mixture, pentanal was not recognized even though the vast majority of pentanal-responsive glomeruli were activated by the mixture (approximately 82% of pentanal-responsive glomeruli were also activated by the mixture; Supplementary Table S1). The poor ability of mice trained on a mixture of equal-partial pressure hexanal/pentanal to recognize pentanal is likely due to two factors. First, many glomeruli that were activated by the mixture were not activated by the pentanal component – approximately 61% of the glomeruli (153 of 249) activated by the mixture were not activated by the pentanal component in isolation. Second, only 12% of the mixture pattern (29 of 249 glomeruli) could be uniquely attributed to the pentanal component – that is, hexanal activated many of the same glomeruli as pentanal. In contrast, 35% of the mixture pattern was uniquely attributable to hexanal (88 of 249 glomeruli). These results suggest that a large number of active glomeruli that are not associated with an odor component and/or little unique representation of an odor component in a mixture response can obscure the component's identity.

Limitations

Because optical imaging can only be used to study the dorsal surface of the mouse olfactory bulb, as much as 3/4 of the bulb was not imaged. The conclusions about activity patterns are therefore based on an incomplete sampling of glomeruli. If, contrary to our conclusions, glomerular activity patterns do explain perception, then there must be a set of glomeruli on the other surfaces that respond to low concentrations of hexanal but not low concentrations of pentanal. This is unlikely, though, as hexanal and pentanal activity patterns are very similar to each other in all imaging studies of the olfactory bulb in both rats and mice (Rubin & Katz, 1999; Xu *et al.*, 2003; Johnson *et al.*, 2004). Alternatively, there might be a small number of glomeruli that contribute disproportionately to odor quality. So far no studies have suggested that such non-linear effects exist, and our own study suggests that a few non-linearly responding glomeruli do not explain perceptual effects (Fig. 8).

Despite the limitation of access only to the dorsal surface, optical imaging is an essential part of efforts to assess the relationship between glomerular activity patterns and behavior. Other imaging techniques, like functional magnetic resonance imaging (fMRI) and 2-deoxyglucose (2-DG), can track activity over the entire bulb, but they cannot be used to address the issue of how individual glomeruli respond to multiple odors. While fMRI can be used to track activity in response to multiple stimuli, its poor spatial resolution does not allow the identification of individual glomeruli. Histological approaches like c-fos and 2-DG staining can often resolve individual glomeruli, but they can only be used to test a single odor condition per animal. Because nearby glomeruli can behave differently, only a high-resolution functional image technique (like intrinsic signal imaging or calcium imaging; Wachowiak & Cohen, 2001; Spors *et al.*, 2006) can determine if non-linearities at the level of individual glomeruli underlie differences in odor perception.

Another potential limitation of this study is that behavior and glomerular activity patterns were examined under different arousal states. It is possible therefore that the pattern of glomerular activity might be different in the awake animal than in the anesthetized animal. The effects of our anesthesia protocol on glomerular activity patterns is, however, likely to be small. First, we used urethane as the primary anesthetic agent. Unlike many other anesthetics, urethane preserves much of the 'awake-like' cortical activity pattern, including odor response specificity of mitral cells and odor-evoked oscillations, although clearly context-associated variations in these responses are absent (Kay & Laurent, 1999; Kay, 2003). Second, the measurements are made early in the olfactory network, at the input level. The effect of anesthetics on neuronal activity increases with the number of synapses from the periphery, with potentially different effects on each subsequent stage. Because the first synapses in the olfactory pathway are at the olfactory nerve input to the glomeruli, anesthesia is not likely to have substantially modified neuronal activity there, although variations in inhalation patterns are undoubtedly present. Consistent with this expectation, a previous study using isoflurane anesthesia and a mixture of two odorants had the same basic imaging findings: that glomerular activity patterns in response to odor mixtures could be predicted by a linear combination of glomerular responses to the odorants presented separately (Belluscio & Katz, 2001). Because two very different anesthetics produced similar results, it is unlikely, albeit possible, that our anesthetic protocol is substantially altering the qualitative properties of glomerular activity patterns as measured by intrinsic signal imaging. Finally, under anesthesia the activity of mitral cells is still a reasonable predictor of monomolecular odor similarities in odor generalization tests (Linster & Hasselmo, 1999; Nusser *et al.*, 2001; Cleland *et al.*, 2002; Kay *et al.*, 2006), suggesting that anesthesia has minimal effects on neuronal activity even one stage beyond the glomeruli.

Conclusion

In conclusion, these results argue that the spatial pattern of glomerular activity in part determines the qualitative properties of a binary odor mixture. When a single odorant in a mixture activates its set of glomeruli it can usually be perceived in a mixture. Small variations in the glomerular activity pattern do not obscure the odorant's identity, but a large number of additional glomeruli can sometimes obscure an odorant's identity even if its full glomerular pattern is activated. One surprise was that low-concentration odorants can sometimes be

identified in a mixture even when the glomerular activity pattern includes few glomeruli that are uniquely activated by the odorant. This suggests that additional processes, such as temporal response profiles (Tabor *et al.*, 2004), intrabulbar or system-wide oscillatory synchrony (Laurent, 1999; Martin *et al.*, 2004; Kay & Stopfer, 2006; Beshel *et al.*, 2007; Martin *et al.*, 2007), non-uniform distribution of downstream targets (Zou *et al.*, 2001) or higher order processing of spatial activation patterns (Di Prisco & Freeman, 1985), are required to fully explain mixture perception.

Supplementary material

The following supplementary material may be found on <http://www.blackwell-synergy.com>

Fig. S1. Overlap of response patterns between aldehydes and degradation products.

Fig. S2. Example of glomerular responses to new and old pentanal, and pentanoic acids.

Fig. S3. Example of glomerular responses to new and old hexanal, and hexanoic acids.

Table S1. Numbers of glomeruli activated by each condition.

Table S2. Numbers of glomerular activations that were different between mixture responses and component responses.

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Abbreviations

2-DG, 2-deoxyglucose; fMRI, functional magnetic resonance imaging; GABA, γ -aminobutyric acid.

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