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# Recombinant Expression and Functional Characterization of Mouse Olfactory Receptor mOR256-17 in Mammalian Cells

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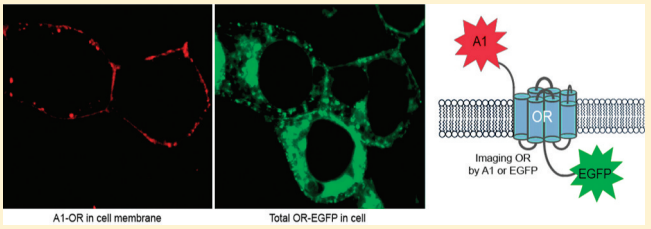
**ABSTRACT:** Olfactory receptors (ORs) constitute the largest family of sensory membrane proteins in mammals. They play a key role within the olfactory system in recognizing and discriminating a nearly unlimited number of structurally diverse odorous molecules. The molecular basis of OR-mediated signal detection and transduction is poorly understood. This is due to difficulties in functional expression of ORs in high yields, preventing structural and biophysical studies at the level of the receptor protein. Here we report on recombinant expression of mouse receptor mOR256-17 yielding $10^6$ ORs per cell in transiently transfected mammalian cells. For quantification and optimization of OR expression, we employed different fluorescent probes. Green fluorescent protein fused to the C-terminus of mOR256-17 allowed quantification of total cellular OR biosynthesis, and post-translational fluorescence labeling of a 12-amino acid polypeptide sequence at the N-terminus permitted the selective visualization and quantification of ORs at the plasma membrane using cell flow cytometry. Our dual-color labeling approach is generally applicable to quantification of membrane proteins for mammalian cell-based expression. By screening a large odorant compound library, we discovered a selective spectrum of potent mOR256-17-specific agonists essential for probing the receptor function for future scaled-up productions.




With more than 1000 intact genes in rodents and approximately 350 in humans, olfactory receptors (ORs) comprise the largest predicted family of G protein-coupled receptors (GPCRs) in mammalian genomes.[1−4] The discovery of OR genes that are presumed to encode receptors with a seven-transmembrane structure[5] has led to the initiation of extensive, ongoing studies of olfaction at a molecular level. In native tissues, ORs are primarily expressed in specialized olfactory sensory neurons (OSN) that are involved in the first step of odor perception.[5,6] When an odorant binds, ORs initiate cascades of signal transduction events in OSN and function together in a combinatorial pattern to detect and discriminate tens of thousands of volatile molecules.[6−9]

In spite of intensive research, many fundamental aspects of olfaction remain elusive.[10−13] The molecular basis of the specificity and sensitivity of ORs is poorly understood, and the quantitative characterization of OR-mediated signaling demands comprehensive structural and functional studies at the receptor protein level. This need is also underlined by the fact that among the hundreds of predicted mammalian ORs, only a few have been functionally verified. In turn, for almost all predicted ORs, the activating odorant molecules remain unidentified. Furthermore, no high-resolution three-dimensional (3D) structure of an OR has been determined, in contrast to those of other mammalian GPCRs.[14−18] Consequently, a reliable structural basis of OR's ligand recognition is unknown. Therefore, only models of ligand binding sites of ORs have been proposed on the basis of sequence comparisons between ORs, by functional studies of mutant OR proteins, and by comparisons with known 3D structures of other GPCRs.[19−21] Finally, quantitative thermodynamic and kinetic constants for interactions between ORs and their activating ligands or their downstream signaling proteins are totally missing. Hence, methods for the efficient production of ORs in their biologically active form are of crucial importance.

Although considerable progress has been made with respect to the expression and structural characterization of membrane proteins in general,[22] for ORs these studies are still at the beginning. Only a few ORs have been heterologously expressed at a functional, albeit low level in the plasma membrane.[23,24] In the majority of the cases, the receptors were retained within the endoplasmic reticulum of the cells, very likely because of inefficient folding and poor coupling to the ER export machinery.[25−27] To overcome these hurdles, several efforts have been undertaken, which only partly improved OR cell surface expression. For instance, the N-terminal addition of the rhodopsin signal sequence[28] and the co-expression of receptor-transporting and expression-enhancing proteins did promote cell surface targeting of some OR proteins.[29,30] Most potent was a short version of receptor transport protein 1 (RTP1), which seems to be the dominant RTP version expressed in olfactory neurons in vivo.[31] As no universal expression system has been discovered yet for the functional production of ORs, the proper combination of target receptor and expression host must be found empirically for each case.[32] HEK293 cells have been shown to be suitable for the

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functional expression of a few ORs[19,20,33,34] and also for large-scale OR production in one case.[35]

Here, we achieved by transient transfection a considerably high yield of functional OR cell surface expression of mouse mOR256-17 in HEK293 cells. This receptor is of interest because, apart from the olfactory epithelium, it is expressed in non-neuronal guidepost cells during critical phases in the embryonic development of the olfactory system, implicating dual receptor functions in axon guidance and olfaction.[36,37] We found that cell surface targeting of mOR256-17 is strictly dependent on the co-expression of RTP1S, reaching high expression levels, suitable for large-scale OR production for structural and biophysical studies. Moreover, we provide a generic method for measuring cell surface expression of ORs in heterologous cells employing post-translational labeling of a 12-amino acid polypeptide fused to the amino terminus of the receptor.[38]

By screening a panel of 250 odorant compounds, we determined the molecular receptive range of mOR256-17 and discovered ligands useful for probing receptor activity during production and purification. The chemical structures of the newly discovered mOR256-17-specific agonists may describe some molecular features of natural ligands.

## MATERIALS AND METHODS

**Plasmids.** The wild-type coding sequence of mouse olfactory receptor mOR256-17 and a C-terminal fusion with the enhanced green fluorescent protein (EGFP) (mOR256-17-EGFP) were subcloned from plasmid pCDNA3-mOR256-17-EGFP (H. Breer, University of Hohenheim, Stuttgart, Germany) and inserted into mammalian expression vector pEAK8 (Edge BioSystems) using the HindIII and NotI restriction sites (NEB Bioconcept). The 12-amino acid polypeptide tag A1 (GDSLDMLEWSLM)[38] was fused to the N-terminus of mOR256-17-EGFP and the neurokinin-1 receptor NK1R-EGFP by two consecutive polymerase chain reactions using the DNA polymerase Phusion (NEB Bioconcept), and cloned into pEAK8 as HindIII/NotI fragments (NK1R-EGFP was obtained following the procedure described elsewhere.[39] The 77-amino acid ACP (ACP)[40] was subcloned from pACP-NK1R,[41] fused to the N-terminus of mOR256-17-EGFP, and inserted into pEAK8 as a HindIII/BamHI fragment. The short form of receptor-transporting protein RTP1[29] and RTP1S[31] was amplified from pCI-RTP1 plasmid DNA (H. Matsunami, Duke University, Durham, NC) and cloned into pEAK8 using the HindIII and NotI restriction sites. All constructs were verified by DNA sequencing.

**Cell Culture, Transfection, and Labeling.** Cell cultures were grown in DMEM/F12 medium (Invitrogen) supplemented with 10% fetal calf serum (FCS) (Invitrogen) and 100 $\mu$g/mL penicillin-streptomycin (Sigma) and were maintained in an incubator at 37 °C with 5% $CO_2$. HEK293T-derived Hana3A cells (H. Matsunami, Duke University) were maintained under selective conditions with 1 $\mu$g/mL puromycin (Sigma). Cells were transfected with plasmid DNA using Lipofectamine 2000 (Invitrogen). A1- and ACP-tagged proteins at the cell surface were labeled by incubating the cells 24 h after transfection for 20 min with a fluorescent coenzyme A (CoA) derivative (5 $\mu$M CoA-Cy5) (NEB Bioconcept), 1 $\mu$M ACP synthase (NEB Bioconcept), and 10 mM $MgCl_2$ in Dulbecco's phosphate-buffered saline (D-PBS) (Invitrogen).

**Confocal Fluorescence Microscopy.** Cells grown on an eight-chamber coverglass (Labtek) were transfected with 750 ng of pACP-OR256-17-EGFP or 750 ng of pA1-OR256-17-EGFP, cotransfected with 375 ng of pACP-OR256-17-EGFP or 375 ng of pA1-OR256-17-EGFP and 375 ng of pRTP1S, or transfected with 750 ng of pA1-NK1R-EGFP. The fusion proteins labeled with CoA-Cy5 and EGFP were imaged using a confocal microscope (LSM510, Carl Zeiss, Jena, Germany) equipped with a 63×, 1.2NA Zeiss water immersion objective and appropriate filter sets operated in multitracking mode.

**Flow Cytometry.** Cells transiently expressing A1-OR256-17-EGFP or co-expressing A1-OR256-17-EGFP and RTP1S were labeled with CoA-Cy5 and diluted in PBS, yielding a density of $10^6$ cells/mL. Fluorescence measurements were taken on a CyAn ADP LX 9-color analyzer (DakoCytomation) employing 488 and 633 nm excitation. The fluorescence of EGFP and Cy5 was measured using 505−530 and 665−720 nm band-pass filters, respectively. Data analysis was performed on viable cells using SummitTM (DakoCytomation).

**Quantification of OR Cell Surface Expression.** Hana3A cells grown on 25 mm diameter glass coverslips were transfected with 2 $\mu$g of pA1-OR256-17-EGFP and 2 $\mu$g of pRTP1S. Twenty-four hours after transfection and 20 min after A1 had been labeled with CoA-Cy5, cells were diluted in PBS, yielding a density of $1.4 \times 10^6$ cells/mL. Fluorescence measurements were taken in a cuvette on an Eclipse (Varian) spectrofluorometer. The emission spectra were recorded from 640 to 720 nm upon excitation at 633 nm. A calibration curve was established by fitting a linear relation between the fluorescence intensities of a set of samples of Cy5 at concentrations ranging from $9 \times 10^{-13}$ to $4.5 \times 10^{-9}$ M. Fluorescence spectra of the cell surface-labeled OR were measured and corrected for unspecific labeling by subtracting the emission spectrum of mock-transfected Hana3A cells treated with CoA-Cy5. By comparison with the calibration curve, the mean number of Cy5-labeled receptors per cell was determined.

**Quantification of OR Responses.** Twenty hours before transfection, Hana3A cells were seeded into 96-well plates (Greiner) at a concentration of $3.5 \times 10^6$ cells/mL of culture medium; 75 ng of pRTP1S, 150 ng of the cAMP response element fused to the secreted alkaline phosphatase (pCRE-SEAP),[21] and either 75 ng of pOR256-17 or 75 ng of calf thymus DNA were used for cotransfection. Odorant compounds diluted in DMEM/F12 without FCS were added 7 h after transfection. Cells were incubated for 16 h at 37 °C with 5% $CO_2$. The culture medium was then mixed with an equal volume of 1 M diethanolamine bicarbonate (pH 9.8) containing 20 mM *p*-nitropheny1phosphate (pNPP) (Sigma) and 1 mM $MgCl_2$ (Sigma). Absorbance was measured at 410 nm using a multiwell absorbance plate reader (Molecular Devices).

## RESULTS

**Expression of mOR256-17 in Heterologous Cells.** EGFP was fused to the C-terminus of mOR256-17 to determine the total amount of OR in individual cells. Furthermore, either of two different polypeptide tags was attached to the N-terminus of mOR256-17: the long ACP tag or the short A1 tag composed of 12 amino acids for post-translational labeling using phosphopantheinyl transferase and non-membrane permeable fluorescent CoA derivatives as substrates. To be labeled, the ACP and A1 tags must be accessible at the

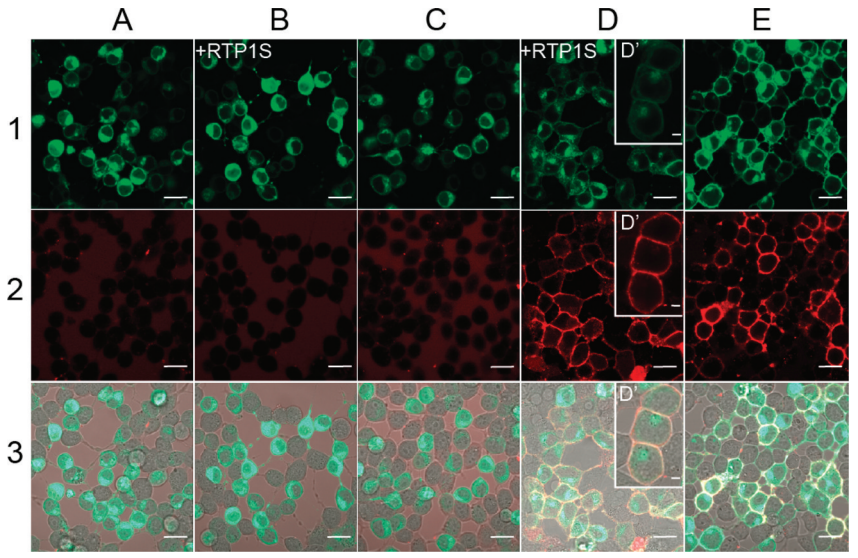


**Figure 1.** Subcellular localization of different mOR256-17 constructs expressed in Hana3A cells. Confocal micrographs show the entire receptor population labeled with EGFP (row 1, excitation at 488 nm and emission at 505−530 nm) and the selective labeling of cell surface receptors with CoA-Cy5 (row 2, excitation at 633 nm and emission at 665−720 nm). The overlay of the green and red fluorescence channel is shown in combination with the transmission channel (row 3): (A) ACP-mOR256-17-EGFP expression, (B) co-expression of ACP-mOR256-17-EGFP with RTP1S, (C) A1-mOR256-17-EGFP expression, (D) co-expression of A1-mOR256-17-EGFP with RTP1S, (D′) co-expression of A1-mOR256-17-EGFP with RTP1S, and (E) A1-NK1R-EGFP expression (control). Scale bars are 20 $\mu$m, except for those in panel D (5 $\mu$m).

extracellular membrane surface, allowing the attachment of fluorescent probes selectively to the membrane-inserted and correctly folded ORs. When performed at defined times after transfection for a specific duration, it is possible to pulse label functional ORs on the cell surface.[42] To investigate the mOR256-17 expression level in different cell types, we transfected the relevant plasmid constructs into Hana3A cells and imaged intracellular and membrane-localized receptors after post-translational labeling using confocal microscopy (Figure 1). Cells expressing A1-NK1R-EGFP, a prototypical GPCR exhibiting a high level of expression in heterologous cells, were used as a quantitative fluorescence standard.[41] The dual-color labeling strategy based on ACP and EGFP tags revealed that an essential prerequisite for achieving cell surface expression of mOR256-17 in heterologous cells was the cotransfection of the gene for the accessory protein RTP1S. Cotransfection of the short version RTP1S enhances the expression of ORs in the plasma membrane and strengthens OR responses in $Ca^{2+}$ ion signaling.[31] Even in Hana3A cells, which were engineered to endogenously express the long RTP1 protein, RTP1S had to be cotransfected to achieve cell surface expression of mOR256-17 (Figure 1). The cotransfection of other plasmids encoding the accessory proteins, RTP2 and REEP1, had no influence on mOR256-17 expression at the plasma membrane (data not shown).

Interestingly, only the receptor comprising the 12-amino acid A1 tag was detected on the cell surface membrane, whereas the receptor fused to the 77-amino acid ACP tag was not integrated into the plasma membrane at a detectable level; instead, it accumulated in intracellular compartments (Figure 1). The intensity of the fluorescence signals of Cy5-A1-mOR256-17 in transiently transfected cells was comparable to that of Cy5-A1-NK1R, indicating that a large number of OR molecules are functionally integrated in the plasma membrane (Figure 1).

**Quantification of the Expression of Functional mOR256-17 at the Cell Membrane.** Flow cytometry of large populations of heterologous cells confirmed our microscopical observation for individual cells that co-expression of RTP1S is essential for plasma membrane expression of mOR256-17. No functional ORs were labeled on the surface of cells expressing A1-mOR256-17-EGFP in the absence of RTP1S (Figure 2A). Co-expression of RTP1S, however, resulted in the distinct appearance of fluorescent receptors in the plasma membrane. Cells expressing mOR256-17-EGFP with or without RTP1S exhibited very similar EGFP fluorescence intensities, indicating that the co-expression of RTPS had no detectable influence on the overall level of expression of mOR256-17 (data not shown). The analysis of mOR256-17 cell surface expression by flow cytometry revealed a broad distribution of Cy5 fluorescence intensities with a distinct high-intensity peak corresponding to one-third of the transfected cell population (Figure 2A). This fraction exhibited a high level of expression of $10^6$ receptors/cell. Finally, we used fluorescence spectroscopy to analyze the average cell surface expression level of mOR256-17 in transiently transfected Hana3A cells labeled with increasing concentrations of Cy5 until saturation was achieved. Following this approach, we determined a mean value of $7 \times 10^5$ receptors per cell (Figure 2B,C).

**Screening of Specific Ligands of mOR256-17.** By screening transiently transfected Hana3A cells with an odorant library of ~250 compounds and using the SEAP reporter activity as a read-out for receptor-mediated odorant responses, we discovered the following mOR256-17-specific agonists: 4-*tert*-butyl-1-cyclohexanone, (−)-carvone, and hexane nitrile specifically, which activated the receptor in a concentration-dependent manner (Figure 3). These odorants can be classified into three chemical lead structures: saturated cyclic compounds, unsaturated cyclic compounds, and noncyclic aliphatic compounds (Figure 3). To investigate the molecular details of mOR256-17-specific odorants and to create an activity map of the odorant structures, we screened compounds with distinct


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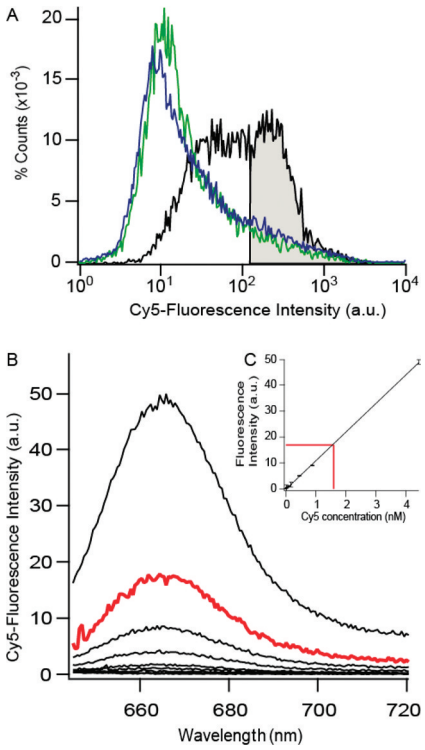


**Figure 2.** Flow cytometry to quantify mOR256-17 expressed on the surface of Hana3A cells. (A) After post-translational labeling with CoA-Cy5, the Cy5 fluorescence was measured on cells expressing A1-mOR256-17-EGFP together with RTP1S (black line) and in the absence of RTP1S (blue line). The shaded area corresponds to a distinct fraction of cells showing a particularly high level of cell surface receptors. Nontransfected Hana3A cells labeled with Cy5 were used as a negative control (blue line). (B) Fluorescence spectrum of Hana3A cells co-expressing OR and RTP1S that were labeled with CoA-Cy5 under saturating conditions (5 $\mu$M) (red). Fluorescence spectra of solutions of CoA-Cy5 at concentrations ranging from 1 pM to 4.5 nM were used for calibration (black). (C) Fluorescence intensities (665 nm) vs concentration as measured in panel B to determine the average concentration of Cy5-labeled ORs per cell (red).

modifications on the three lead structures for their potency in activating mOR256-17.

Centered around the chemical structure of 4-*tert*-butyl-1-cyclohexanone, we have tested different sizes of carbon cycles and various alkyl substituents at different positions on the ring. We found two additional OR-activating compounds, 4-*tert*-butyl-1-cyclohexanol and 4,4-dimethyl-1-cyclohexanone, both sharing a bulky substituent at position 4 and a six-membered carbon ring (Figure 3A). We observed that the $EC_{50}$ value increased 2-fold when the carbonyl group was substituted with an alcohol group, and it increased 5-fold when the *tert*-butyl group was replaced with a dimethyl group (Figure 3). These results indicate that mOR256-17 accepts different polar groups but is more specific for a carbonyl group and exhibits a preference for bulky aliphatic groups. Furthermore, the compounds sharing a substituted hexyl ring at positions 2 and 3 exhibited no SEAP activity, pointing to the prerequisite for a bulky group in position 4 for activating mOR256-17 (Figure 3). This structure−activity relation is supported by the observation that cyclohexane and molecules without a hexyl ring did not activate the receptor (Figure 3). However, norbornanone, which carries a bridged ring structure, induced a weak reporter response at high concentrations. In contrast, camphor, which also has a bridged ring structure, did not activate the receptor (Figure 3).

The second most potent agonist, (*R*)-(−)-carvone, has a substituted six-membered carbon cycle that is structurally related to 4-*tert*-butyl-1-cyclohexanone, but it possesses a carbon−carbon double bond in the ring and a bulky alkenyl group at position 2 (Figure 3A). The bulky alkenyl group of the carvone cycle seems to replace the *tert*-butyl group of 4-*tert*-butyl-1-cyclohexanone. Additionally, we have observed different $EC_{50}$ values obtained for (*R*)-(−)-carvone and (*S*)-(+)-carvone revealing a stereoenantiomer selection of mOR256-17 for the former (Figure 3). In the case of limonene, only the *R* enantiomer was recognized by the receptor with a weak SEAP response at a high $EC_{50}$ value, while the *S* enantiomer did not activate the receptor (Figure 3). To investigate the relevance of an unsaturated carbon cycle for activation of the receptor, we have tested a series of benzene derivatives (Figure 3). However, none of them generated a detectable SEAP activity, indicating that only specific ring structures interact with the receptor. Finally, we also discovered that aliphatic odorants, such as hexane nitrile, specifically activate mOR256-17 (Figure 3). However, they are less potent agonists than cyclic compounds. The structure of hexane nitrile has challenged us to consider aliphatic compounds as mOR256-17-specific compounds. Therefore, we analyzed molecules with different carbon chain lengths and functional groups (Figure 3A). However, in this last series of odorant compounds, no molecules were found to activate mOR256-17 (Figure 3).

## DISCUSSION

Although research has progressed in detecting downstream OR signaling,[43−45] the molecular mechanism of olfaction at the receptor level remains poorly understood. The development of simple and efficient methods for high-yield production of recombinant native ORs in combination with functional assays is therefore a prerequisite for fostering structural and functional investigations of ORs. In this study, we have achieved high levels of expression of mouse olfactory receptor mOR256-17 in HEK293-derived mammalian cells. We yielded an average level of surface expression of $7 \times 10^5$ ORs/cell. One-third of the transfected cell population expressed even up to $10^6$ ORs/cell. This expression level is to the best of our knowledge the highest so far observed for ORs in mammalian cells using transient transfection and is close to that of a strongly expressing GPCR, the neurokinin1 receptor NK1. This is an important finding considering the frequently encountered difficulties of OR production in heterologous cells.[10,46]

The high level of OR expression in HEK293 cells has only been achieved thus far for a synthetic human codon-optimized OR.[35] Our approach did not involve receptor modifications on the DNA level for efficient OR production. Recombinant expression of ORs has previously also been attempted in bacterial and insect systems, which yielded only small quantities of the full-length receptors.[47,48] A recent study reported significant synthesis of full-length OR in *Escherichia coli*, however, without providing evidence of functional activity.[49] The use of cell systems lacking the mammalian post-translational machinery might result in the absence of critical protein modifications. For instance, glycosylation is an important modification of most GPCRs that correlates to the


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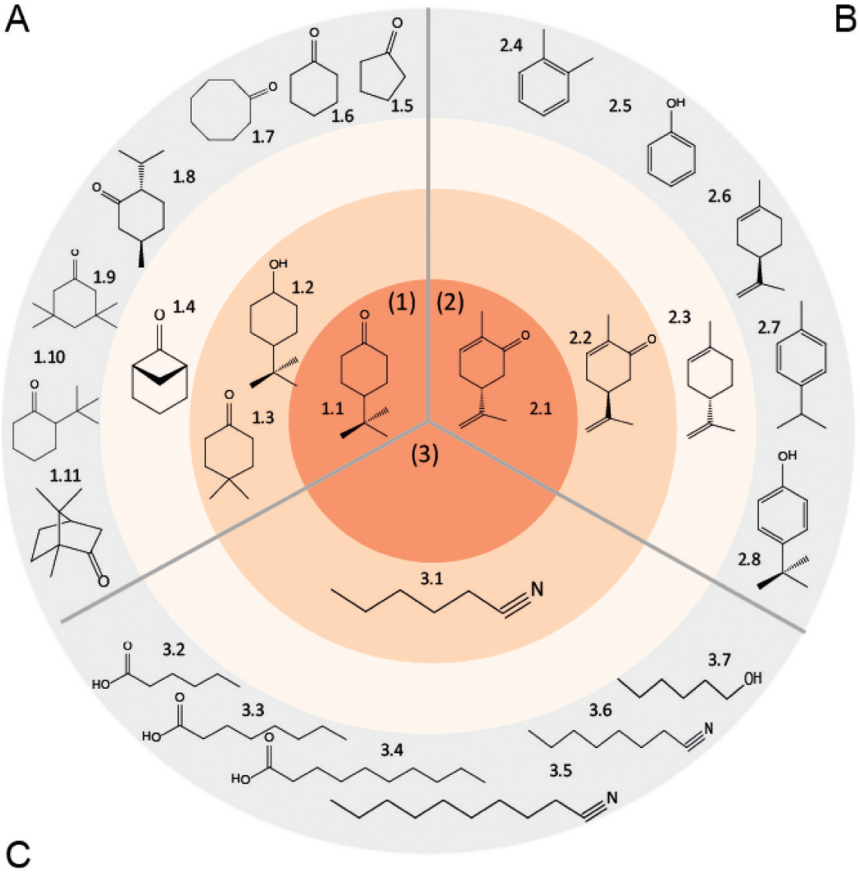
A




B

| | (1) Saturated cyclic compounds | EC50 [μM] |
|---|---|---|
| 1.1 | Tert-butyl-4-cyclohexanone | 6.9 ± 0.6 |
| 1.2 | Tert-butyl-4-cyclohexanol | 14.8 ± 0.7 |
| 1.3 | Dimethyl-4-4-cyclohexanone | 33.2 ± 9.8 |
| 1.4 | Norbornanone | 118.2 ± 8.8 |
| 1.5 | Cyclopentanone | n. a. |
| 1.6 | Cyclohexanone | n. a. |
| 1.7 | Cyclooctanone | n. a. |
| 1.8 | Menthone (+/-) | n. a. |
| 1.9 | Tetramethyl-2,2,6,6-cyclohexanone | n. a. |
| 1.10 | Tert-buthyl-2-cyclohexanone | n. a. |
| 1.11 | Camphor (+/-) | n. a. |
| | **(2) Unsaturated cyclic compounds** | **EC50 [μM]** |
| 2.1 | Carvone (-) | 6.7 ± 1.8 |
| 2.2 | Carvone (+) | 13.4 ± 0.9 |
| 2.3 | Limonene (-) | 62.0 ± 7.3 |
| 2.4 | O-xylene | n. a. |
| 2.5 | Phenol | n. a. |
| 2.6 | Limonene (+) | 223.6 ± 199 |
| 2.7 | P-cymene | n. a. |
| 2.8 | Tert-buthyl-4-phenol | n. a. |
| | **(3) Aliphatic compounds** | **EC50 [μM]** |
| 3.1 | Hexane nitrile | 29.6 ± 9.1 |
| 3.2 | Hexanoic acid | n. a. |
| 3.3 | Octanoic acid | n. a. |
| 3.4 | Decanoic acid | n. a. |
| 3.5 | Decane nitrile | n. a. |
| 3.6 | Octane nitrile | n. a. |
| 3.7 | Hexanol-1 | n. a. |

C

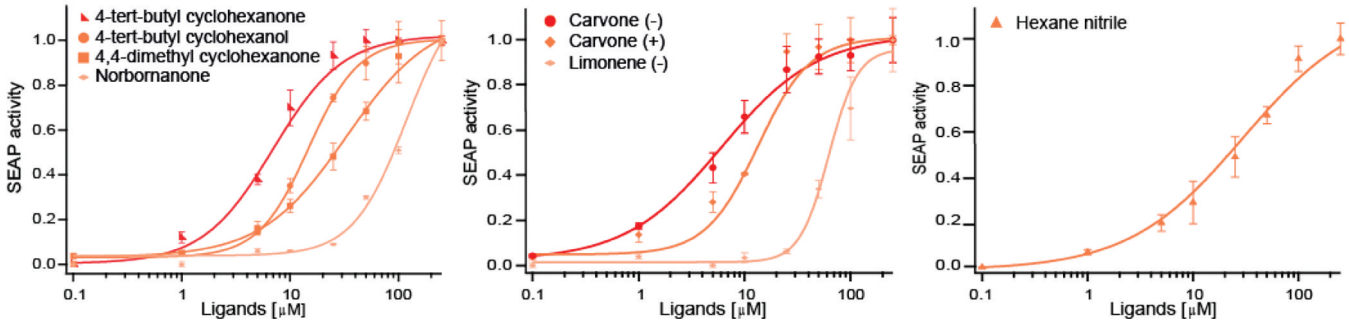



**Figure 3.** Molecular features of odorants activating mOR256-17. (A) Chemical structures of three classes of mOR256-17-specific agonists. (1) Saturated cyclic compounds, (2) unsaturated cyclic compounds, and (3) noncyclic aliphatic compounds: $EC_{50} \leq 10$ $\mu$M (dark orange), $EC_{50}$ = 10−40 $\mu$M (orange), $EC_{50} > 40$ $\mu$M (light orange), and no activation (light blue). (B) List of mOR256-17-specific odorants and $EC_{50}$ values. (C) Normalized dose−response profiles of representative ligands of each structural class of odorant (left, saturated cyclic compounds; middle, unsaturated cyclic compounds; right, noncyclic aliphatic compounds) measured on the wild-type receptor.

cellular localization and function of the receptors.[50] ORs have conserved N-linked glycosylation sites at their N-termini that are structural determinants for OR trafficking.[26] Studies focusing on other GPCRs, like rhodopsin and the AT1 angiotensin receptor, have shown that loss of glycosylation may lead to a decreased receptor function or stability.[51,52] These findings indicate that the production of ORs in mammalian cells might be crucial for functional expression and purification. Apart from these obvious advantages, mammalian expression systems also offer the possibility of probing OR function directly in the same heterologous cell type used for protein production.[20,21,33,53] This is not possible in bacterial or insect cell expression systems or in the recently emerging cell-free OR production systems.[54−56]

The transport of ORs to the plasma membrane is a highly regulated process. The olfactory-specific chaperones RTP1, RTP2, and REEP have been shown qualitatively to enhance the cell surface functional expression of a number of ORs in heterologous cells, but a generally applicable solution for achieving high-yield heterologous cell surface expression of ORs of different species does not exist.[29,34,57] We have found that the N-terminal fusion of a rhodopsin signal sequence, which is widely used to promote cell surface expression of ORs of differenct species in mammalian cells,[28] has no detectable influence on the surface expression of mOR256-17. Therefore, we expressed native mOR256-17 without recombinant modifications. The only requirement for achieving a high level of mOR256-17 expression in the plasma membrane was the co-expression of RTP1, whereas no enhancing influence was observed for RTP2 and REEP1.

For analyzing the functional activity of mOR256-17 during the production process, the knowledge of its cognate ligand is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essential. Therefore, we screened a large odorant library and have discovered six mOR256-17-specific agonists. Five of these share some structural similarities: a six-carbon ring structure (either saturated or unsaturated) with a keto or alcohol group and a compact bulky, hydrophobic residue at the 4 or 5 ring position. One odorant comprises a saturated aliphatic chain together with a nitrile group structurally unrelated to the other principal agonists. Two of the activating odorants are potent agonists with $EC_{50}$ values in the micromolar range, which compare well to the ranking of effective odorant agonits in other studies.[21,34,58] This preference for a distinct odorant structure has already been observed for other ORs: rat OR-I7 responds exclusively to aldehydes,[59] and mOR42-3 and mOR42-1 accept only aliphatic dicarboxylic acids.[60] Even higher ligand specificity has been found for some ORs. For example, hOR17-4 responds to bourgeonal, lilial, and florolozone,[61] and M71 responds to acetophenone and benzaldehyde.[62] Other studies indicate that certain ORs accept a wide range of agonists even with structurally unrelated odorants.[21,63] The knowledge of ligand structures that activate individual receptors is critical in view of current efforts to gain some basic understanding of the principle of olfactory coding at the receptor level.[8,11,34]

The ectopic expression of a number of specific ORs in some non-olfactory human tissues[64] indicates other potential biochemical functions of certain ORs apart from sensing odorants. Experimental proof for this hypothesis has been documented in spermatozoa[65,66] and in prostate cancer cells.[67] Functional studies at a molecular level that require large amounts of receptor to elucidate additional functions of mOR256-17 have been lagging. This OR has been proposed to perform homophilic interactions at axonal terminals between neighboring neurons and to play a role in the directed neuronal wiring of the olfactory system.[68] Furthermore, during embryonic development, the receptor is expressed not only in a large number of olfactory sensory neurons but also in adjacent nonsensory cells, pointing to its potential role in the development of the olfactory system.[36] The functional overexpression of mOR256-17, as exemplified in this study, makes the receptor amenable for biochemical and biophysical studies. Our thorough description of mOR256-17 activating ligands will be essential for probing its function in future scale-up productions. The dual-color labeling approach described here will be generally useful for quantifying membrane proteins in mammalian expression systems and optimizing their cellular protein yields.

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## ABBREVIATIONS

OR, olfactory receptor; mOR256-17, mouse olfactory receptor 256-17; GPCR, G protein-coupled receptor; RTP1, receptor-transporting protein; PPtase, phosphopantetheine transferase; ACP, acyl carrier protein; A1, peptide tag as a substrate for PPtase; CoA, coenzyme A; EGFP, enhanced green fluorescent protein; pCRE-SEAP, cAMP-response-element-secreted alkaline phosphatase reporter plasmid.

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