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Sensitivity-dependent Hierarchical Receptor Codes for Odors

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Abstract

In order to comprehend the strategy of odor encoding by odorant receptors, we isolated 2740 mouse receptor neurons from four olfactory epithelial zones and classified them in terms of their sensitivities and tuning specificities to a chiral pair of odorants, *S*(+)-carvone (caraway-like odor) and *R*(–)-carvone (spearmint-like odor). Our approach revealed that the majority of receptors at the lowest effective stimulus concentration represented the principal odor qualities characteristic of each enantiomer by means of the principal odor qualities of the odorants for which the receptors were most sensitive. The chiral-non-discriminating receptors became 3.7 times of *R*(–)-carvone-sensitive receptors in the subpopulations when the stimulus concentration was increased 10-fold. More than 80% of the responsive receptors (an estimated 70 $\pm$ α types) exhibited overlapping sensitivities between the enantiomers. The signals from the non-discriminating receptors may be reduced to decode the characteristic odor identity for *R*(–)-carvone in the brain over an adequate range of stimulus strengths. The information processing of odors appears to involve the selective weighting of the signals from the most sensitive receptors. An analysis of the overall receptor codes to carvones indicated that the system employs hierarchical receptor codes: principal odor qualities are encoded by the most sensitive receptors and lower-ranked odor qualities by less sensitive receptors.

Key words: calcium imaging, odorant, odor discrimination, odor quality, olfactory receptor neuron, sensory information processing

Introduction

The mammalian olfactory system can discriminate subtle differences in the molecular structures of a great number of volatile organic compounds, presented either as pure chemicals or blended as complex mixtures. This remarkable capability is exemplified by the discrimination of a pair of enantiomeric carvones which possess the same molecular structures except for the chiral portion, but evoke distinct odor qualities. A spearmint-like fresh herbal odor is induced by *R*(–)-carvone (*rCa*), whereas a caraway-like fresh herbal scent appears in *S*(+)-carvone (*sCa*) (Friedman and Miller, 1971; Leitereg *et al.*, 1971; Boelens *et al.*, 1993). The basic question is what mechanism enables us to discriminate different odors. The importance of the molecular features of an odorant has been indicated by psychochemical theory (Braun and Kröper, 1937; Moncrieff, 1949; Amoore, 1970; Beets, 1982; Ohloff, 1986), by the responses of olfactory receptor neurons (ORNs) or functionally expressed odorant receptors (ORs) (Sato *et al.*, 1994; Bozza and Kauer, 1998; Malnic *et al.*, 1999; Touhara *et al.*, 1999; Kaluza and Breer, 2000; Kajiya *et al.*, 2001; Bozza *et al.*, 2002) and by the responses of glomeruli or neurons in the olfactory bulb (Mori *et al.*, 1992, 1999; Rubin and Katz, 1999, 2001; Johnson and Leon, 2000; Uchida *et al.*, 2000; Linster *et al.*, 2001; Wachowiak and Cohen, 2001; Spors and Grinvald, 2002).

The discovery of a family of ~1000 mammalian genes encoding ORs (Buck and Axel, 1991) led to an understanding of the response properties of individual ORs (Krautwurst *et al.*, 1998; Zhao *et al.*, 1998; Malnic *et al.*, 1999; Touhara *et al.*, 1999; Wetzel *et al.*, 1999; Araneda *et al.*, 2000; Kajiya *et al.*, 2001; Bozza *et al.*, 2002), the homogeneity of ORs expressed in single ORNs (Chess *et al.*, 1994; Malnic *et al.*, 1999; Touhara *et al.*, 1999; Rawson *et al.*, 2000; Kajiya *et al.*, 2001), the alternative expression of ORs in one of the four olfactory epithelial zones (Ressler *et al.*, 1993; Buck, 1996), the distribution of 1296 ORs in chromosomes (Zhang and Firestein, 2002), candidate odorant-binding sites in ORs (Singer and Shepherd, 1994; Singer, 2000; Floriano *et al.*, 2000), the OR-type-specific convergent projections from the olfactory epithelium to the bulb (Ressler *et al.*, 1994; Mombearts *et al.*, 1996; Wang *et al.*, 1998; Tsuboi *et al.*, 1999; Serizawa *et al.*, 2000) and the OR-type-dependent clusters of pyramidal cells in the olfactory cortex (Zou *et al.*, 2001). Odor encoding in the periphery has been interpreted in terms of overlapping combinatorial receptor codes (Malnic *et al.*, 1999). However,

a simple combinatorial receptor code may not be sufficient to represent actual odor qualities. The olfactory system may employ additional strategies to encode and decode odor information.

We suppose that the brain genetically functions to find common information and unique information between different subjects, based on an informational factorization at receptors. We suppose that the identity of an odorant can be decomposed into a unique odor quality, transduced by specific binding to unique ORs, plus one or more additional odor qualities common to multiple odorants. The additional odor qualities would be transduced by various subsets of common ORs with highly overlapped sensitivities to multiple odorants. We postulate that the relative strengths of the different odor qualities elicited by an odorant are primarily dependent on the relative sensitivities of different classes of ORs to that odorant. This is in contrast to a simple combinatorial coding model which assigns equal weights to all ORs activated by an odorant. To test our hypothesis regarding odor encoding in ORs, we clarified the overlap and differences among the receptors activated by subsets of odorants, including the carvone enantiomers *sCa* and *rCa*, which possess unique and shared elements of odor quality.

Our analysis of the sensitivity profiles of ORs responsive to enantiomeric carvones reveals that the most sensitive receptors which occupy minor subpopulations can represent the principal odor qualities of the odorant. Here, we describe sensitivity-dependent hierarchical receptor codes for odors. The most sensitive receptors encode the principal odor qualities and the less sensitive receptors encode supplementary odor qualities of the odorant. The receptors preferentially sensitive to either *sCa* or *rCa* could represent the unique odor qualities of caraway or spearmint, respectively; those equally sensitive to both enantiomers could represent the fresh herbal quality common to both. In the hierarchical receptor codes, multiple supplementary odor qualities encoded by common receptors or less sensitive receptors are reduced in an overall odor identity representation by signal weighting governed by the most sensitive receptors.

Materials and methods

Determination of odorant tuning specificities of ORNs

In order to understand the concrete receptor codes for the odor qualities of interesting odorants, we employed a previously reported experimental strategy (Malnic *et al.*, 1999). The odorant responsiveness of the ORs was examined in isolated ORNs using an intracellular calcium imaging assay (Ca-imaging assay) on living cells (Sato *et al.*, 1994; Malnic *et al.*, 1999; Touhara *et al.*, 1999) and the amino-acid sequences of the ORs were identified in the assayed ORNs using single-cell RT-PCR (Malnic *et al.*, 1999). These methods and recent modifications are briefly described as follows. Mice (BALB/c, CBA, or NZB) were anesthetized by Ketalar 50 (50 mg/ml ketamine, 5 μl/g, i.p.; Sankyo Pharmaceutical) and killed. The animals were treated in accordance with the guidelines for the care and use of laboratory animals of the National Institute of Health and of the AIST-Kansai Animal Experiment Committee. The ORNs on a lateral surface of a small piece of one of the four olfactory epithelial zones of mice were isolated on coverslips using the tissue-printing method (Hirono *et al.*, 1992) after sequential enzymatic treatments with trypsin, trypsin inhibitor and DNase I. The DNase treatment was added to remove olfactory mucus and damaged ORNs from the epithelial pieces. The isolated ORNs were loaded with fura-2 and then exposed to different odorants (Aldrich) for 4 s. The cellular responses were monitored in intracellular Ca^{2+} increases (Ca^{2+} responses) by observing the fura-2 fluorescence decreases at 510 nm, excited at 380 nm. Ca^{2+} responses to 6–14 stimuli sequentially applied at intervals of ~35 s were recorded every 10–15 min.

To determine the relative sensitivity of ORNs/ORs to various test odorants, we compared the relative response amplitudes of the odorant-induced Ca^{2+} responses. The odorant-induced Ca^{2+} increases are mediated by three pathways: Ca^{2+} influxes via cyclic-AMP-dependent channels (Kurahashi, 1990; Sato *et al.*, 1992; Leinders-Zufall *et al.*, 1998) and voltage-dependent Ca^{2+} channels (Hirono *et al.*, 1991), and Ca^{2+} release (Sato *et al.*, 1991; Zufall *et al.*, 2000). All pathways are theoretically activated more intensively as the receptor potentials of the ORNs become greater. In the typical odorant-induced Ca^{2+} responses, whose maximum intracellular Ca^{2+} concentrations were tens to hundreds of nanomolar (Malnic *et al.*, 1999), reproducible Ca^{2+} influxes were predicted even in insufficient recovery of the intracellular Ca^{2+} concentration, because the extracellular Ca^{2+} concentration was millimolar which could be sufficiently high to produce the differential concentration for driving the Ca^{2+} influxes. In fact, we previously showed that four successively repeated identical stimulations at intervals of ~35 s evoked responses with amplitudes of 100, 98, 80, and 77% of the initial ones in insufficient recovery levels (Sato *et al.*, 1994). Furthermore, fluorescence intensity excited at 380 nm monotonically decreases with increasing Ca^{2+} concentration. Although the responses were gradually reduced in the fluorescence changes during the recordings, we could observe Ca^{2+} responses to the most sensitive odorants with response amplitudes of >60% of the initial responses in most ORNs. Considering these properties, we were able to compare the relative sensitivity of ORs by comparing the net fluorescence decreases normalized by the baseline fluorescence after the subtraction of the background signals in the somata.

We employed the following criteria to judge whether the fluorescence changes were odorant-induced responses or artifacts. The Ca^{2+} responses in the soma of depolarized

healthy neurons should be mediated mainly by the depolarization-induced activation of voltage-dependent Ca^{2+} channels, intracellular Ca^{2+} release and the Ca^{2+} extrusion system. These mechanisms predict a rapid increase of Ca^{2+} concentration, followed by a slow decay of the Ca^{2+} concentration, in the soma of ORNs. The kinetics of the Ca^{2+} concentration change should be determined by the kinetics of the relative transduction components, which are characteristically differentiated cell by cell. In fact, the odorant-induced responses showed a rapid initial decrease of fluorescence with reasonable onset latency, followed by a relatively slow exponential recovery to baseline in a cell-dependent manner. The initial fluorescence decrease could be fit to a logistic curve ($b/(1 + c{\cdot}\exp(-at))$) and the time constants of the fluorescence recovery could be reproducible with a statistical variation in each ORN. The fluorescence changes exhibiting these kinetics were subjected to analysis. Responses which started with slow fluorescence decreases, or whose time constants of exponential recovery differed from the averages by more than two standard deviations in each ORN, were attributed to artifacts, spontaneous changes or incomplete activations of the signal cascade and were rejected.

The odorant tuning specificities of individual ORNs were determined in terms of their relative sensitivities to the test odorants, at least by the sets of the most sensitive odorants. At a fixed concentration, the relative sensitivity of an ORN was defined as low for odorants that evoked responses with amplitudes of <60% of the largest response across all odorants. The 'first' (most sensitive) odorants were defined to induce responses at the lowest concentration among all test odorants with amplitudes of >60% of the maximum at the same concentration. The 'second' odorants were the second-most sensitive odorants, less sensitive than the first odorants and more sensitive than the third odorants, in this definition. The validity of this classification of ORN types is supported by the conservation of relative sensitivities of M71-expressing ORNs to two odorants (Bozza *et al.*, 2002). Previous studies have shown that most individual ORNs express only a single OR gene (Chess *et al.*, 1994; Malnic *et al.*, 1999; Rawson *et al.*, 2000). Furthermore, the relative sensitivities of functionally expressed exogenous ORs to subsets of odorants were coincident with those of ORNs in which the mRNAs of the ORs were amplified after the physiological assays (Touhara *et al.*, 1999; Kajiya *et al.*, 2001). Based on these results, we made the assumption that the odorant specificities of ORNs represent those of ORs with the cAMP-mediated signal amplifier.

It is necessary to minimize the intensity and time of the exposure of the ORNs to ultraviolet light and also to minimize intracellular Ca^{2+} increases, in order to prevent the intracellular fura-2 and the mRNAs of ORs from photo-bleaching and degrading in the ORNs before performing the single-cell RT-PCR for OR identification. The success rate of single-cell RT-PCR was reduced by a factor of 2.3 after the Ca-imaging assays (Malnic *et al.*, 1999). Considering all these factors, we employed a weak excitation light illumination, an interstimulus interval of ~35 s and single trials for each stimulus with the following two exceptions. When the preceding stronger stimulus severely prevented the following weak stimulus from evoking a response clear enough to evaluate the relative sensitivity, the weak stimulus was again applied to the ORNs in the next recording. When the odorant sensitivities severely decreased during a series of recordings, a set of candidates for the most sensitive odorants were again assayed in the single recording. Because all of the ORNs responded to 2 s 145.6 mM KCl stimulation (*hk*) and most of them responded to 4 s 2 mM IBMX (*ibmx*), which generates the receptor potential via cAMP increase, cell viability was tested using these stimuli in the first set of recordings and near the end of the experiment.

Chemicals

All chemicals for the odorants were commercially purchased from Aldrich. The stimulants used were: carvone (*Ca*), 5-isopropenyl-2-methyl-2-cyclohexenone; (–)menthone (*mn*), (2*S*,5*R*)-2-isopropyl-5-methylcyclohexanone; *R*(+)-pulegone (*pu*), (*R*)-*p*-menth-4(8)-en-3-one; isopulegol (*ip*), 2-isopropenyl-5-methylcyclohexanol; menthol (*me*), 2-isopropyl-5-methylcyclohexanol; *R*(+)-limonene (*lim*), (*R*)-1-methyl-4-(1-methylethyl)-cyclohexene; isoamyl acetate (*am*), 3-methylbutyl acetate; vanillin (*va*), 4-hydroxy-3-methoxybenzaldehyde; *o*-vanillin (*ova*), 3-methoxysalicyl-aldehyde; geraniol (*ge*), 3,7-dimethyl-(*E*)-2,6-octadiene-1-ol; nerol (*ne*), 3,7-dimethyl-(*Z*)-2,6-octadiene-1-ol; hexanoic acid (*mc6*); heptanoic acid (*mc7*); octanoic acid (*mc8*); nonanoic acid (*mc9*); 1-hexanol (*mh6*); 1-heptanol (*mh7*); 1-octanol (*mh8*); 1-nonanol (*mh9*); indole (*in*), 1-H-indole; triethylamine (*ta*); isovaleric acid (*iv*), 3-methylbutylic acid; potassium chloride (*hk*); and IBMX (*ibmx*), 3-isobutyl-1-methylxanthine.

The supplied reagents of *S*(+)-carvone (*sCa*) and *R*(–)-carvone (*rCa*) contained small amounts of impurities. In *rCa*, the major impurities were α-terpineol and *cis*-2,6-dimethyl-5,6-epoxy-1,7-octadien-3-ol, whose peak areas in gas chromatography equipped with a flame ionization detector were 0.17 and 0.15% of the total peak areas, respectively. The remaining 99.68% was occupied by the peak of *rCa*. In *sCa*, the major impurities consisted of a mixture of α-terpineol + γ-terpineol, DH-isocarveol, neoiso-DH-carveol, a mixture of β-elemene + DH-carvone, a mixture of DH-carveol + *trans*-6-*p*-menthen-2-ol + *cis*-1,2-*p*-menthadien-8-ol, longifolene and DH-isocarvone, whose peak areas in gas chromatography were 1.13, 0.46, 0.36, 0.31, 0.21, 0.21 and 0.17%, respectively. The peak area for *rCa* occupied 96.74% of the total peak areas. Although we cannot exclude the possibility that these or other minor ingredients, not the carvones, might have induced the responses in a minority of assayed ORNs, we assumed that they did not severely distort the assay of OR responsiveness

for determining the most sensitive odorants in the present study.

Isolation of OR cDNAs from single ORNs

Each ORN was transferred to a tube and the cDNAs were prepared from the 3′ ends with pd$(T)_{25–30}$ or Anchor T primer (TATAgAATTCgCggCCgCTCgCgA$(T)_{24}$) and then amplified as described previously (Matsunami and Buck, 1997; Malnic *et al.*, 1999), but with a substitute SuperScript II (Gibco BRL) in some ORNs. Briefly, we performed two-step PCR: the first PCR for all cDNA using the AL1 primer (ATTggATCCAggCCgCTCTggACAAAATATgA-ATTC-$(T)_{24}$ or Anchor T primer, and the second PCR using OR-specific degenerate primers. The primers used to amplify a region of the transmembrane domain 3 (TM3)-TM6 were P26 (GCITA(C/T)GA(C/T)CGI-TA-(C/T)GTIGCIATITG) and P27 (ACIACIGAIAG(G/A)-TGIGAI(G/C)C(G/A)CAI-GT). The purified PCR products for the TM3–TM6 region were subcloned into pCR 2.1 or pCR II-TOPO vector (Invitrogen) and sequenced using a gel DNA-sequencer (DSQ-2000L; Shimadzu Co., Japan). The dendrogram was generated using the Clustal X program (Thompson *et al.*, 1997). The amino acid sequence of the TM3–TM6 region of each OR was used for the sequence comparisons.

Behavioral assay for evaluating odorant discrimination capability of mice

We employed a Y-maze behavioral assay for determining whether or not mice can discriminate *sCa* from *rCa*. The animals were treated in accordance with the guidelines for the care and use of laboratory animals of the National Institute of Health and of the AIST-Kansai Animal Experiment Committee. The basic design of the Y-maze was similar to that previously reported (Yamazaki *et al.*, 1999). In order to simplify the apparatus, we used negative pressure generated by a dry vacuum pump downstream of the Y-maze instead of positive pressure by a fan upstream of the Y-maze and omitted gates to control the starts and stops of each mouse's run. The Y-maze was made of glass tubes 80 mm in inner diameter (i.d.). The negative pressure was weakened between the Y-maze body and the vacuum pump by adjusting the opening of the leak line so that the flow rate of influx fresh air was 0.7 ml/min at each arm of the maze. The air influxes and effluxes passed through a 10 mm i.d. glass-tube port centered at each terminal cap of the maze. At the glass-tube port of each Y-maze arm, the influx air was odorized by being passed through a small stainless steel mesh basket holding cotton balls immersed with diluted odorants. Each mouse ran along the body of the maze for 18 cm from the starting area and selected one arm of 45 cm length for the subsequent run. The reward for odor concordance was a drop of water in a small glass funnel placed inside the terminal cap of the arm of the maze. If the mouse went straight to the funnel on the discordance odor side, the terminal cap with the funnel was removed before the mouse started to drink the water. When identical odors were presented at both arms, one of the two sets of odorized cotton balls had been selected for the concordance before the series of the assay. The terminal caps with the cotton balls were randomly exchanged between two arms. The glass funnels were also sometimes exchanged between the two terminal caps. These randomizations made the mouse pick each arm presenting the identical odor at an even chance. The odorants were diluted to target concentrations in propylene glycol. Identical odorant concentrations were prepared for both arms. The concordance odorant was fixed to *sCa* for carvone enantiomer discrimination in this study.

Results

Classification of odorant tuning specificities of ORNs

In our physiological assay system, the order of the relative sensitivities of individual ORNs among various odorants was conservative in repeated stimulations, at least for determining the most sensitive odorants, even though the sensitivities gradually decreased during the measurements. Figure 1 shows an example of the reproducibility of fluorescence changes normalized as shown in the Materials and Methods. The traces indicate changes in fura-2 fluorescence intensity excited at 380 nm, where a fluorescence decrease corresponds to a Ca^{2+} increase. The order of letters (A–D)

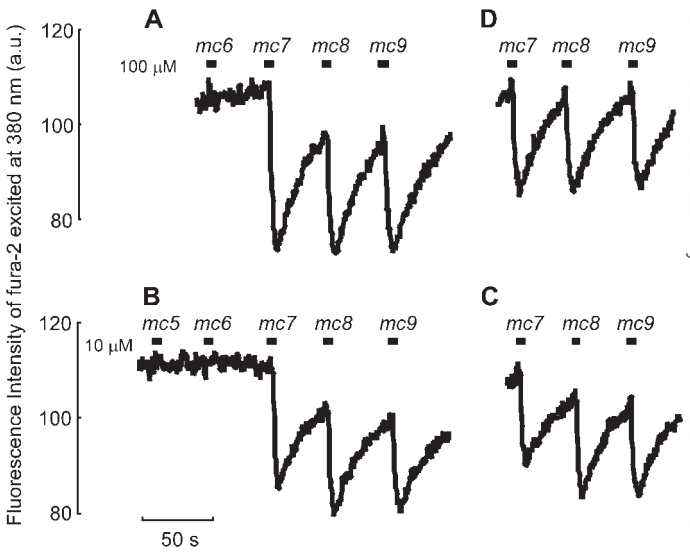

Figure 1 Reproducibility of odorant responses of isolated olfactory receptor neurons. The intensity of fluorescent light emission (510 nm) from a single fura-2-loaded neuron was monitored during continuous exposure to 380 nm light. A fluorescent decrease (in arbitrary units) indicated an increase in intracellular free calcium. The neuron was exposed to odorants for 4 s, as indicated by the bar, at the indicated concentrations. The order of the response amplitudes was sufficiently conservative among the tested odorants to determine the most sensitive odorants, although the responses gradually decreased during the recordings. The order of letters **(A–D)** indicates the order of measurements.

represents the order of recordings. At a concentration of 100 μM, the ORN responded to *mc7*, *mc8* and *mc9* with identical net fluorescence changes, indicating the identical Ca^{2+} increases at the maximum (Figure 1A). As shown in Figure 1B,C, differences in the relative sensitivities appeared clearer in the response amplitude at a low odorant concentration than at a high concentration. The response reduction rate in the identical stimuli generally became greater with increasing number of responses between two recordings. At 100 μM, all net response amplitudes in fluorescence changes similarly decreased to 62% of the initial responses after several recordings (Figure 1A,D). At 10 μM, the responses to the three odorants were reduced into 83, 91 and 90% of the respective responses in the preceding recording, but the order of response amplitude was conservative between the two successive recordings (Figure 1B,C). Single or two successive recordings for object odorants helped us determine whether the relative sensitivities were clearly different or not. Based on these observations, we set the relative response amplitude to a borderline at 60% of the largest responses in order to separate relative sensitivities from those with clear differences.

First, we determined the odorant tuning specificities of the carvone-responsive ORNs for a series of test odorants. The test odorants were selected to test our hypothesis regarding odor identity decomposition into unique odor qualities by unique ORs and multiple common odor qualities by common ORs. In addition to the carvone enantiomers *sCa* and *rCa*, we tested odorants chosen for the similarities of their odor qualities to the odors of the carvones. For example, *R*(+)-pulegone (*pu*), (–)menthone (*mn*), isopulegol (*ip*), menthol (*me*), isoamyl acetate (*am*), nerol (*ne*), *R*(+)-limonene (*lim*) and normal aliphatic alcohols (*mh6–9*) with a 6–9 carbon chain were selected for the common fresh and/or herbal odor qualities possessed by both carvones. The first four odorants were also similar to *rCa* in that they had a minty odor. All of the 11 odorants above, as well as vanillin (*va*), *o*-vanillin (*ova*) and geraniol (*ge*), present somewhat sweet odors, as do both carvones. We also included some compounds with very different odors: indole (*in*), triethylamine (*ta*) and isovaleric acid (*iv*). For a statistical comparison with our previous data, normal aliphatic acids (*mc6–9*) with a 6–9 carbon chain were also included from our previous series.

Most ORs for carvones were responsive to multiple odorants, as previously reported for other ORs (Sato *et al.*, 1994; Zhao *et al.*, 1998; Malnic *et al.*, 1999; Touhara *et al.*, 1999; Araneda *et al.*, 2000; Kajiya *et al.*, 2001; Bozza *et al.*, 2002). The responses of an ORN expressing an OR, *car-b85*, are shown as odorant-induced intracellular Ca^{2+} increases (Figure 2). The odorant tuning specificities were determined by relative response amplitudes among the odorants. The cell viabilities were confirmed at the beginning of the measurements (Figure 2A) and at almost the end (Figure 2F) by KCl and IBMX stimulations. The criteria for responses enabled us to identify small responses, such as those to 1 μM *rCa* or *sCa* (Figure 2E). The lowest (threshold) responsive concentration for an ORN was 1 μM, where

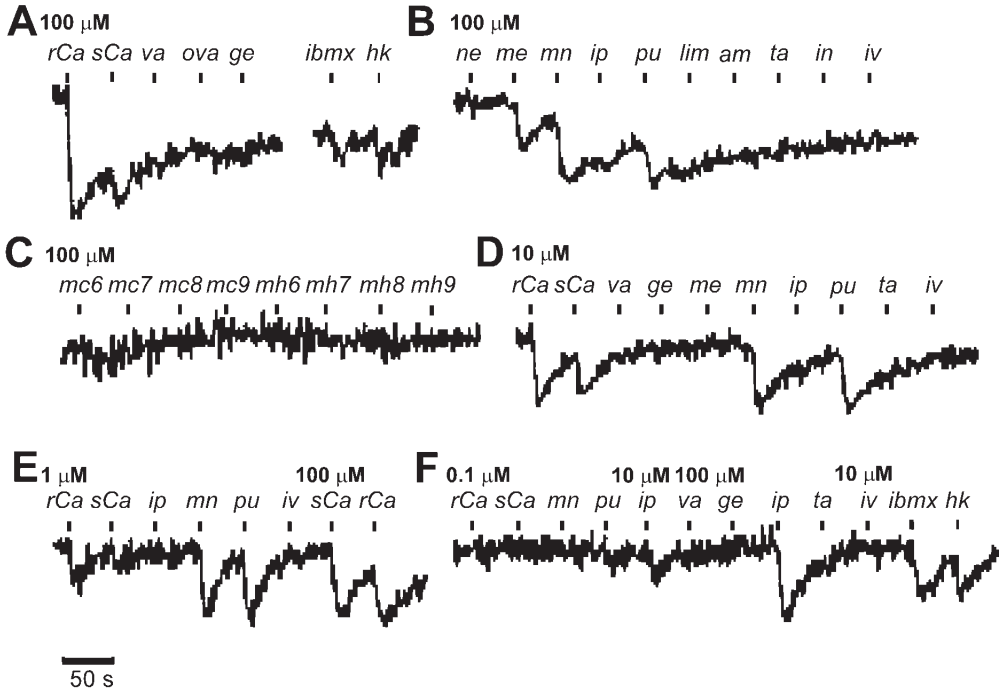

Figure 2 Odorant responsiveness of one type of isolated ORN. The measurements were undertaken for another neuron under the same conditions as in Figure 1. This neuron, which expressed the OR *car-b85*, responded to multiple odorants in the relative sensitivity of *pu*, *mn* > *sCa*, *rCa* > *ip*, *me*. The order of letters **(A–F)** indicates the order of measurements.

rCa, *sCa*, *mn* and *pu* induced responses with relative net fluorescence decreases of 44, 25, 95 and 100%. Based on the relative response amplitudes, the sensitivity of the ORN was determined as *pu*, *mn* > *rCa*, *sCa*. At 10 μM, *ip* induced a response in the ORN with a relative response amplitude of 48% (Figure 2F), normalized to a 10-μM-*pu* response (Figure 2D), while 100 μM *me* also induced a response of 58% of that evoked by 100 μM *mn* (Figure 2B). Taken together, the sensitivity among the test odorants was *pu*, *mn* > *rCa*, *sCa* > *ip*, *me*. The response amplitudes of *sCa* and *rCa* at 100 μM were reduced to 63 and 62% of the initial ones (Figure 2A,E), similar to the reduction in *mc*-induced responses shown in Figure 1. This result indicated that the sensitivity decrease was not too severe to compare the relative response amplitudes in our criteria. Consequently, the odorant tuning specificity of the ORN expressing the OR, *car-b85*, was determined as *pu*/*mn sCa*/*rCa* > *ip*/*me*. In the definition in this study (described in Materials and methods), *pu*/*mn* were the first odorants (the most sensitive odorants) and *sCa*/*rCa* were the second odorants (the second-most sensitive odorants). All time constants of the recovery phases of the responses varied within two standard deviations (27.2 s) from the average of 45.9 s.

Three ORNs expressing the identical ORs in the amino acid sequences showed similar odorant tuning specificities (Figures 3 and 4). The ORNs expressing ORs, *car-b153* and *car-b158*, came from the same preparation and the third ORN expressing an OR, *car-b86*, came from a different preparation. The three ORNs were identical in having the odorant tuning specificities of *sCa* > *pu*. Small fluorescence decreases induced by 100 μM *rCa* in all three cells were not considered valid responses but incomplete activations, because the time constants of the recovery phases differed from the averages by 15 standard deviations in individual ORNs (Figure 3; see Materials and methods). The sensitivity of the *car-b86* ORN was lower than those of the other two ORNs by approximately half an order of magnitude. The response of the *car-b86* ORN to 100 μM *pu* was 50% of that to 100 μM *sCa* and no clear responses were evoked by 10 μM *sCa*, whereas the responses of the ORNs with *car-b153* or *car-b158* to 100 μM *pu* were 88 and 100% of those to 100 μM *sCa*, respectively; clear responses were evoked only by *sCa* at 10 μM. The ratio of the net fluorescence changes of IBMX versus KCl in the *car-b86* ORN at the beginning of the measurements was smaller than those of the *car-b153* and the *car-b158* ORNs at the beginning of the measurements and similar to those of the two ORNs at the end of the measurements (Figure 3A,F,E′). This ratio is an indicator of signal amplification by the cyclic-AMP second messenger system in individual ORNs. Thus, the sensitivity of the *car-b86* ORN might be reduced throughout its receptive range, compared to the *car-b153* and the *car-b158* ORNs, but with little change in tuning specificity. Consequently, the odorant tuning specificities of the same types of ORs/ORNs could be quite similar except for differences in absolute sensitivities in some cases, as reported previously (Bozza *et al.*, 2002). Based on these observations, we classified the odorant responsiveness of ORs/ORNs by the first odorants and, supplementarily, the second odorants.

To adequately sample nearly all types of carvone ORNs, we assayed 2740 ORNs which is ~2.7 times the estimated ~1000 types of ORs (Buck and Axel, 1991). Out of 2740 ORNs, 263 ORNs (9.6%) were responsive to *sCa* and/or *rCa*. Out of 263 carvone ORNs, 234 ORNs were responsive to *sCa* and 222 ORNs were responsive to *rCa*. First, the odorant tuning specificities of ORNs were classified into four primary classes in terms of their most sensitive odorants among the carvones and other odorants, i.e. *sCa*-sensitive (n = 59, 22% of 263 ORNs), *rCa*-sensitive (n = 49, 19%), *sCa*/*rCa*-equi-sensitive (n = 100, 38%) and other-odorant-sensitive (n = 55, 21%). They could be further divided into >41 subclasses by the combination of the first and second odorants in the tuning specificities (data not shown). However, most odorants, except for *sCa* or *rCa*, were not tested against all ORNs. For example, *pu* and *mn* were not tested in 105 carvone ORNs (40% of 263). Therefore, some additional subclasses may emerge and the subpopulation of subclasses may change after exhaustive assays have been completed.

Another possible source of variation of the ORN subpopulation of each subclass might be heterogeneous sampling of the four OR expression zones in the olfactory epithelium. We roughly estimated the largest variation of sampling rates among the four epithelial zones to be a factor of three for zone 1 versus zone 3, where the expected sampling number of ORNs was 320. Because the grand average of the subpopulations of OR types in a zone was 250 for a total of 1000 types of ORs throughout the four zones, the ORNs assayed in this study were expected to cover most types of ORs. We concluded that the largest subpopulation of ORs is the *sCa*/*rCa* equi-sensitive type and the second largest, the *sCa*-sensitive type. Our finding that the number of *sCa*/*rCa*-discriminating ORs was only about one-half the number of *sCa*/*rCa*-non-discriminating ORs indicates the need for an additional mechanism for combining receptor signals to produce the characteristically distinct perceived odors of the carvones.

In order to estimate how many ORs are responsive to *sCa* and *rCa*, we tried to clone ORs from ORNs by single-cell RT-PCR using degenerate primers, after completing the physiological assays for odorant responsiveness. We obtained OR cDNA products from 28/103 ORNs responsive to carvones. They included four pairs and two trios of the same ORs, resulting in a total of 20 types of ORs. Because most of the ORN subclasses were subjected to single-cell RT-PCR in a random manner (data not shown), we employed an OR-type multiplicity factor of 28/20 in this study. This ratio produced an estimated 70 ± α types of carvone ORs from a 9.6% subpopulation of responsive ORNs in 1000 ORs. The

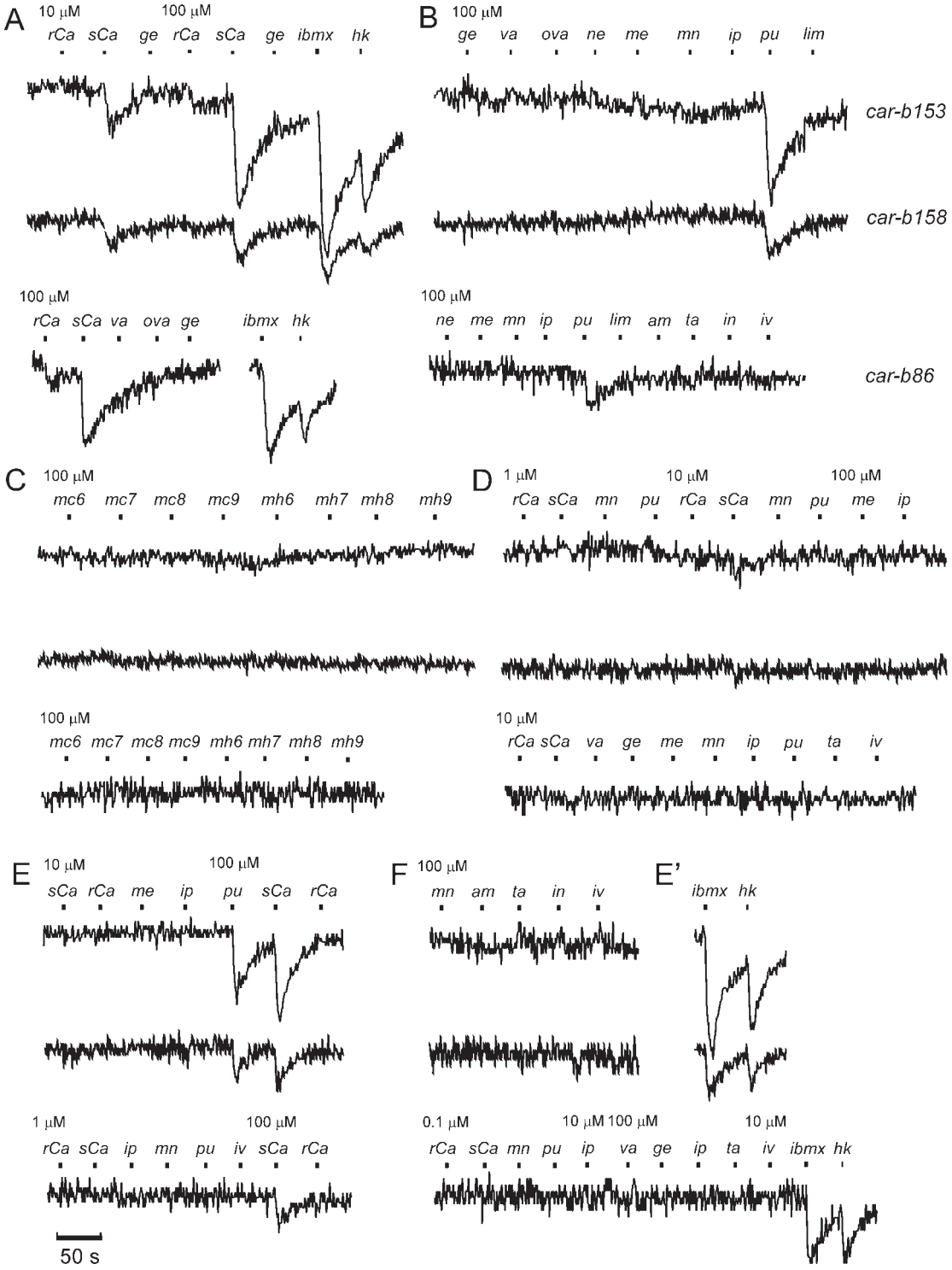

Figure 3 Odorant responsiveness of the three isolated ORNs expressing identical ORs. The odorant responsiveness of three ORs, *car-b86*, *car-b155* and *car-b158*, were shown to be the same as in Figure 1. The three ORNs that expressed the identical ORs *car-b86*, *car-b155* and *car-b158*, were all identical in having the tuning specificities of *sCa* > *pu*. The sensitivity of the ORN expressing the OR *car-b86* was lower than those of the other ORNs.

RT-PCR success rate was 5/6 with no Ca-imaging assays. These rates before or after the Ca-imaging assays were similar to those of 18/26 and 14/47 reported previously (Malnic *et al.*, 1999). This accordance indicates that our single-cell RT-PCR would amplify a large variety of ORs as well as in the previous study.

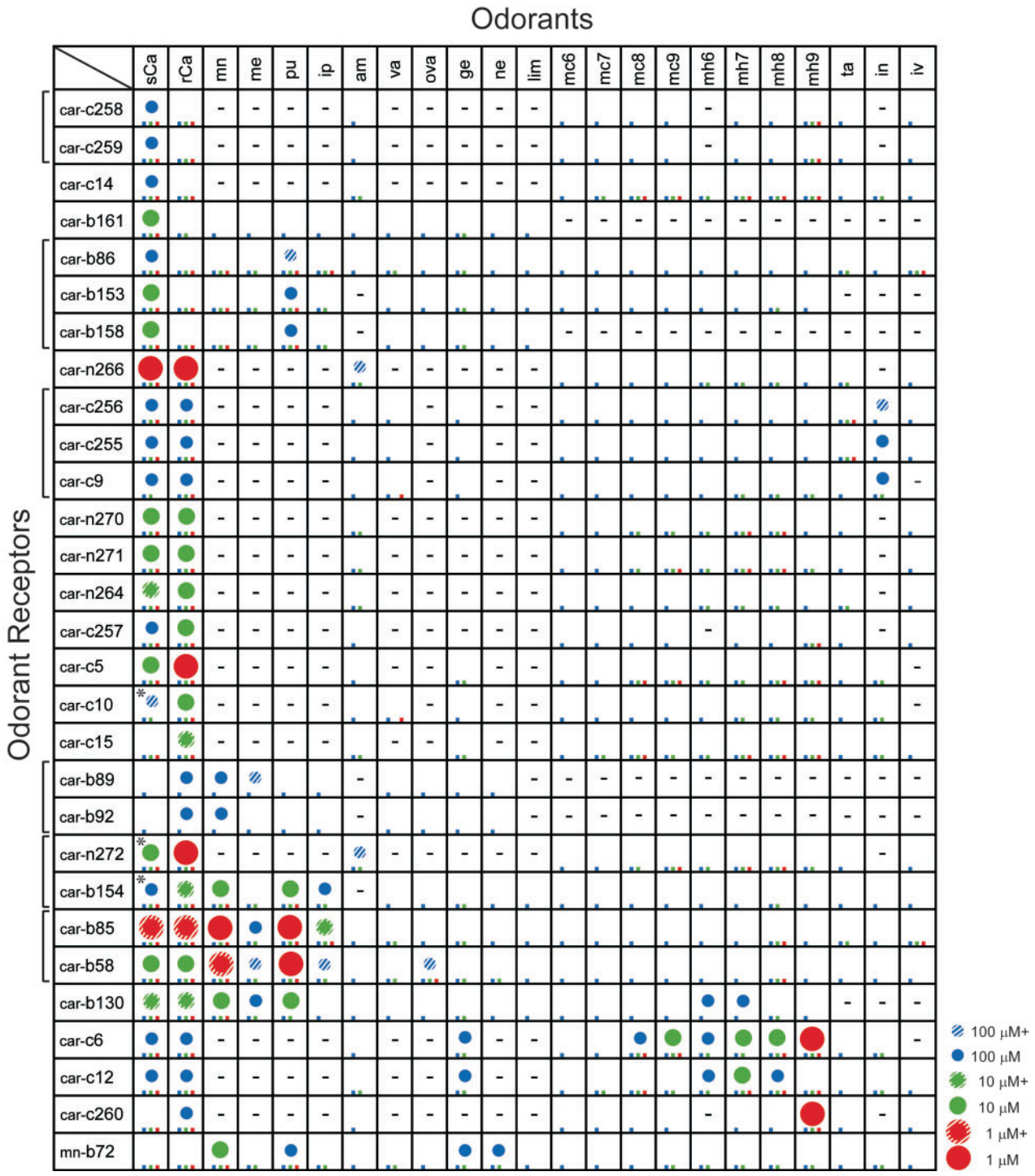

Figure 4 The sensitivity profiles of 28 carvone ORs and one menthone OR. The ORs' threshold concentrations for odorants are indicated by different colored circles. The odorant concentrations tested are shown in small squares on the bottom in each matrix: left blue, 100 μM; middle green, 10 μM; right red, 1 μM. The threshold concentrations marked by plus symbols indicate responses with amplitudes of <60% of the maximum among all responses at that concentration. The ORs within single brackets were the same in the DNA sequence, respectively. The sensitivities marked by asterisks are explained in the Results.

Sensitivity profiles of carvone-responsive ORs among test odorants

Figure 4 shows the sensitivity profiles of 28 *sCa*- or *rCa*-responsive ORNs (20 types) and one *mn*-sensitive ORN that was responsive to neither *sCa* nor *rCa*. The sensitivities are demonstrated by the threshold concentration for each

odorant. In most cases, the set of ORs with identical sequences showed quite similar sensitivity profiles for test odorants, as described above. As shown previously (Malnic *et al.*, 1999), one odorant is recognized by multiple ORs, in a manner in which different odorants are recognized by different combinations of ORs with some overlaps. In this study, we focused the difference in the classified subpopulations of sensitive ORNs as described in the next section.

The TM3–TM6 regions of 18 types of carvone ORs were compared with 97 ORs previously identified in mice (Figure 5). Seven of the 18 carvone ORs were grouped in the same cluster, indicating greater similarity to one another than to other ORs. Out of a possible 153 pairs among 18 ORs, 125 pairs (82%) possessed 35–45% amino acid identities among the TM3–TM6 regions and two pairs showed higher identities of >77%.

Additionally, we would like to mention an interesting observation: three ORs marked by asterisks in Figure 4—*car-c10*, *car-n272* and *car-b154*—showed a synergistic effect of *rCa* and *sCa*. The indicated thresholds were observed for *sCa* after *rCa* stimulation with an interstimulus interval of ~35 s. When *sCa* was applied prior to *rCa*, the threshold concentrations of *sCa* were elevated by more than one order of magnification. This phenomenon suggests the existence of an intermediate activation state, in addition to the resting states and activated states of some ORs.

Receptor codes for odors of enantiomeric odorants *sCa* and *rCa*

How is odor quality encoded in odorant receptors? To discover the strategy used to encode odor qualities in ORs, we evaluated overlap rates of the activated receptors between each of the carvones and the test odorants and compared them with overlaps of submodal odor qualities of the odorants (Table 1). The receptor overlap rates were highest between *sCa* and *rCa* (>80%), followed by *pu*, *mn* and *ip* in decreasing order. Based on the uniformly weighted

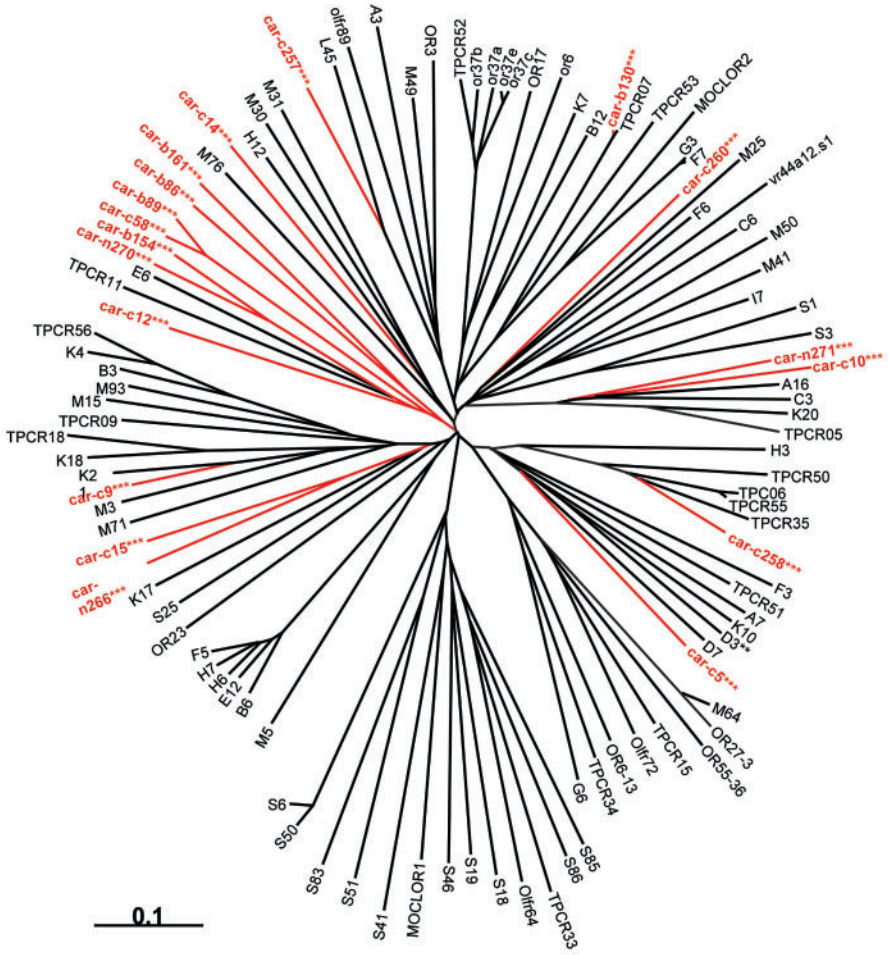

Figure 5 The relationship among the carvone ORs and the other murine OR family members. Amino acid sequence similarities among the 25 carvone ORs (colored in red and marked with triple asterisks) responsive to enantiomeric carvones were compared with the other 97 murine ORs for the TM3–TM6 regions using the Clustal X program. Some of the carvone ORs were clustered in neighborhoods, indicating that they are more similar to one another than to other ORs. D3 (with double asterisks) was reported to be responsive to the carvones.

Table 1 Responsiveness of carvone-responsive ORNs to test odorants

ORNs	Odorants											
	sCa	*rCa*	*pu*	*mn*	*me*	*ip*	*am*	*va*	*ova*	*ge*	*ne*	*lim*
% assays of 234 *sCa*-ORNs	(100)	(100)	(59)	(59)	(59)	(59)	(81)	(82)	(59)	(75)	(51)	(59)
% responsive ORNs	100	82	52	39	14	24	7	2	7	15	16	1
% assays of 222 *rCa*-ORNs	(100)	(100)	(55)	(55)	(55)	(55)	(81)	(81)	(56)	(74)	(48)	(56)
% responsive ORNs	87	100	51	43	14	24	8	3	7	18	17	2
Perceptive odor qualities	fresh	fresh	fresh	fresh	fresh		fresh				fresh	fresh
	herbal	herbal	herbal			herbal						
	sweet[a]	sweet	sweet	sweet[a]		sweet	sweet	sweet	sweet	sweet	sweet	
		spearmint	minty	minty	minty	minty						
	caraway		camphor	woody	camphor[a]	citrus[a]	banana	vanilla	vanilla	rose	fruity	orange
			resinous		cooling		fruity	creamy	pungent	floral		citrus

	Odorants										
	mc6	*mc7*	*mc8*	*mc9*	*mh6*	*mh7*	*mh8*	*mh9*	*ta*	*in*	*iv*
% assays of 234 *sCa*-ORNs	(94)	(94)	(96)	(94)	(94)	(94)	(94)	(91)	(74)	(65)	(77)
% responsive ORNs	1	3	7	9	13	19	19	19	9	4	3
% assays of 222 *rCa*-ORNs	(93)	(93)	(95)	(93)	(93)	(93)	(93)	(90)	(76)	(64)	(77)
% responsive ORNs	1	3	8	9	14	21	21	22	10	4	2
Perceptive odor qualities						fresh	fresh	fresh			
					herbal	herbal	herbal				herbal[b]
					sweet	sweet	sweet	sweet			
	goat-like	rancid	repulsive	nut-like	woody	woody	rose	rose[b]	fishy	repulsive	putrid
	sweaty	sweaty	sweaty	waxy	cognac	violet	waxy	floral	ammonia	floral[b]	sweaty
	fatty, etc.	fatty, etc.	fatty, etc.	fatty, etc.	Scotch	fatty	fatty, etc.	fatty	pungent		cheese

Assayed *sCa*- or *rCa*-ORNs for the other odorants are shown as percentages in parentheses. The percentage of subpopulations of *sCa*- or *rCa*-ORNs responsive to odorants are shown. The abbreviations of the odorants are shown in Materials and methods. The perceived odor qualities shown in the bottom panel were obtained from references (Arctander, 1969: Boelens *et al.*, 1993), The Good Scents Company (http://www.execpc.com) and The Chemfinder Web Server (http://chemfinder.cambrigesoft.com).

[a]We were able to perceive the odor qualities.

[b]Odor qualities appear in extreme dilution.

summation of ORN signals in a simple combinatorial receptor coding scheme, the observed overlap of >80% most likely leads to expected, almost-identical odors for both odorants, which are inconsistent with the perceived characteristic odors of *sCa* and *rCa*.

More detailed comparisons are described as follows. The principal strategies of odor encoding and decoding may be similar across mammalian species. Because the submodal odor qualities have not been determined in mice, the odor qualities of the odorant were substitutively represented by those perceived by humans. The odorants *sCa*, *rCa* and *pu*, whose receptors overlap with the carvone receptors ranging from 51 to 87% in cross-comparison, commonly possess three submodal odors of 'fresh', 'herbal' and 'sweet'. However, the odorants *mh7* and *mh8*, which also possess 'fresh', 'herbal' and 'sweet' odors, shared common receptors in the receptor codes with carvones only at 19 or 21%. Such a smaller receptor overlap rate might represent the perceived strengths of 'fresh', 'herbal' and 'sweet' odors in *mh7* and *mh8* to a weaker degree than in *sCa*, *rCa* and *pu*. In contrast, the receptor overlap rate between the carvones and *va*, which presents a more intense 'sweet' odor than the carvones, was only 2%. This small overlap rate may be attributed to an existence of different 'sweet' odor qualities or a difference between species. The overlaps of the carvone receptors were, respectively, 13 and 21% with the receptors for *mh6* and *mh9*, aliphatic alcohols that also possess a 'sweet' odor and a 'herbal' or 'fresh' odor. Additionally, the odorants *mn*, *ip*, *am*, *ova*, *ge* and *ne*, showing receptor overlap rates ranging from 7 to 43%, also present a 'sweet' odor. This finding suggests that the common ORs for these odorants may partially contribute to a common 'sweet' odor quality among the odorants.

In contrast, the fraction of carvone ORNs that were responsive to the aliphatic acids *mc6* or *mc7* (with no clear odor qualities shared with the carvones) was less than one-sixth of the fraction responsive to the aliphatic alcohols *mh6* or *mh7* (with three common odor qualities, as described above). This was so, despite the similarity of the molecular structures of *mc* and *mh*, except for the different terminal

functional groups of carboxyl versus hydroxyl. These results could lead to the following hypothesis: as the overlapped subpopulation of carvone ORNs among test odorants increased, the common odor qualities increased in number and/or intensity. In other words, a common odor quality among multiple odorants may be encoded by subsets of common ORs in the manner of a weighted and integrated population. Furthermore, a small surplus in the receptor overlap rate (4%) was observed in the *rCa–mn* pair with respect to the *sCa–mn* pair. It is likely that the surplus carvone ORs may contribute to a 'minty' odor characteristic of *rCa* but not to that of *sCa*. We observed some ORs that were *mn*-sensitive and *sCa*/*rC*a-non-responsive (Figure 4). Such *mn*-unique ORs should contribute to the composition of an *mn*-unique odor quality.

Three odorants (*mc8*, *mc9* and *ta*) that smelled quite differently (repulsive, nut-like and fishy, respectively) from the carvones induced responses commonly in ~10% of the carvone ORNs and most of their sensitivities were not particularly high. This result leads to the hypothesis that more sensitive ORs contribute strongly to the representation of the respective odor qualities and less sensitive ORs contribute only weakly. If the ORN sampling procedure in our experiments was relatively unbiased, then the odorant sensitivities of our assayed ORNs should provide a good estimate of the subpopulation of carvone ORs in the entire olfactory epithelium. Based on this assumption, we compared the actual numbers of the classified ORNs sensitive to *sCa* or *rCa* at 1 or 10 μM.

Our analysis revealed that a difference between the receptors classified by the most sensitive odorants characteristically appeared at the lowest effective stimulus concentrations and the *sCa*/*rCa*-non-discriminative ORNs became dominant at 10 times the lowest concentration (Table 2). At 1 μM, *rCa* activated four *rCa*-sensitive ORNs, two *sCa*/*rCa*-equi-sensitive ORNs and one of each of the other five types of ORNs, whereas *sCa* activated two *sCa*/*rCa*-equi-sensitive ORNs, two *sCa*-sensitive ORNs, two *rCa*-sensitive ORN and one of each of the other three types of ORNs. Thus, the four *rCa*-sensitive ORNs were the largest subpopulations among the classified *rCa*-most-sensitive ORNs, whereas the two *sCa*-sensitive ORNs and the two *sCa*/*rCa*-non-discriminative ORNs were the largest subpopulations among the classified *sCa*-most-sensitive ORNs. The *rCa*-sensitive ORNs activated by1 μM *sCa* were not included in the most sensitive types, because the responses were smaller in the percentage maximum amplitude than those of the *sCa*/*rCa*-equi-sensitive ORNs. Interestingly, the principal odor qualities of the target odorants were represented by the principal (common) odor qualities of the most sensitive odorants of the most sensitive ORs which occupied the largest subpopulation at the lowest effective stimulus concentration, although the murine OR codes applied to the odor qualities perceived by humans. This observation led to the hypothesis that the signals of the most sensitive ORs of the largest subpopulation may govern the signal processing for the odor identity representation in the brain.

By increasing the stimulus concentration up to 10 μM, 19 *sCa*-sensitive ORNs and three *rCa*-sensitive ORNs were recruited by *sCa*, 14 *rCa*-sensitive ORNs and six *sCa* -sensitive ORNs were recruited by *rCa*, in addition to 39 *sCa*/*rCa*-equi-sensitive ORNs. The largest subpopulation of the *sCa*/*rCa*-equi-sensitive ORNs, which were one subclass of the most sensitive ORNs for *sCa*, could explain the stronger 'herbal' or 'sweet' quality, which is common to *sCa* and *rCa*, in the odor of *sCa*, compared to *rCa*. However, based on the simple combinatorial receptor coding scheme, it is difficult to understand the fact that the *rCa*-sensitive ORNs, which were approximately one-third of the *sCa*/*rCa*-equi-sensitive ORNs activated by 10 μM *rCa*, formed a 'minty' odor of *rCa* more intensively than the 'herbal' quality of *rCa*. These results strongly indicate that the signals from the most sensitive ORs govern olfactory information processing by selectively composing OR signals that represent principal odor qualities of the target odorants, instead of a simple combinatorial integration of receptor signals to synthesize odor qualities. This also suggests that 1-μM-*rCa*-activated signals may feed into an inhibitory circuit that suppresses the less sensitive and different submodal signals in the brain. The receptors most sensitive to the odorant provide the signaling framework that defines the unique odor quality of the target odorant and the multiple subsets of ORs less-sensitive to the odorant contributes to the supplementary odor qualities of the target odorant. Thus, the sensitivity-dependent hierarchical receptor codes can well describe odor identity encoding and decoding in the olfaction.

Carvone enantiomer discrimination in mice

sCa and *rCa* have been reported to be discriminative in humans, monkeys and rats (Friedman and Miller, 1971; Leitereg *et al.*, 1971; Boelens *et al.*, 1993; Laska *et al.*, 1999; Linster *et al.*, 2001), but not in mice. In order to determine whether mice can discriminate *sCa* from *rCa*, we conducted behavioral assays using a Y-maze. One NZB mouse and one CBA mouse were initially trained to discriminate amyl acetate from mineral oil. After the concordance trials occupied >80% of each trial set (12 or 24 trials), the mice were tested to discriminate *sCa* from *rCa*. The concordance trials immediately increased to >80% (Figure 6). This value was significantly different from even chance in the χ^2 test ($P < 0.05$), indicating that the mice could discriminate *sCa* from *rCa*. The mice successively performed significant discriminations for more than eight sets of trials, even when the odorant concentrations were gradually reduced to 1/4000 v/v step by step. The NZB mouse demonstrated a decrease in the concordance trial rate at 1/40 000 v/v and the following 1/400 000 v/v (Figure 6A). The mouse could not

Table 2 Subpopulations of sensitive ORNs classified by the most sensitive odorants

First odorants	Second odorants	1 μM *sCa*		1 μM *rCa*	Classified ORNs: *sCa*/*rCa*
sCa (1)	*rCa* (10)	1	>		2/0
sCa (1)	*rCa*/*pu*/*mn* (10)	1	>		
sCa/*pu* (1)	*rCa* (100)	1	>		1/0
sCa/*rCa*/ (1)	[*am*, *ge*/*in*] (100)	2	=	2	2/2
sCa/*rCa*/*pu* (1)	*ov* (100)	1	=	1	1/1
rCa (1)	*sCa* (1+, 10)	2	<	4	2/4
rCa/*pu* (1)	*sCa*/*mn* (10)		<	1	0/1
rCa/*mn* (1)	*sCa*/*pu*/*ip*/*me*/*mh*/*ne* (100)		<	1	0/1
pu/*mn* (1)	*rCa* (1+)		<	1	0/1
ge (1)	*rCa* (1+)		<	1	0/1
First odorants	Second odorants	10 μM *sCa*		10 μM *rCa*	Classified ORNs: *sCa*/*rCa*
sCa (10)	[–, *pu*] (100)	10	>		19/3
sCa (1, 10)	*rCa*/[–, *pu*/*mn*, *pu*/*mn*/*me*] (10, 10+, 100)	9	>	3	
sCa/*mn*/[–, *ip*] (10)	*rCa*/[*me*, *pu*/*me*, *pu*/*me*/*ip*] (100)	3	>		3/0
sCa/*pu* (1)	*rCa* (100)	1	>		1/0
mh (1)	*sCa*/[*mc*, *mc*/*rCa*, *rCa*] (10)	4	>		4/0
mh/*ge*/*ne* (1)	*sCa*/*mc* (10)	1	>		1/0
sCa/*rCa* (10)		17	=	17	39/39
sCa/*rCa* (10)	[*mn*, *pu*/*mn*, *pu*/*me*/*ip*] (10+, 100)	7	=	7	
sCa/*rCa* (1, 10)	*am*/[–, *mn*, *ge*/*in*] (100)	3	=	3	
sCa/*rCa* (10)	*am*/*mh*/[–, *ge*, *mc*] (100)	3	=	3	
sCa/*rCa* (10)	*mh*/[–, *mn*/*ip*/*mc*, *mc*] (100)	3	=	3	
sCa/*rCa* (1, 10)	*ge*/[–, *in*] (100)	4	=	4	
sCa/*rCa* (10)	others (100)	2	=	2	
sCa/*rCa*/*pu*/*mn*/*me* (10)	*ge* (100)	1	=	1	1/1
sCa/*rCa*/*pu* (1, 10)	[–, *ov*] (100)	3	=	3	3/3
sCa/*rCa*/*mn* (10)	[*pu*, *pu*/*ip*/*me*/*ne*] (10+, 100)	3	=	3	3/3
sCa/*rCa*/*mh* (10)	[*pu*/*ip*, *mc*/*am*] (100)	2	=	2	2/2
sCa/*rCa*/others (10)	[–, *mh*/*ne*, *mn*/*ip*] (100)	3	=	3	3/3
pu/*mn* (1, 10)	[*rCa*, *rCa*/*sCa*, *me*/*mh*] (1+, 10, 10+)	4	=	4	4/4
mn (1)	*sCa*/*rCa*/*pu*/[–, *ip*] (10)	2	=	2	2/2
ge (1, 10)	*rCa* (1+, 10+)	2	=	2	2/2
rCa/*pu* (1)	*sCa*/*mn* (10)	1	=	1	1/1
rCa (10)			<	1	6/14
rCa (1, 10)	*sCa* (1+, 10, 10+, 100)	5	<	9	
rCa (10)	*sCa*/[*pu*, *pu*/*mn*, *mh*] (10, 100)	1	<	4	
rCa/*pu*/*ov* (10)	*mn* (10+)		<	1	0/1
rCa/*mn* (1, 10)	*sCa*/[*ge*, *pu*/*ip*/*me*/*mh*/*ne*] (100)		<	2	0/2
pu (1, 10)	*rCa*/[*sCa*, *sCa*/*mn*, *mn*/*ov*, –] (10, 10+)	4	<	5	4/5

The odorant tuning specificities were classified by the combination of the most sensitive odorants and the second-most sensitive odorants. The observed numbers of ORNs responsive to *sCa* or *rCa* at 1 μM or 10 μM are summarized in each subclass. The threshold concentrations in micromol for the odorants are shown in parentheses. The threshold concentrations with a plus symbol indicate that the odorants evoked responses with amplitudes <60% of the largest responses at the concentration. One of the sets of odorants in brackets or one of the threshold concentrations in parentheses corresponds to those of the single ORNs. A minus symbol indicates no response. One to all of the odorants shown in brackets induced responses in the ORNs. The summated subpopulations of ORNs in each type classified by the most sensitive odorants are shown for *sCa* versus *rCa* in the right-hand column. Other aspects are the same as in Figure 4.

discriminate *sCa* from the identical *sCa* at 1/100 v/v or 1/2000 v/v in this Y-maze assay. However, the NZB mouse demonstrated a significant concordance trial rate for discriminating *sCa* from *rCa* at 1/80 000 v/v after the *sCa* versus *sCa* trials (Figure 6A).

The CBA mouse also demonstrated significant results in the Y-maze behavioral assays for discriminating *sCa* from *rCa* (Figure 6B). The CBA mouse lost discriminative performance for a pair of *sCa* and *rCa* at 1/400 000 v/v and 1/4 000 000 v/v. The concordance trial rates recovered to

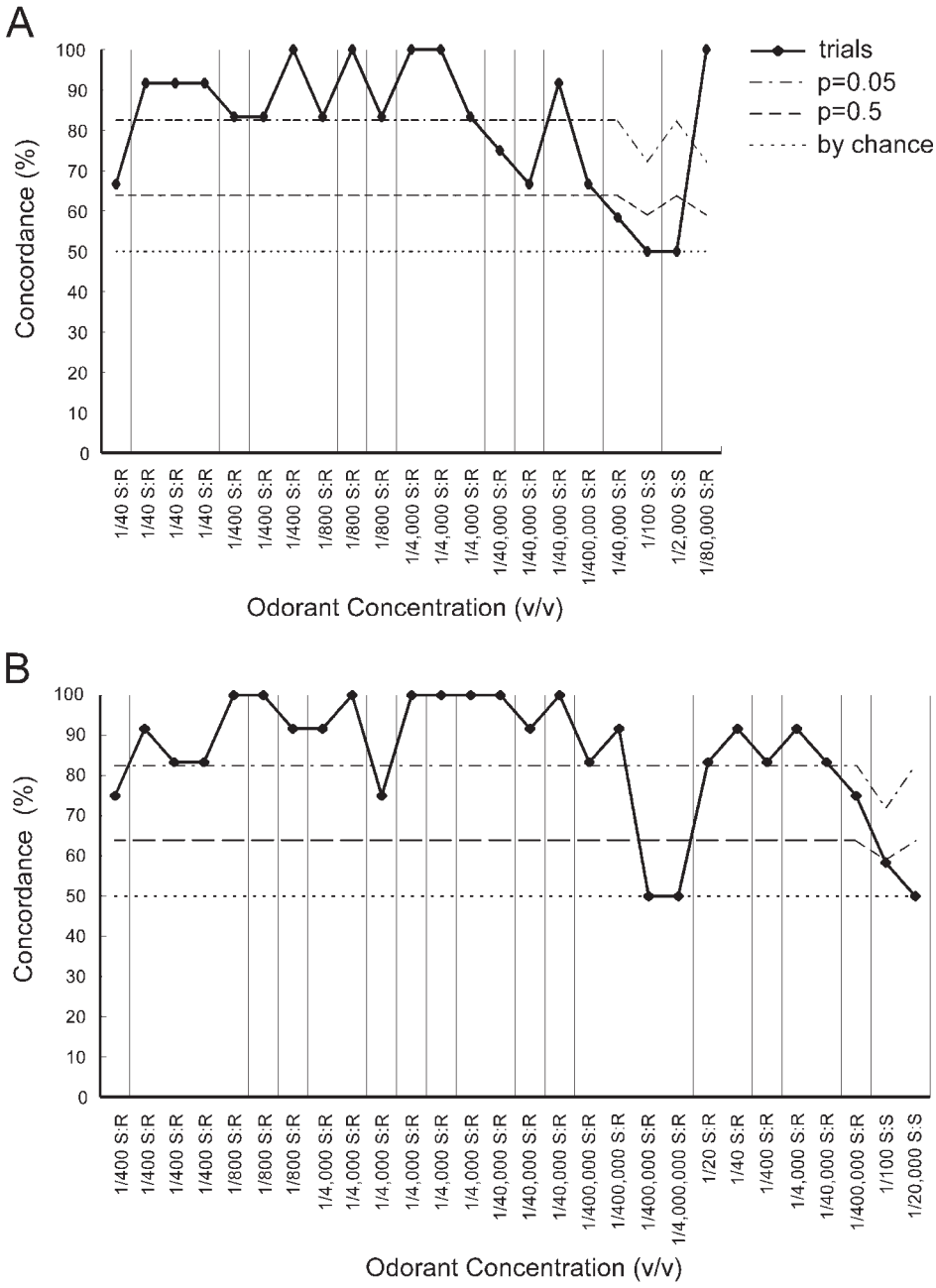

Figure 6 The behavioral assays for evaluating odor discrimination capability in mice. Each mouse could discriminate *sCa* (S) from *rCa* (R) at various concentrations, but not between identical odors of *sCa* presented in each arm of the Y-maze. The test odorant pairs were arranged in the order of the assays and shown by the initials on the horizontal axis: S:R for *sCa* from *rCa* and S:S for *sCa* from *sCa*. The concentrations of odorants diluted in propylene glycol were identical in both arms, as shown by 'v/v' at the front of the odorant pairs. Each data point consisted of 12 or 24 trials. **(A)** NZB strain. **(B)** CBA strain.

>80% by elevating the concentrations up to 1/20 v/v. After the mouse demonstrated >80% concordance performances, the significance of concordance again showed a decrease to near the level of $P = 0.5$ (χ^2 test) at a concentration of 1/400 000 v/v. The concordance trial rates for the following identical pair trial of *sCa* further decreased to the level of even chance. These results were preliminary in evaluating the lowest concentration for discrimination between *sCa* and *rCa*. However, the results demonstrated that mice can discriminate *sCa* from *rCa* at concentrations >1/4000 v/v, where most carvone ORs of mice might be activated intensively.

Discussion

Odorants seem difficult to discern because of their small molecular size and slight structural differences across

many compounds. How is odor quality encoded during olfactory reception? The biological systems are generally hierarchically structured and derived from layers of simpler functional modules. Salient coding pathways in neural systems may be predetermined by genetic hard-wiring and modified via experience-driven plasticity. In the present study, we explored the structure of pathways encoding odor quality at the peripheral level by undertaking a detailed analysis of the receptor codes for a chiral pair of molecules, the *S*(+)- and *R*(–)-carvone, in the sensitivities and the subpopulations classified by the odorant tuning specificities of ORNs. In our data analysis and interpretations, we have made the working assumption that there is a general correspondence between human ORs and respective murine ORs regarding odorant tuning specificity and, simultaneously, that there are no large differences in OR members among the three mouse strains CBA, BALB/c and NZB. The principal strategies of odor encoding and decoding may be similar across mammalian species. Surprisingly, the hierarchical receptor code hypothesis can explain the human-perceptive odor qualities characteristic of the carvone enantiomers in murine receptor codes. This result suggests that our assumptions may be not greatly different from nature. As learned from the Human Genome Project, the expected number of human ORs is about one-third that of murine ORs, after omitting pseudogenes. The large family size of murine ORs may indicate that odor discrimination capabilities should be generally higher in mice than in humans. The validity of our working hypothesis is still subject to future verification by functional assays of human ORs.

Differential responses to carvone enantiomers have been observed previously in the assays of ORNs/ORs (Krautwurst *et al.*, 1998; Ma and Shepherd, 2000), in the assays of limited subsets of glomeruli in the olfactory bulb (Taniguchi *et al.*, 1992; Rubin and Katz, 1999, 2001) and entirely in the olfactory bulb at a single concentration (Linster *et al.*, 2001). However, the differences in their global receptor codes have remained unclear. We attempted to screen nearly all types of ORs/ORNs responsive to the carvone enantiomers by assaying ORNs numbering more than twice the expected 1000 types of ORs from all four epithelial zones. We found that 9.6% of the cells responded to one or both carvones. More than 80% of carvone-responsive ORs demonstrated overlapping sensitivities between the enantiomers. This finding is difficult to reconcile with a simple combinatorial scheme in which the responses of all ORs are equally weighted. Such a scheme predicts enantiomers with closely similar, not clearly distinctive, odor qualities at moderate stimulus concentrations. Their odors would be dominated by the common odor quality encoded by the non-discriminating ORs, which occupy some 40% of carvone ORs and outnumber the discriminating ORs 2-fold. Contrary to this prediction, the present study demonstrates that mice can discriminate *sCa* from *rCa* at relatively high concentrations in the Y-maze assay.

Furthermore, the receptors that were activated at the lowest effective stimulus concentration represented the principal odor qualities of each enantiomer by means of the principal odors of the most sensitive odorants for the receptors. These characteristic receptors in the receptor codes became a minority in the subpopulation at 10 times the lowest effective concentration. The combinatorial scheme predicts a dramatic change in the principal odor qualities of *rCa*, corresponding to the shift of the major subclass along the odorant concentration increase. To produce the divergent and constant perceptual odor qualities of the two chiral forms, we postulate that the olfactory system imposes non-uniform weighting on the OR inputs, granting higher priority to the more specific, more sensitive, chiral-discriminating ORs. This would allow minor subpopulations of chiral-discriminating ORs to govern the odor quality and counterbalance numerically superior sets of chiral-non-discriminating ORs. In general, we hypothesize that single-odorant-sensitive ORs contribute primarily to unique odor qualities and that multiple-odorant-equi-sensitive ORs contribute to the encoding of common odor qualities, and that odor identity is represented by composing OR signals for submodal odors of the target odorant in a sensitivity-dependent hierarchical manner.

The number of 1-μM-*rCa*-sensitive ORs was twice that of the 1-μM-*sCa*-sensitive ORs, although the total sample size was small. If the human OR system is an analogous to the murine OR system except for the large member size and a small subpopulation of ORs most sensitive to *rCa* strongly contributes to the sensitivity to *rCa*, one can easily understand the higher sensitivity to *rCa* compared to *sCa* observed in the perceptual threshold (Polak *et al.*, 1989). Various specific anosmias are defined as the significant elevation of sensory thresholds for particular odorants (Amoore and Steinle, 1991). In our hierarchical sensitivity model, specific anosmias arise naturally as the genetic disruption of odor-identity-governing ORs, or as a reduction in their weighted central integration. Interestingly, the defect degrees of human specific anosmias were almost twice as great in *rCa* in the logarithmic threshold than in *sCa* (Pelosi and Viti, 1978). It is more difficult to explain specific anosmias to odorants with tens of responsive receptors in terms of the combinatorial model of odor coding.

Odorant receptor signals converge onto OR-type-specific glomeruli in the olfactory bulb. The convergent pathways genetically form a stereotypic map of OR signal inputs to the brain. Odorant-characteristic glomerular maps activated near the threshold concentration in the olfactory bulb have also been reported for the other odorants (Wachowiak and Cohen, 2001). They also reported significant overlaps of glomerular maps between different odorants at higher concentrations, as shown here. The signals generated by ORs most sensitive to the most intense compounds in a complex

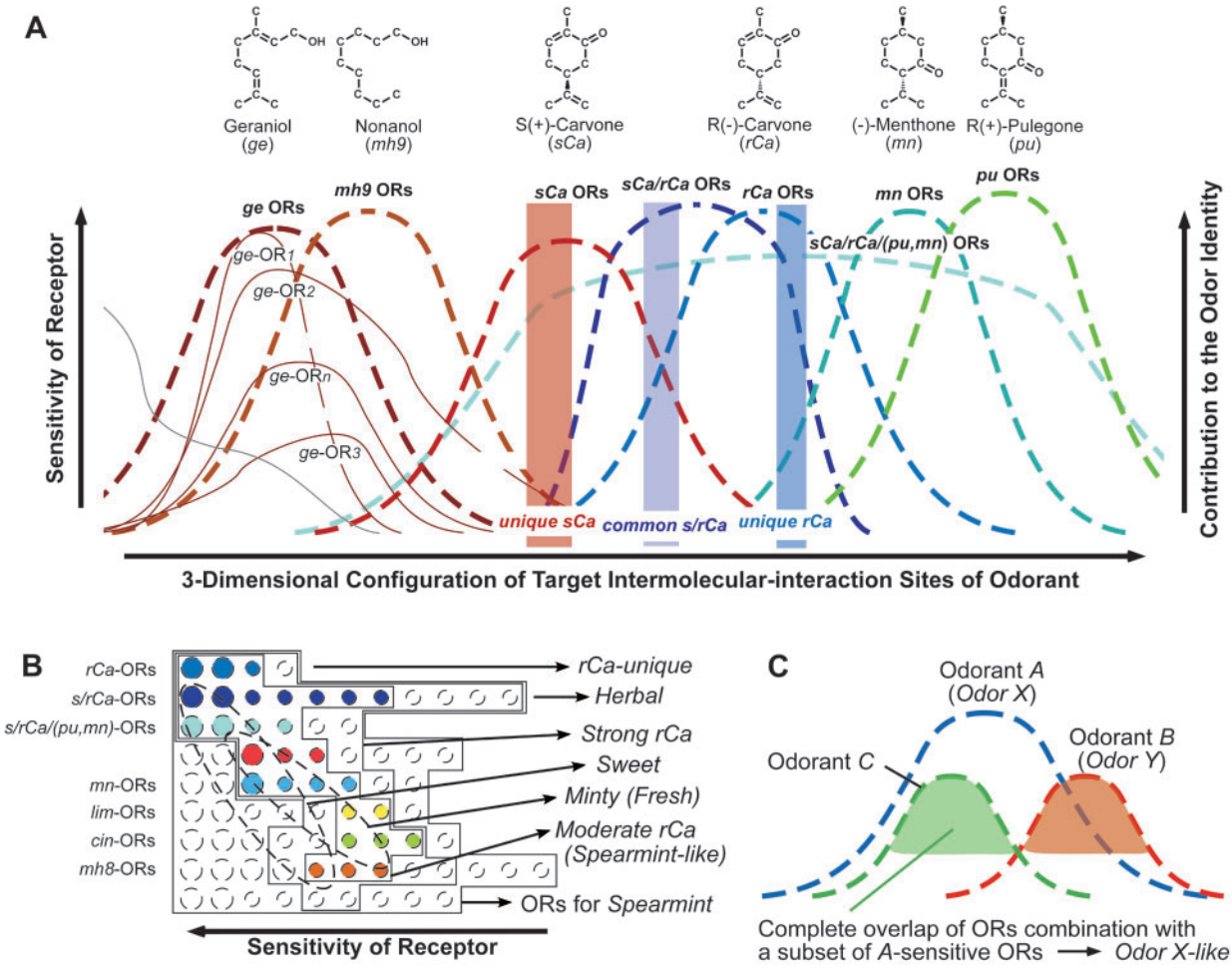

Figure 7 Schematic diagram of odor encoding by odorant receptors. **(A)** ORs are grouped into given-odorant-sensitive subclasses based on the tuning specificities. The structures of the odorants are shown with omissions of most hydrogen atoms; one of the possible molecular structures of nonanol is shown. **(B)** The signals of the sensitive ORs may primarily serve to integrate genetically selected signals for the respective odor qualities. **(C)** An explanation of the mechanism of loss of characteristic odors, such as in anosmia. See the Discussion for detailed explanations.

odor stimulus should arrive first at neurons anywhere in the main olfactory pathway. Filtering by inhibitory networks in the central pathways should favor the first input signals over the later ones from the relatively less-sensitve ORs. These first input signals from the most sensitive ORs are expected to play an important role in maintaining the characteristic odor representation over a wide range of stimulus concentrations. The fast and slow inhibitions in the piriform cortex (Satou *et al.*, 1982a, b) may play an important role in such a signal filtering. The possible mechanisms for emphasizing odorant-unique signals include the dendrodendritic synaptic inhibition in the olfactory bulb (Yokoi *et al.*, 1995). The dynamic optimization of odor representations as mitral cell activity patterns (Friedrich and Laurent, 2001; Spors and Grinvald, 2002) might also be related to the enhancement system of characteristic signals for given odorants.

The subpopulations of pyramidal cells, which receive signals from a single type of OR and formed a few stereotypical clusters, were calculated as 2.4 and 3.7% (Zou *et al.*, 2001). The subpopulations of ~30 times the 0.1% corresponding to that of a single OR suggest that the pyramidal cells in each cluster may summate the signals of different types of ORs by synchronized inputs (Kashiwadani *et al.*, 1999) and/or by the association fiber system (Haberly, 2001). The pyramidal cells may represent submodal odor qualities, such as the primary odors investigated by Amoore and other groups. The signal summations of selected ORs may be organized by odorant tuning specificity.

Almost half of the carvone-responsive ORNs could not discriminate enantiomeric structures, while the remaining half could do so. Single carvone-responsive ORs could recognize diverse combinations of odorants with various sensitivities. Furthermore, we have found that aliphatic-odorant-sensitive ORNs/ORs could discriminate the molecular length of an odorant at a resolution of a single carbon atom in a dose-dependent manner (Sato *et al.*, 1994; Malnic *et al.*, 1999). This suggests that target interaction

sites should include two sites around the major axial terminals of an odorant molecule. Considering these results, each OR may interact with and recognize odorant molecules at multiple sites in such a manner that different odor molecules select different combinations of interaction sites in a given OR's odorant-binding pocket. Some receptor sites tightly bind different parts of a given odorant molecule, endowing the receptor with a high sensitivity and specificity for the unique three-dimensional profile of intermolecular interactions with that odorant. Other sites may not bind the given odorant, but may loosely bind other odorants, conferring lower sensitivity to those stimuli. This principle of 'tight-and-loose profiling' results in well-defined odorant tuning curves, or tuning surfaces in a multidimensional odorant space. This hypothesis is also supported by psychochemical studies (Braunand Kröper, 1937; Moncrieff, 1949; Amoore, 1970; Beets, 1982; Ohloff, 1986).

A schematic diagram of receptor codes for odors is presented in Figure 7. Most hydrogen atoms are omitted and one of the possible molecular structures of nonanol is shown. The ORs are classified into subfamilies such as the *sCa*-sensitive, *rCa*-sensitive, or *sCa*/*rCa*-equi-sensitive types by the most sensitive odorants (Figure 7A). Each subfamily consists of multiple members whose tuning specificities are partially different. Each OR is tuned to a specific three-dimensional configuration of intermolecular interaction sites. Sensitivity decreases as differences from the tuned intermolecular interaction profiles increase. Consequently, one OR can discriminate one pair of odorants while another cannot. Odorant-unique ORs contribute mainly to the generation of an odorant-unique odor quality, according to their specific sensitivities. Multiple odorant equi-sensitive ORs contribute to the generation of common odor qualities among the multiple odorants. Common ORs could also assist in emphasizing the signals of unique ORs via inhibitory circuits.

Figure 7B illustrates the same hypothesis in a different way. As the concentration of odorant increases, each odor quality becomes more intense by recruiting new members of respective OR subclasses, in addition to the signal growth in each OR. Each of submodal odor qualities is represented by composing the signals of multiple different ORs. The principal odor qualities of the odorant are encoded by the most sensitive ORs and additional less-sensitive similar tuning-type ORs. As shown in Figure 7C, if all ORs most sensitive to odorant *C* are more sensitive to odorant *A* (*odor X*) and their subpopulation is small, we may recognize odorant *C* as an indeterminate odor (i.e. *odor X*-like). If the odorant is biologically very important for the animals to discriminate from other odorous stimulants, a subset of ORs of lower-ranked sensitivity to the odorant may play a dominant role in composing odor representation signals instead of the most sensitive receptors. In color vision, the hierarchical receptor code may explain 'yellow' as the principal color common to the long-wave-sensitive receptor and the medium-wave-sensitive receptor, based on the significantly overlapping wavelength range between the two cones (Bowmaker and Dartnall, 1980). Various 'red' colors are also explained as those principally encoded by the long-wave-sensitive cones which are most sensitive to 'red'.

We possess three remote sensory systems—vision, audition and olfaction. We have also developed recording systems that help us to recognize, remember and share transient experiences pertaining to the first two modalities. However, we have yet to develop an olfaction-compatible information recording system that shares transient odor information. In order to develop olfactory informational devices that have the same sophistication as video and audio systems, it is important to gather knowledge on the detailed profiles of odor receptor codes in the olfactory system. Data gathered by the type of extended approach applied in this study may facilitate the future development of innovative devices for recording and presenting olfactory experiences in a universal coordinate system.

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