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Combinatorial Receptor Codes for Odors

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Summary

The discriminatory capacity of the mammalian olfactory system is such that thousands of volatile chemicals are perceived as having distinct odors. Here we used a combination of calcium imaging and single-cell RT–PCR to identify odorant receptors (ORs) for odorants with related structures but varied odors. We found that one OR recognizes multiple odorants and that one odorant is recognized by multiple ORs, but that different odorants are recognized by different combinations of ORs. Thus, the olfactory system uses a combinatorial receptor coding scheme to encode odor identities. Our studies also indicate that slight alterations in an odorant, or a change in its concentration, can change its "code," potentially explaining how such changes can alter perceived odor quality.

Introduction

The mammalian olfactory system possesses enormous discriminatory power. Humans are thought to have a poor sense of smell compared to other animals, and yet they can perceive a vast number of volatile chemicals. Odorants, typically small organic molecules of less than 400 Da, can vary in a number of parameters, including size, shape, functional groups, and charge (Amoore, 1970). They include a panoply of diverse aliphatic acids, alcohols, aldehydes, ketones, and esters; chemicals with aromatic, alicyclic, polycyclic, and heterocyclic ring structures; and innumerable substituted chemicals of each of these types, as well as combinations of them. Remarkably, these molecules are not only detected by the olfactory system, but also discriminated by it.

Human studies have provided information about olfactory perception that is both surprising and puzzling. They have demonstrated that even a slight change in the structure of an odorant can cause a dramatic shift in its perceived odor (Beets, 1970; Polak, 1973). For example, when the hydroxyl group of octanol is replaced by a carboxyl group to give octanoic acid, its perceived odor changes from orange and rose-like to rancid and sweaty (Arctander, 1969). Curiously, the perceived quality of an odorant can also differ with a change in its concentration. Indole, for example, has a putrid odor when concentrated but is perceived as floral when diluted. Sensitivity to odorants also varies, with some odorants detectable at a much lower concentration than others (Cain, 1988). In addition, there are individual differences in olfactory perception. Androstenone, a pig pheromone, is a striking example; at a low concentration, androstenone has a mild, pleasant odor to some, a disgusting urinous odor to others, and still others cannot smell it at all (Amoore, 1970).

Neither the mechanisms by which the olfactory system accomplishes its perceptual feat nor the bases of these perplexing features of olfactory perception are well understood. Studies of rodent olfactory systems have, however, provided information about the structure and cellular functioning of the mammalian olfactory system, as well as molecular tools that now permit queries into the molecular bases of olfactory perception. Volatile odorants that enter the nose are detected by millions of olfactory sensory neurons (olfactory neurons) (Shepherd, 1988; Buck, 1996). These neurons transmit signals to the olfactory bulb of the brain, which, in turn, sends signals to the primary olfactory cortex. From there, olfactory information is relayed both to higher cortical areas and to the limbic system, thereby allowing for both the conscious perception of odors and their emotional and motivational effects.

The detection of odorants is mediated by ~1000 different G protein–coupled odorant receptors (ORs) that are encoded by a multigene family (Buck and Axel, 1991; Levy et al., 1991; Lancet and Ben-Arie, 1993; Ngai et al., 1993). ORs share characteristic sequence motifs, but they vary in sequence, consistent with an ability to recognize diverse ligands. Several findings indicate that each olfactory neuron expresses only one OR gene. First, individual OR gene probes hybridize to only ~0.1% of olfactory neurons in situ (Nef et al., 1992; Strotmann et al., 1992; Ressler et al., 1993; Vassar et al., 1993). Second, by reverse transcriptase–polymerase chain reaction (RT–PCR) analysis of small numbers of olfactory neurons, a single neuron expresses only one allele of a given OR gene (Chess et al., 1994). Finally, using single-cell RT–PCR, only one OR species can be identified per olfactory neuron (C. Dulac and R. Axel, personal communication).

In the nose, neurons expressing a given OR are confined to one of four OR expression zones, where they are randomly interspersed with neurons expressing other ORs (Ressler et al., 1993; Vassar et al., 1993; Strotmann et al., 1994). In the olfactory bulb, the axons of neurons expressing the same OR converge at fixed sites in only a few of the bulb's ~2000 glomeruli (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996). This suggests that olfactory information is first roughly organized into four large sets in the nose and then reorganized in the olfactory bulb into a sensory map, which is identical in different individuals. In both the nose and bulb, information derived from different ORs is strictly

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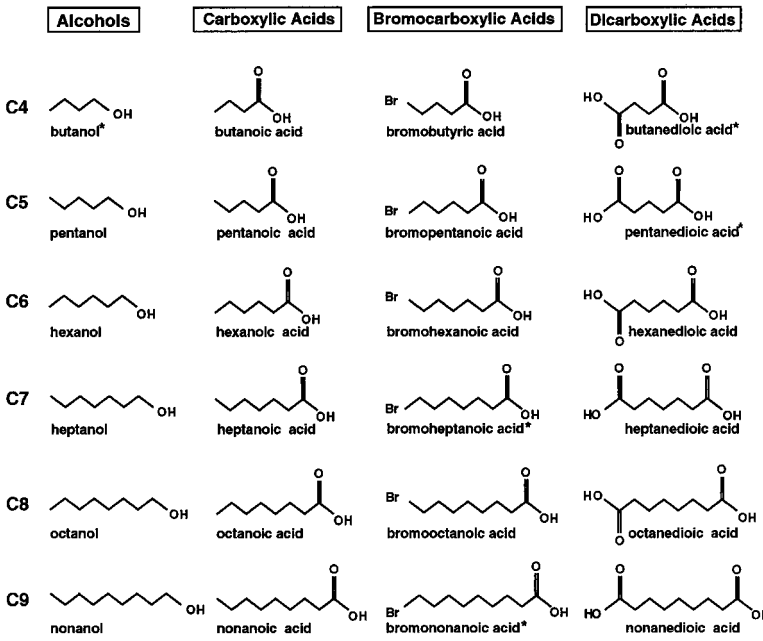

Figure 1. Aliphatic Odorants

The test odorants used were aliphatic alcohols with straight carbon chains ranging from 4 to 9 carbons in length (C4–C9), and the corresponding aliphatic carboxylic acids, bromocarboxylic acids, and dicarboxylic acids. *, not tested.

segregated: each olfactory neuron in the nose and each glomerulus in the olfactory bulb appear to be dedicated to input from one OR type.

Given functional observations that single olfactory neurons (Sicard and Holley, 1984; Firestein et al., 1993; Sato et al., 1994), and individual olfactory bulb glomeruli and output neurons (Adrian, 1950; Leveteau and MacLeod, 1966; Mori et al., 1992; Friedrich and Korsching, 1997) are stimulated by multiple odorants, the arrangements of OR inputs in the nose and bulb suggest that each OR recognizes multiple odorants. However, despite intensive efforts to determine the odorant specificities of individual ORs, until very recently (Krautwurst et al., 1998), ligands for only two ORs had been reported (Raming et al., 1993; Zhao et al., 1998). Attempts to obtain functional expression of ORs in heterologous cell types have largely failed, apparently because ORs cannot reach the plasma membrane.

To circumvent this problem, we developed an approach that allows one to analyze the ligand specificities of ORs in the olfactory neurons that express them and thereby reliably identify ORs that recognize specific odorants. We then used this approach to identify receptors for a series of aliphatic odorants with related structures but varied odors. The results of these studies provide insight into the molecular bases of odor discrimination and the strategies used by the olfactory system to distinguish a vast number of different odorants. They also shed light on several intriguing features of olfactory perception in humans.

Results

A Method to Identify Receptors for Specific Odorants

To identify ORs that recognize specific odorants, we used a combination of calcium imaging and single-cell RT–PCR. In response to odorants, olfactory neurons exhibit transient increases in intracellular calcium that can be detected by calcium imaging with the calcium indicator fura-2 (Restrepo and Boyle, 1991; Hirono et al., 1992, 1994; Restrepo et al., 1993). We used this method to monitor responses of individual mouse olfactory neurons to a series of aliphatic odorants. The test odorants included carboxylic acids with straight carbon chains ranging in length from 4 to 9 carbon atoms, and the corresponding aliphatic alcohols, bromocarboxylic acids, and dicarboxylic acids shown in Figure 1.

Olfactory neurons from the dorsal nasal septum (zone 1) were first deposited on coverslips and loaded with fura-2. The intensity of 510 nm fluorescent light emitted from individual neurons illuminated with 380 nm light was then recorded during sequential exposure to individual test odorants (Figure 2A). Responsiveness to 87.4 mM KCl, which depolarizes olfactory neurons and causes an increase in intracellular Ca^{2+} ($[Ca^{2+}]i$), was used to establish cell viability. In preliminary experiments with mouse olfactory neurons, $[Ca^{2+}]i$ was 10–50 nM in resting cells and was increased to 20–170 nM by KCl and up to 124 nM by odorant. In this range of $[Ca^{2+}]i$, decreases in the intensity of emitted light under 380 nm illumination are roughly linear with increases in $[Ca^{2+}]i$ (Grynkiewicz et al., 1985). Each odorant was first tested at a concentration of 100 μM, and odorants that elicited a response at 100 μM were retested at 10 μM; if effective at 10 μM, they were further tested at 1 μM. Previous studies have established that the odorant responses recorded from individual neurons using this approach are reproducible and not subject to variability (see Experimental Procedures).

We next used a two-step, single-cell RT–PCR procedure to identify the OR genes expressed by individual neurons. In the first step, oligo-dT-primed cDNAs were prepared from the 3′ 0.5–1.0 kb of mRNAs in each cell, and the complex mix of cDNAs was amplified by PCR. In the second step, an aliquot of this 1° PCR product was

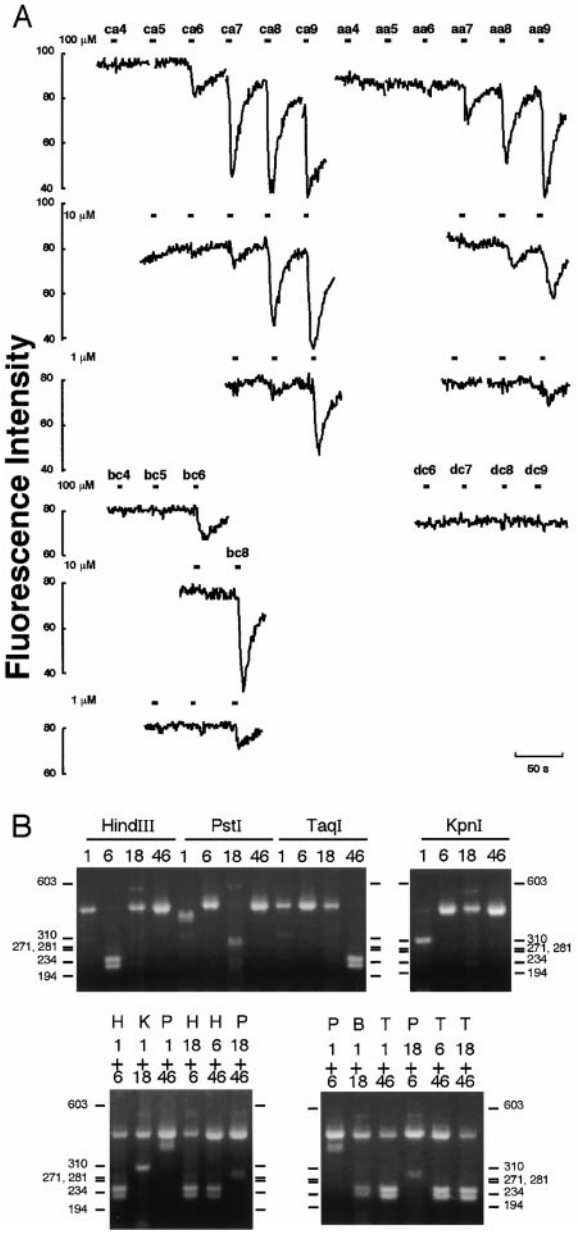

Figure 2. Methods Used to Identify ORs Expressed in Single Olfactory Neurons Responsive to Specific Odorants

(A) 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 relative decrease in the intensity of emitted light (Fluorescence Intensity [in arbitrary units]) indicates an increase in intracellular free calcium. The neuron was exposed to a series of aliphatic odorants, each at 100 μM. Odorants that elicited a response at 100 μM were subsequently retested at 10 μM, and at 1 μM if a response was observed at 10 μM. Exposure to each odorant was for 4 s, as indicated by the bar under each odorant abbreviation. ca, carboxylic acid; aa, aliphatic alcohol; bc, bromocarboxylic acid; dc, dicarboxylic acid. The number in each abbreviation indicates the number of carbon atoms in the odorant. This neuron, which expressed OR S19, responded to four of the carboxylic acids, three of the aliphatic alcohols, and two of the bromocarboxylic acids.

(B) Aliquots of mixed cDNAs (the 1° RT–PCR products) amplified from four different neurons, from which ORs S1, S6, S18, and S46 were obtained (Figure 3), were mixed (1:1) in all six possible combinations (or unmixed) and subjected to PCR with TM3/TM6 OR primers. Aliquots of each PCR product were digested with restriction enzymes that would cleave the OR cDNA previously identified in one of the donor neurons represented in each mix, but not the other, and then electrophoresed on agarose gels. The enzymes used were HindIII (H), PstI (P), TaqI (T), KpnI (K), or BglI (B) (BglI was not tested for the unmixed PCR reaction). The results indicate that in each mix both of the expected OR cDNAs were amplified (see Experimental Procedures), showing that amplification of one OR cDNA does not hinder amplification of another.

subjected to PCR with degenerate primers matching conserved amino acid sequence motifs in mammalian ORs. The OR cDNA product of this 2° PCR reaction was then isolated and sequenced.

Previous findings suggest that each olfactory neuron expresses only one OR gene (see above). To further examine this issue, and confirm that we could identify the OR expressed in a single neuron, we conducted a series of control experiments. Using nonresponsive control neurons, which should express an unbiased assortment of OR genes, we obtained OR cDNA PCR products from 18/26 cells using primers that match motifs in and around OR transmembrane domains 3, 6, and 7 (TM3, TM6, and TM7). We obtained OR cDNAs from twice as many cells with TM6/TM7 primers as with TM3/TM6 primers, even though we previously found that the TM3/TM6 primers would amplify a large variety of ORs (for example, ~500 different OR genes contained in human genomic clones [J. Brenman et al., unpublished data]). Since the 1° PCR reaction amplifies cDNAs prepared from the 3′ ends of mRNAs, this difference is likely to reflect the fact that the 3′ untranslated regions of different OR mRNAs vary in length (J. P. Montmayeur and L. B. B., unpublished data).

Direct sequencing of OR PCR products from the control cells (and from 24 neurons in earlier experiments) gave a single OR cDNA sequence per cell. Sequencing of two OR PCR products combined at ratios of 1:1 to 1:40 showed that a second OR cDNA could be detected if present at 1/20 the concentration of the major species. We also cloned TM6–TM7 OR cDNAs from two neurons and hybridized the inserts of 60 clones from each to a probe prepared from one insert of each set. All inserts from each set hybridized to the OR probe from the appropriate set, but none hybridized to the OR probe from the other set (data not shown).

If a neuron expressed two OR genes, would this method detect the expression of both genes? To address this question, we made six pairwise combinations of the 1° RT–PCR products obtained from four neurons that had previously yielded OR cDNAs and amplified the mixes, or each alone, with OR primers. Digestion of each OR PCR product with enzymes that cut one or the other of the cDNAs showed that each mix yielded the two expected OR cDNA species, one cut with one restriction enzyme, and the other cut with the second enzyme (Figure 2B). Thus, if there were two OR cDNAs in the mixed cDNAs amplified from a single neuron, both would be detected in the final OR PCR product.

To assess whether a neuron might yield different OR cDNAs with different primers, we sequenced two OR PCR products obtained with different primer pairs from

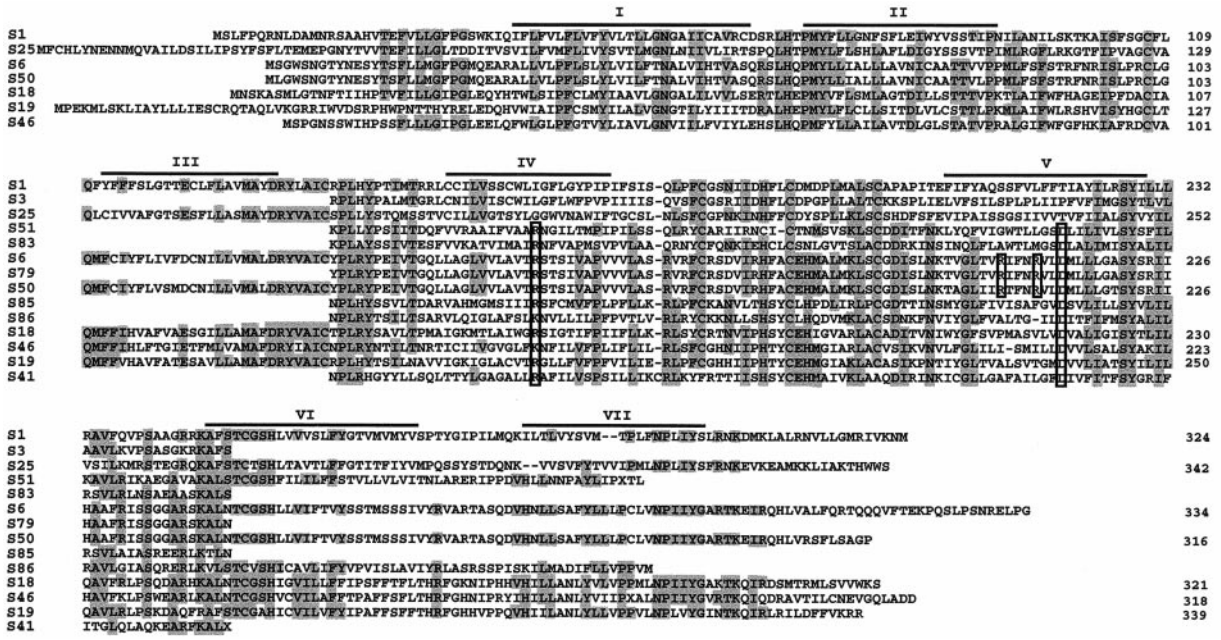

Figure 3. The Amino Acid Sequences of ORs Identified in 14 Neurons that Recognized Aliphatic Odorants

The sequence of the TM3–TM6 or TM3–TM7 region of each OR was obtained from the OR cDNA segment isolated from a single neuron. Additional sequences for seven ORs were obtained from genomic clones. Like other ORs, these proteins have seven potential transmembrane domains (I–VII) and exhibit sequence motifs characteristic of the OR family (e.g., MXXDRY/FXAIC at the end of TM3 and TCXXH at the beginning of TM6). Eleven of the ORs have charged residues (boxed residues) in TM4 and TM5 at positions where charged residues are rarely seen in ORs, raising the possibility that these residues play important roles in ligand interactions.

each of 11 control neurons. For each neuron, we obtained the same OR cDNA species with different primer pairs. Finally, to exclude the unlikely possibility that, by chance, a single OR gene was amplified from genomic DNA from each neuron, we split the initial RT reactions of single neurons into three aliquots, amplified total cDNAs and then OR cDNAs from each, and sequenced the three OR products from each of five cells. In each case, all three were the same. Together, these control experiments, as well as those of others (C. Dulac and R. Axel, personal communication), indicate that each olfactory neuron expresses only a single OR gene and that the OR gene expressed by one neuron can be determined using the two-step RT–PCR technique.

Receptors for Aliphatic Odorants

Using calcium imaging, 98 of 647 mouse olfactory neurons that were examined responded to one or more of the aliphatic test odorants in Figure 1. We obtained OR cDNA products from 14/47 neurons analyzed using TM3/TM6 or TM3/TM7 primers. Two or more OR cDNAs obtained with different primer pairs were sequenced for 13/14 neurons. An additional ten neurons gave OR cDNAs with TM6/TM7 primers, but the small size of the TM6–TM7 segment precluded further analyses.

Figure 3 shows the amino acid sequences of the 14 ORs for which we identified TM3–TM6 or TM3–TM7 segments. For seven of the ORs, we obtained additional full-length protein sequences from matching genomic clones (Figure 3). All 14 ORs are novel members of the mouse OR family. They contain amino acid sequence motifs typical of ORs, and they exhibit the diversity characteristic of the OR family (Buck and Axel, 1991).

The dendrogram in Figure 4 compares the TM3–TM6 regions of the 14 aliphatic ORs with the same region in 73 ORs previously identified in mammals. Amino acid sequence identity among the 14 aliphatic ORs is 19%–100% in the TM3–TM6 region, a particularly variable region in ORs (Buck and Axel, 1991). Eleven of the 14 aliphatic ORs are grouped in Figure 4, indicating greater similarity to one another than to most other ORs. Although this group of 11 ORs is itself diverse (Figure 5), all members of the group are unusual in having charged residues at specific positions in TM4 and TM5, transmembrane domains previously proposed to be involved in OR–odorant interactions (Buck and Axel, 1991; Lancet and Ben-Arie, 1993). The other three ORs that recognized aliphatic odorants, and all seven ORs identified in nonresponsive control neurons, are more highly related to previously identified ORs (Figure 4). These analyses show that, as a group, aliphatic odorants are recognized by a diverse array of ORs that includes both highly related and divergent receptors.

A Single Receptor Recognizes Multiple Odorants

Figure 6 shows the response profiles of the 14 neurons in which we identified ORs, and thus the recognition profiles of the 14 ORs. These profiles make several important points about the mechanisms underlying odor discrimination. The first is that a single OR can recognize multiple odorants. Most (12/14) of the ORs recognized more than one test odorant. On average, each OR recognized four test odorants. Each OR might, of course,

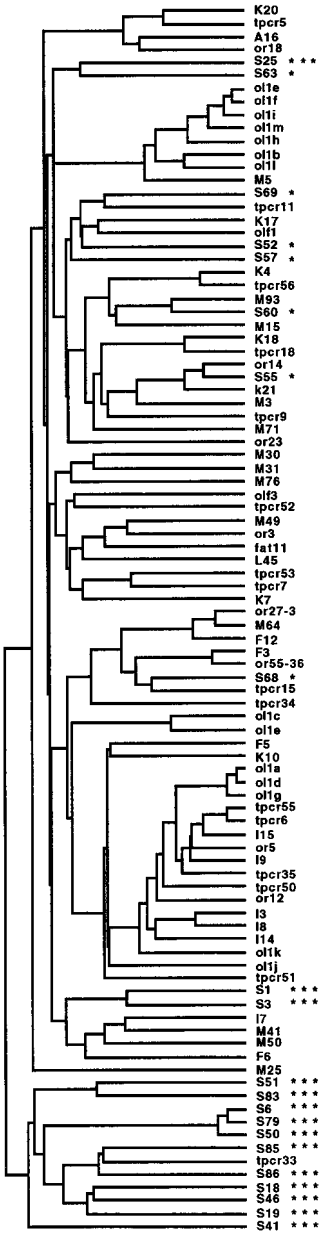

Figure 4. Relationships among the ORs that Recognized Aliphatic Odorants and Other OR Family Members

Amino acid similarities among the 14 ORs identified and other ORs. The amino acid sequences of 14 ORs (***) identified in neurons that responded to the aliphatic test odorants, and 7 ORs (*) identified in nonresponsive control neurons, were compared with 73 members of the OR family previously identified in mouse, rat, or human. The TM3–TM6 regions of the ORs were compared using the PILEUP program (GCG). Eleven of the receptors for aliphatic odorants are grouped, indicating that they are more similar to one another than to most other ORs.

recognize additional odorants that were not tested. These results are consistent with previous observations that a single olfactory neuron can respond to multiple odorants (Sicard and Holley, 1984; Firestein et al., 1993; Sato et al., 1994).

The identification of multiple odorants that are recognized by a single OR allows one to ask whether those

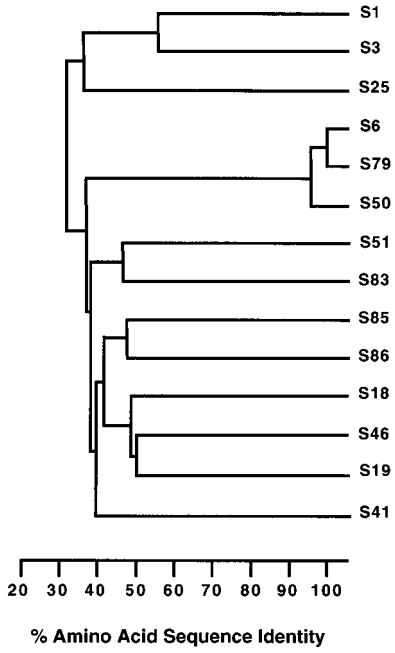

Figure 5. Amino Acid Sequence Identities among the 14 ORs that Recognized Aliphatic Odorants

Comparisons of the TM3–TM6 regions of the 14 ORs that recognized aliphatic odorants show that some are highly related, but as a group they are diverse in sequence (19%–100% amino acid sequence identity). The lowest homology (19%) was between S25 and S41. Bars connecting different groups of ORs indicate the maximum percent identity between pairs of ORs from the two groups.

odorants share a discernible structural feature that might be recognized by the OR. One feature that appears to be important for most of the ORs we identified is the length of the odorant's carbon chain (Figure 6). All but one of the ORs recognized only odorants of several consecutive carbon chain lengths. For example, five ORs recognized only odorants with seven, eight, or nine carbon atoms (C7–C9 odorants), and one OR recognized only C5–C7 odorants. Similarly, only 1/47 responsive neurons recognized odorants of all lengths tested (i.e., C4–C9), and the majority (38/47) responded only to odorants of 2–4 consecutive carbon chain lengths, consistent with previous observations (Sato et al., 1994).

The functional groups of the odorants also appeared to be important determinants of recognition for the aliphatic ORs (Figure 6). None of the ORs recognized odorants belonging to all four classes of test odorants (carboxylic acids, aliphatic alcohols, bromocarboxylic acids, and dicarboxylic acids). In fact, five ORs recognized odorants of only one class. The remaining ORs recognized odorants that belonged to two or three classes, but the combination of odorant classes recognized by different ORs varied. Similar variability was seen in the total population of responsive neurons. These results suggest that there is considerable variation in the rules that govern odorant recognition by different ORs, even ORs that recognize structurally related odorants. This diversity in the recognition properties of ORs is likely to be of central importance to the olfactory system's ability to detect and discriminate a wide variety of structurally diverse odorants.

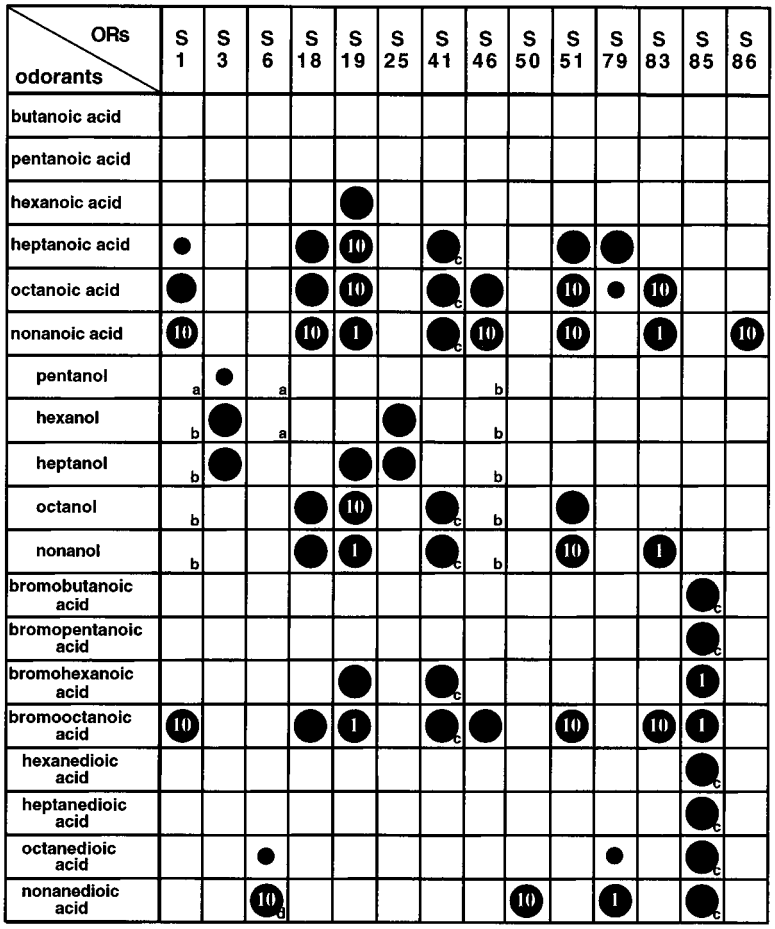

Figure 6. The Recognition Profiles of 14 Olfactory Neurons and the ORs They Expressed

Test odorants are shown on the left, and the ORs identified in the responsive neurons are shown on top. Filled circles indicate responses to 100 μM odorants, with smaller circles indicating a relatively weak response (less than half the change in fluorescence intensity elicited by KCl). Responses that were also obtained at 1 or 10 μM odorant are indicated by a 1 or 10 inside the filled circle. a, not tested; b, tested at 10 μM, but not 100 μM; c, not tested at 10 μM or 1 μM; d, not tested at 1 μM.

A Single Odorant Is Recognized by Multiple Receptors

A second important point made by the OR recognition profiles is that a single odorant can be recognized by multiple receptors (Figure 6). The 14 neurons in which we identified ORs responded to a total of 17 odorants. Most (11/17) of the odorants were recognized by two or more ORs. Since most of the odorants were also recognized by neurons from which we did not obtain OR cDNAs, they are probably recognized by additional ORs in vivo.

One might imagine that ORs that interact with the same odorant would have similar protein sequences. In fact, three of four ORs that detected nonanedioic acid (S6, S50, and S79) are highly related. In the TM3–TM6 region, S6 and S79 are 100% identical, and S50 is 96% identical to S6/S79 (Figure 5), a finding that, parenthetically, also provides support for the reliability of the assay system used. However, the fourth OR (S85) that recognized nonanedioc acid is only 33% identical to the others. Similarly, one OR that recognized octanoic acid (S1) is only 22%–30% identical to the other seven ORs that recognized the same odorant, and the other seven are only 27%–50% identical to each other. Thus, a single odorant can be recognized by a diverse set of ORs, some of which may be highly related to one another.

One might also predict that ORs that are highly related would recognize the same odorant. The fact that S6, S50, and S79 all recognized nonanedioc acid indicates that ORs that are nearly identical may indeed recognize the same odorant. For each of these ORs, nonanedioic acid stimulated the most robust (or the only) cellular response, and it elicited a response at 1–10 μM as well as 100 μM, suggesting a relatively high affinity interaction. Neurons 6 and 79 also responded weakly to octanedioc acid, while S50 did not, suggesting that highly related ORs might have related, but distinct, recognition profiles. However, even though Southern blotting and genomic clone analyses indicated that there is a single S6/S79 gene (and a separate S50 gene), neuron 79 responded to two odorants that S6 did not (Figure 6), and while S6 responded less well to 10 μM than 100 μM nonanedioc acid, S79 responded equally at the two concentrations. These differences, as well as those between S50 and S6/S79, could conceivably result from heterogeneity in the level of expression of an OR gene (or another transduction molecule) in different neurons, or in the same neuron at different stages of development (olfactory neurons live for 30–60 days), differences in the health of isolated neurons, or the existence of separate S6 and S79 genes that we could not detect.

The number of neurons, and the number of identified ORs, that recognized different odorants varied considerably in our studies. For example, 1/47 neurons responded to bromopentanoic acid, whereas 22/47 neurons responded to nonanoic acid. As a group, dicarboxylic acids

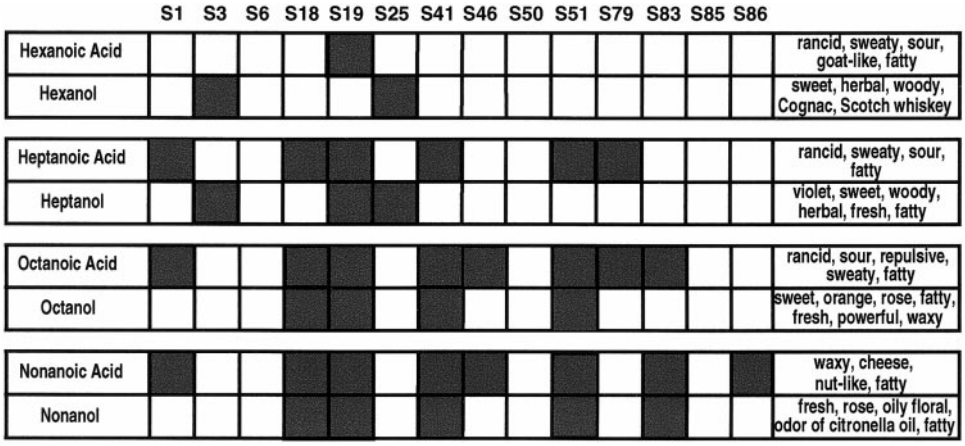

Figure 7. Comparison of the Receptor Codes for Odorants that Have Similar Structures but Different Odors

Aliphatic acids and alcohols with the same carbon chains were recognized by different combinations of ORs, thus providing a potential explanation for why they are perceived as having strikingly different odors. Perceived odor qualities shown on the right were obtained from Arctander (1969), The Good Scents Company (http://www.execpc.com/~goodscnt//index.html), and The Chemfinder Web Server (http://chemfinder.camsoft.com).

were recognized by far fewer neurons (4/47) and ORs (4/14) than odorants of the other three classes. For all four odorant classes, there appeared to be a rough correlation between carbon chain length and the number of neurons, and identified ORs, that recognized an odorant. For example, aliphatic alcohols with 5, 6, 7, 8, and 9 carbon atoms stimulated 3, 7, 12, 16, and 18 neurons and were recognized by 1, 2, 3, 4, and 5 of the identified ORs, respectively.

Different Odorants Are Recognized by Distinct Combinations of Receptors

The third important point made by the OR recognition profiles in Figure 6 is that different odorants are recognized by different combinations of ORs. Even though individual ORs recognized multiple odorants in our studies, all of the odorants that were recognized by more than one OR were recognized by a unique combination of ORs. Each odorant that elicited a response in more than one of the 47 responsive neurons was similarly recognized by a different combination of neurons. For example, all eight of the ORs that recognized bromooctanoic acid also recognized other odorants, but no other odorant was recognized by this set of eight ORs (Figure 6). A number of odorants were recognized by overlapping, but nonidentical, sets of ORs.

These data provide direct evidence that different odorants are encoded by different combinations of ORs, with each OR serving as one component of the composite "receptor codes" for many odorants. They further show that odorants that have related structures can be recognized by overlapping, though nonidentical, sets of ORs and thus have overlapping, but distinct, receptor codes.

The results shown in Figure 6 also suggest that a change in the concentration of an odorant can result in a change in its receptor code. Most neurons that responded to an odorant at 100 μM were retested with that odorant at 10 μM and, if appropriate, at 1 μM. In some cases, a neuron continued to respond at a lower concentration, while in others it did not (Figures 2A and 6). A 10- to 100-fold decrease in the concentration of an odorant invariably resulted in a decrease in the number of ORs that recognized the odorant with sufficient affinity to elicit a cellular response. For example, bromooctanoic acid was "recognized" by eight ORs at 100 μM, five ORs at 10 μM, and two ORs at 1 μM. This trend was also observed in the total responsive neuron population in which, for example, 21, 16, and 5 neurons responded to bromooctanoic acid at 100, 10, and 1 μM, respectively. It appears that an increase in the concentration of an odorant leads to the recruitment of additional ORs and a consequent change in the receptor code.

Receptor Codes for Rancid versus Floral Odorants

It is well known that a slight change in the structure of an odorant can lead to a profound change in its perceived odor (Beets, 1970; Polak, 1973). The carboxylic acid and aliphatic alcohol test odorants we used are excellent examples of this phenomenon. Although they differ only in functional group (carboxyl versus hydroxyl) (Figure 1), the acids and alcohols of corresponding carbon chain lengths have strikingly different odors (Figure 7) (Arctander, 1969). All of the carboxylic acids have unpleasant odors. Many are perceived as rancid, sour, and sweaty, and some are also described as goat-like or repulsive. In contrast, the alcohols are described as pleasant, with herbal, woody, floral, and/or fruity scents.

Figure 7 compares the ORs that recognized carboxylic acids and aliphatic alcohols with the same carbon chains. In every instance, the acid and alcohol with the same carbon chain were recognized by different combinations of ORs, but one or more ORs usually recognized both. In the case of nonanoic acid and nonanol, for example, all five ORs that recognized the alcohol also recognized the acid, but the acid was also recognized by three other ORs. In the case of heptanoic acid and heptanol, only one OR recognized both, while five ORs recognized only the acid and two ORs recognized only

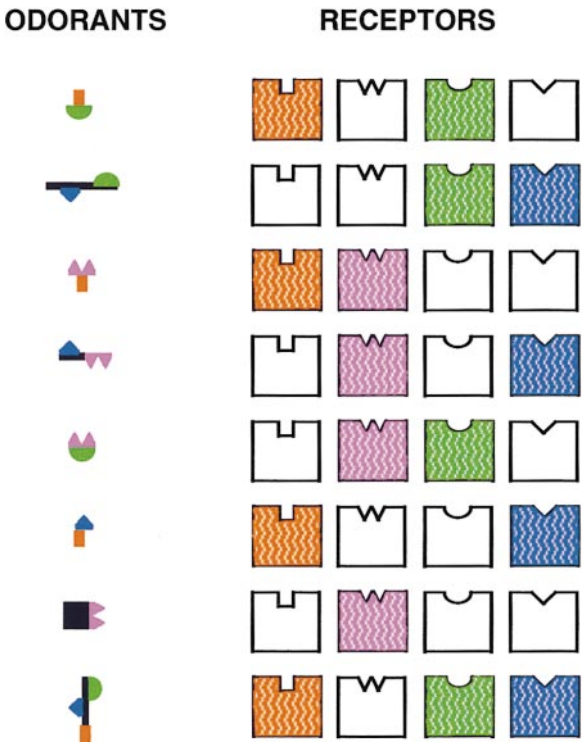

Figure 8. Combinatorial Receptor Codes for Odorants

In this model, the receptors shown in color are those that recognize the odorant on the left. The identities of different odorants are encoded by different combinations of receptors. However, each OR can serve as one component of the combinatorial receptor codes for many odorants. Given the immense number of possible combinations of ORs, this scheme could allow for the discrimination of an almost unlimited number and variety of different odorants.

the alcohol. In the total responsive neuron population, differences were also seen. For example, of 21 neurons that responded to octanoic acid and 16 that responded to octanol, 9 neurons responded to both. These comparisons again emphasize that changes in odorant structure can result in changes in the receptor code for an odorant. They further suggest that a change in an odorant's receptor code can give rise to a striking change in perceived odor.

Discussion

To link odorant receptors with specific odorants, we developed a novel approach that uses calcium imaging and single-cell RT–PCR to identify odorant receptors expressed by olfactory neurons that respond to specific odorants. Our results indicate that individual mouse olfactory neurons express only one OR gene each. Thus, the response profile of a neuron to a series of test odorants reflects the recognition properties of the OR expressed in that cell. Using this method, we characterized ORs that recognize aliphatic odorants with related structures but varied odors. Our results demonstrate that a single OR can recognize multiple odorants, and a single odorant can be recognized by multiple ORs, but that different odorants are recognized by different combinations of ORs. They further indicate that a single odorant can be recognized by highly related ORs as well as divergent ORs, and that odorants that are almost identical in structure can be recognized by different, but often overlapping, sets of ORs. Finally, our studies indicate that slight changes in the structure of an odorant or changes in its concentration result in changes in the combination of receptors that recognize the odorant. The implications of these findings for odor discrimination and perception are discussed below.

Combinatorial Receptor Codes for Odors

These studies provide evidence that the mammalian olfactory system uses a combinatorial receptor coding scheme to encode odor identity and to discriminate odors (Figure 8). In this scheme, different odorants are encoded by different combinations of ORs, but each OR may serve as one component of the unique combinatorial receptor codes for many odorants. Different ORs that recognize the same odorant might recognize different structural features of the odorant, as shown in Figure 8.

Given that there are ~1000 OR genes in the genome, this combinatorial receptor coding scheme should permit the discrimination of a vast number of diverse odorants. Even if each odorant were encoded by only three ORs, the number of odorants that could theoretically be discriminated would be nearly one billion. The combinatorial receptor codes might have another advantage. Since the maintenance of connections between olfactory neurons and the brain is likely to require at least occasional odor-induced neuronal activity (Brunjes, 1994), the use of individual ORs to recognize a variety of odorants could serve to maintain the components of the code for an odorant, even in its absence, thus assuring perceptual fidelity over time as well as the ability to perceive novel odors.

Here, we embarked on an initial exploration of the molecular bases of odor discrimination. We identified two structural features of aliphatic odorants that appear to be important to their recognition by ORs: the length of the odorant's carbon chain, and its functional group. Individual receptors recognized only odorants with several consecutive carbon chain lengths and odorants with certain functional groups, but not others. A similar restriction was previously seen for the I7 ORs in rat and mouse (Krautwurst et al., 1998; Zhao et al., 1998). Interestingly, even though *C. elegans* and mammals use different strategies to organize chemosensory information (Chou et al., 1996), a *C. elegans* receptor for the odorant diacetyl also discriminates among a number of related odorants but recognizes two of them (Sengupta et al., 1996; Zhang et al., 1997).

Our studies show that individual mammalian ORs perform very fine discriminations in ligand binding. A single OR can distinguish between odorants that differ in carbon chain length by one carbon atom, or between odorants that have the same carbon chain but a different functional group.

Molecular Codes and Perception

The data presented here also suggest explanations for several perplexing features of olfactory perception in humans. The basic structure of the olfactory system and the ORs used to detect odorants are similar in mouse and humans. Thus, information regarding the encoding

and discrimination of odors in mouse can be presumed to apply to humans as well.

One intriguing feature of olfactory perception is the dramatic effect that can be wrought by a small change in odorant structure (Beets, 1970; Polak, 1973). The acids and alcohols we tested that have the same carbon chain differ by only a single functional group, but they have vastly different odors (Arctander, 1969). The carboxylic acids are described as unpleasant, with rancid, sour, sweaty, repulsive, or goat-like odors. In contrast, the alcohols are perceived as pleasant or fresh, with herbal, woody, orange, or rose scents. Comparisons of the ORs that recognized acids and alcohols with the same carbon chain in our studies showed that, though some ORs recognized both, the corresponding acid and alcohol were invariably recognized by different combinations of ORs. This suggests that changes in the perceived quality of an odorant that result from an alteration in its structure may be a direct result of changes in its receptor code.

Some odorants are perceived as having different odors at different concentrations. A striking example is thioterpineol, whose odor is described as "tropical fruit" at a low concentration, as "grapefruit" at a higher concentration, and as "stench" at a still higher concentration (R. Boden, personal communication). Our studies indicate that, at different concentrations, an odorant can be recognized by different combinations of ORs. Thus, a change in the concentration of an odorant can change its receptor code, and this, in turn, may lead to a change in odor quality.

Humans can detect some odorants at a much lower concentration than they can others (Cain, 1988). One possible explanation for this phenomenon is suggested by our finding that different odorants can be recognized by different numbers of ORs and by different percentages of olfactory neurons. Aliphatic alcohols of increasing carbon chain length were recognized by increasing numbers of ORs (and neurons) in our studies, while the detection threshold for aliphatic alcohols in rats and humans decreases with increasing carbon chain length (Cain, 1988). Thus, the size, or complexity, of an odor code might be an important determinant of how easily an odorant can be detected, perhaps reflecting the cumulative intensity of signals transmitted to the olfactory bulb.

Differences in the sizes of odor codes might also be relevant to the existence of selective perceptual deficits (specific anosmias) to some odorants, but not others (Amoore, 1970). If an odorant is recognized by only one OR, mutations in that OR would result in specific anosmia for the odorant. If an odorant is recognized by multiple ORs, specific anosmia would not occur unless all of the relevant ORs were mutated. In this case, mutation of one OR that recognizes the odorant would, however, change its code, perhaps giving rise to perceptual differences among individuals, which are known but not understood.

What is the molecular basis of odor quality? Interestingly, a single odorant is often perceived as having several different odor "qualities." For example, heptanoic acid is described as rancid, sour, and sweaty, and octanol is perceived as both orange and rose-like (Figure 7). When two odorants are mixed, both odorants can often be perceived in the mix (Laing and Francis, 1989). This implies that a perceived odor quality can result from a subset of the ORs that recognize the mix. This, in turn, raises the possibility that, in the extreme case, a single OR might convey an odor quality. One way to explore this question would be to ask whether there are ORs that recognize only odorants with a particular odor quality. For example, the two ORs that recognized only aliphatic alcohols in our experiments (S3 and S25) might be candidates for ORs that convey a "woody" or "sweet" quality.

Odor Coding beyond the Nose

Each olfactory neuron in the nose and each glomerulus in the olfactory bulb appear to be dedicated to input from a single receptor type. Although neurons expressing different ORs are interspersed in the nose, there is a stereotyped sensory map in the bulb in which input from different ORs is mapped onto specific glomeruli (Ressler et al., 1993, 1994; Vassar et al., 1993, 1994; Mombaerts et al., 1996). Given that the code for an odorant consists of a combination of ORs, this arrangement suggests that the individual components of an odor code are anatomically segregated in both the nose and bulb—in the nose into different neurons, and in the bulb into different glomeruli. Our studies provide experimental evidence for previous proposals that the code for an odorant in the nose is a dispersed ensemble of neurons expressing different ORs, whereas in the bulb, where many glomeruli are activated by one odorant (Stewart et al., 1979; Jourdan et al., 1980; Guthrie et al., 1993), it is a specific combination of glomeruli whose spatial arrangement is identical in different animals (reviewed in Buck, 1996).

It is not yet known how signals derived from different ORs are represented in the olfactory cortex and other areas of the brain, nor is it known how the individual components of an odor code are decoded to yield the perception of an odor. The representation of an odor code may be modified, for example, by intrabulbar circuits that "sharpen" it (Yokoi et al., 1995) or that impose differential temporal patterns on signals transmitted to the cortex (Laurent, 1997). Signals derived from olfactory neurons in the nose are ultimately delivered to a number of different brain areas, including neocortical areas involved in the conscious perception of odors, and limbic areas, such as the amygdala and hypothalamus, which are thought to mediate the emotional and physiological effects of odors. It is not known whether all of these areas receive information derived from the entire OR repertoire. It may be, for example, that higher cortical areas that mediate the conscious perception of odors receive information derived from all ORs, while the hypothalamus receives relatively direct input only from ORs that signal the presence of chemicals whose recognition is of particular value to the perpetuation of the species, such as rotten food or a pheromone.

Experimental Procedures

Calcium Imaging

Calcium imaging was performed as previously described (Sato et al., 1994), but with an Argus-50 image processor (Hamamatsu Photonics). Briefly, olfactory neurons from the dorsal region nasal septum of BALB/c mice were deposited on coverslips, loaded with fura-2, and then exposed sequentially, for 4 s at intervals of ~35 s, to

different odorants (Aldrich) at 100 μM. Light emission (510 nm) was recorded under 380 nm illumination. Only neurons that exhibited increases in intracellular free calcium ([Ca^{2+}]i) in response to 87.4 mM KCl, and were thus viable, were analyzed. Of 647 KCl-responsive neurons, 98 responded to test odorants. High-amplitude odorant responses in Figure 6 were those in which the change in fluorescence intensity exceeded 50% of that induced by KCl.

Previous studies established the reproducibility of odorant responses recorded from individual neurons using this approach. When odorants that had elicited a response were retested at the same concentration at the end of the odorant series, responses were often reduced in amplitude, but responses to the same odorants were always seen, except when the initial response had been very weak. Retesting of a few neurons with selected odorants in the present studies gave the same result. In many cases, a neuron also responded to an odorant at more than one concentration (see below; Sato et al., 1994). Repeated applications of the same odorant at 40 s intervals gave responses that were 100%, 98%, 80%, and 77%, respectively, of the initial response, further indicating that the brief odorant exposures used in this assay system do not elicit significant adaptation (Sato et al., 1994).

Isolation of OR cDNAs from Single Neurons

Each neuron was transferred to a tube, and cDNAs were prepared from the 3′ ends of mRNAs in the cell and then amplified as described previously (Matsunami and Buck, 1997), but with a 30 min reverse transcription step. In a second reaction (50 μl), 1 μl of a 1/100 dilution of the first PCR reaction, together with 0.2 mM each dNTP, 2 μM each primer, and 2.5 U of Taq polymerase (Boehringer Mannheim) in 1× buffer (Boehringer Mannheim), was subjected to 45 cycles of PCR (1 min, 95°C; 3 min, 40°C; and 3 min, 72°C). The primers used to amplify TM3–TM6 OR segments were P26 (GCITA(C/T)GA(C/T)CGITA(C/T)GTIGCIATITG) and P27 (ACIACIGAIAG(G/A)TGIGAI(G/C)C(G/A)CAIGT), which match conserved regions around the end of TM3 (AYDRYVAIC) and the beginning of TM6 (TC(A/G)SHLSVV), respectively, in rodent ORs (Buck and Axel, 1991). The following degenerate primers were used to amplify TM6–TM7 OR segments: TM6: P41:AA(GA)(TG)CITTI(AGT)(AC)IACITG(CT)G(GC)IT CICA; TM7: P42: TC(TC)(TC)TIGTI(TC)TI(AG)(TC)IC(TC)GATAIATIA TIGG(GA)TT, or W68:TCI(TC)T(GA)TTIC(TG)IAGIG(TA)(GA)TAIAT(GA) AAIGG(GA)TT,or W69:TC(TC)TT(GA)TTIC(TG)IAGIG(TA)(GA)TAIA(TC) IA(GC)IGG(GA)TT, or W70:TCIT(GC)(GA)TTIC(TG)IA(GA)I(GC)A(GA)T AIATIATIGG(GA)TT, or P8:(GA)TTIC(TG)IA(AG)I(GC)(TA)(GA)TAIAT (AG)AAIGG(GA)TT. Anchor sequences were added to primer 5′ ends to allow direct sequencing of amplified DNAs. PCR products were gel purified (agarase [NEB]) and sequenced (ABI) using an anchor sequence primer. To obtain full-length OR sequences, a BALB/c mouse genomic library (Clontech) was screened at 80°C (Ressler et al., 1993) using isolated OR TM6–TM7 segments as probes. DNAs from purified phage clones (Sambrook et al., 1989) were sequenced with primers matching specific ORs.

The dendrogram in Figure 4 was generated using the PILEUP computer program of the Genetics Computer Group (GCG package). The TM3–TM6 region of each OR (corresponding to amino acids 130–238 of S1) (between the primers P26 and P27) was used for the sequence comparisons. The amino acid sequence identities shown in Figure 5 were calculated by hand and by the Distances program of the GCG package.

Control Analyses of OR cDNAs Amplified from Single Neurons

TM6–TM7 PCR products obtained from two different neurons (S1 and S46) were cloned into a plasmid vector. The inserts of 60 clones derived from each PCR product were amplified with TM6/TM7 primers, size fractionated on agarose gels, blotted onto nylon membranes, and then hybridized to probes prepared from the insert of one (also sequenced) clone from each set of 60 clones (which was also sequenced).

Mixed (1:1) pairs of the 1° RT–PCR products (or unmixed control samples) obtained from four neurons (S1, S6, S18, and S46) in which ORs had been identified were used in a 2° PCR reaction with the TM3–TM6 OR primers. The products were digested with restriction enzymes that cut only one of each of the two receptors expected within a mix, leaving the second one intact (Figure 2B). The expected sizes for the digested fragments were as follows: undigested products (430); S1/PstI (360/70); S1/KpnI (295/135); S6/HindIII (223/207); S18/BglI (226/204); S18/PstI (255/175); and S46/TaqI (222/208).

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