

Agonists for 13 Trace Amine-Associated Receptors Provide Insight into the Molecular Basis of Odor Selectivity

David M. Ferrero,[†] Daniel Wacker,[‡] Miguel A. Roque,[†] Maude W. Baldwin,[§] Raymond C. Stevens,[‡] and Stephen D. Liberles^{*†}

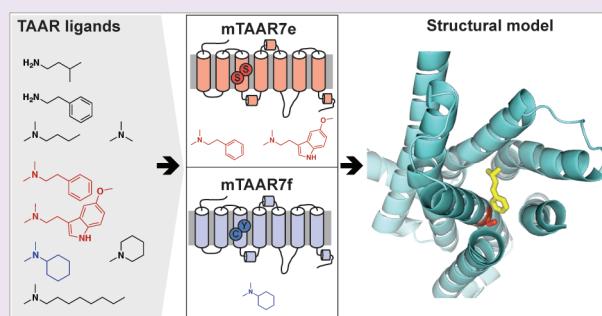
[†]Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115, United States

[‡]Department of Molecular Biology, The Scripps Research Institute, La Jolla, California 92037, United States

[§]Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, Massachusetts 02138, United States

S Supporting Information

ABSTRACT: Trace amine-associated receptors (TAARs) are vertebrate olfactory receptors. However, ligand recognition properties of TAARs remain poorly understood, as most are “orphan receptors” without known agonists. Here, we identify the first ligands for many rodent TAARs and classify these receptors into two subfamilies based on the phylogeny and binding preference for primary or tertiary amines. Some mouse and rat orthologs have similar response profiles, although independent *Taar7* gene expansions led to highly related receptors with altered ligand specificities. Using chimeric TAAR7 receptors, we identified an odor contact site in transmembrane helix III that functions as a selectivity filter. Homology models based on the β_2 adrenergic receptor structure indicate spatial proximity of this site to the ligand. Gain-of-function mutations at this site created olfactory receptors with radically altered odor recognition properties. These studies provide new TAAR ligands, valuable tools for studying receptor function, and general insights into the molecular pharmacology of G protein-coupled receptors.



The initial event in mammalian olfaction is the detection of odor molecules by dedicated sensory neurons in the nose. Olfactory sensory neurons, in particular, use two families of G protein-coupled receptors (GPCRs), odorant receptors (ORs) and trace amine-associated receptors (TAARs), to effectively convert chemical signals from the environment into electrical signals that are transmitted to the brain.^{1,2} In addition, rare olfactory sensory neurons use a noncanonical odor detection mechanism that relies on membrane guanylate cyclase-D instead of GPCRs.¹

The olfactory system uses a combinatorial coding scheme, in which each receptor detects multiple odors and each odor activates multiple receptors.³ Consistent with this scheme, many olfactory receptors are broadly tuned to detect a large number of structurally related chemicals,^{4,5} although some are narrowly tuned for particular odors.⁶ While many OR agonists have now been identified,^{4–8} our current understanding of the ligand specificity among olfactory receptors is based on studies involving only a small number of ORs.^{5,9–11} The odor binding pocket in these ORs is composed of highly variable amino acid side chains in transmembrane (TM) helices III, V, and VI.^{5,9,10}

In contrast, the structural basis for odorant recognition by TAARs remains uncharacterized, mainly because of a lack of identified agonists. The TAARs comprise an evolutionarily conserved family of receptors found in diverse vertebrates, including 15 in mouse (mTAARs), 17 in rat (rTAARs), 6 in human, and 112 in zebrafish.^{12–17} TAARs do not share

sequence similarity with ORs but instead are distantly related to biogenic amine receptors, a medically important class of GPCRs.^{12–17} In mammals, most TAARs retain amine recognition motifs conserved in biogenic amine receptors,^{12,18} including an aspartic acid in TM helix III that forms a salt bridge with the ligand amino group. These observations suggested that rodent TAARs would be amine receptors, but ligands for them remained largely unknown.

We previously identified the first ligands for mTAAR3, mTAAR4, mTAAR5 and mTAAR7f, and each indeed detects a different combination of volatile amines.¹⁹ In addition, ligands were reported for TAAR1, the only receptor in this family that is not an olfactory receptor, and rTAAR4 (then called TA-2).^{19–22} Identified TAAR agonists include biogenic amines secreted into urine, a rich source of chemosignals for rodents.^{19,23,24} A TAAR4 agonist, 2-phenylethylamine, is a carnivore odor that repels rodents,²³ and a TAAR5 agonist, trimethylamine, is a sexually dimorphic mouse odor.¹⁹ The biosynthesis of these naturally occurring TAAR ligands can be dynamic, varying with age, sex, or physiological state.^{19,24} Furthermore, some TAAR ligands trigger innate behavioral responses in mice.^{23,25}

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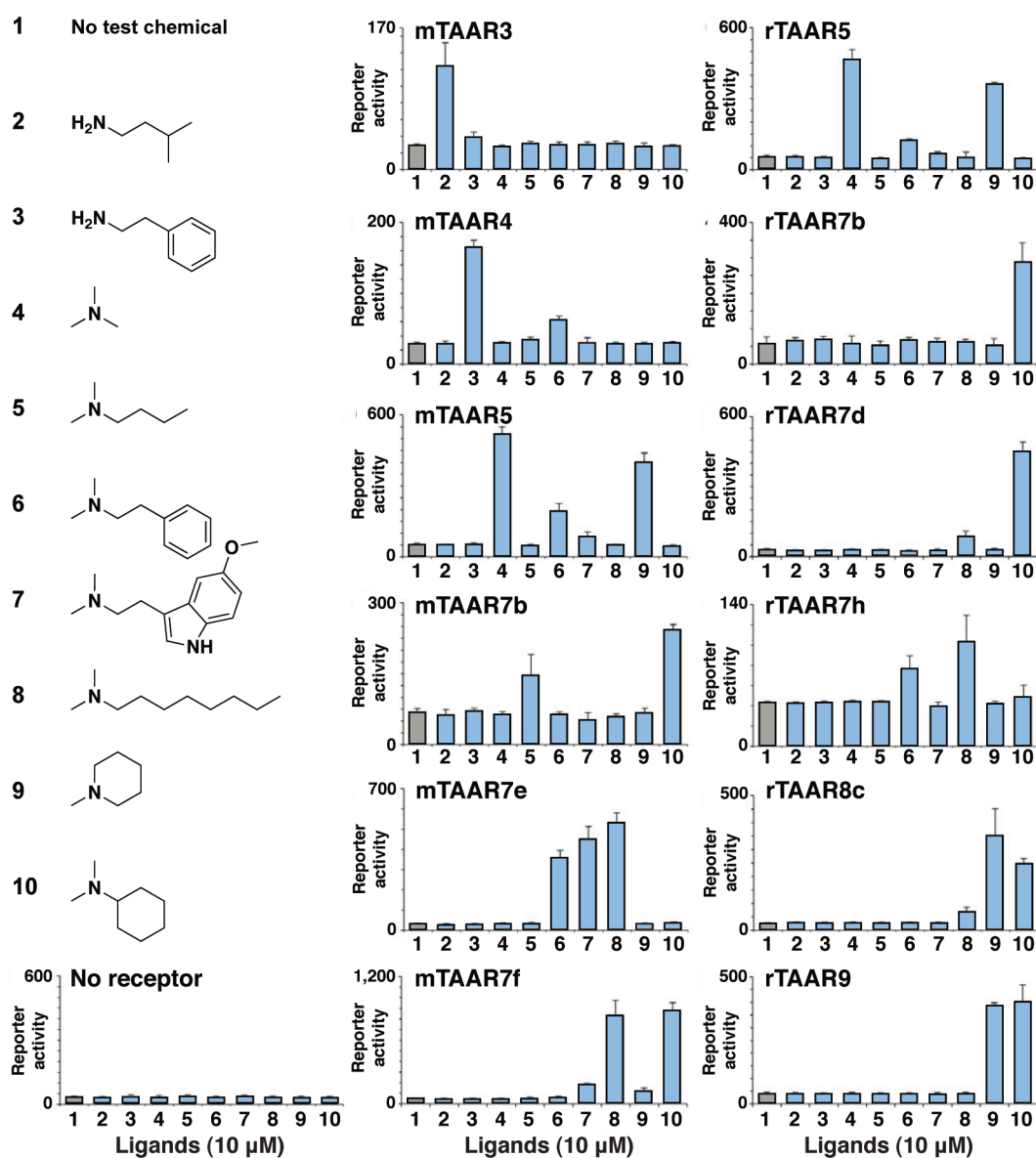


Figure 1. Thirteen TAARs detect volatile amines. HEK293 cells were transfected with TAAR and reporter plasmids, incubated with ligands (10 μ M), and assayed for reporter activity (triplicates \pm the standard deviation). Test conditions were as follows: (1) no ligand, (2) isoamylamine, (3) 2-phenylethylamine, (4) trimethylamine, (5) *N,N*-dimethylbutylamine, (6) *N,N*-dimethyl-2-phenylethylamine, (7) 5-methoxy-*N,N*-dimethyltryptamine, (8) *N,N*-dimethyloctylamine, (9) *N*-methylpiperidine, or (10) *N,N*-dimethylcyclohexylamine. Twelve TAARs indicated and rTAAR3 (Figure 1 of the Supporting Information) responded to at least one ligand shown, but no responses were observed in control cells transfected with the reporter plasmid alone. On the basis of these data, 6 of 14 mouse olfactory TAARs and 7 of 16 rat olfactory TAARs respond selectively to amines.

Here, we set out to identify agonists for additional mouse and rat TAARs. We examined odor response profiles using a previously established reporter gene assay based on cAMP-dependent odor transduction in olfactory sensory neurons.^{19,23} Briefly, TAAR plasmids were transfected into HEK293 cells along with a cAMP-dependent reporter gene encoding secreted alkaline phosphatase (CRE-SEAP). TAARs were expressed both in unmodified form and as fusion proteins with an N-terminal sequence of bovine rhodopsin ("Rho tag") that promotes cell surface expression of some chemosensory receptors.¹¹ Transfected cells were incubated with test chemicals, and phosphatase activity was quantified with a fluorescent substrate as a reporter for TAAR activation. In initial experiments, we tested 38 different odorant mixtures containing 244 structurally diverse test chemicals (2–5 μ M) for the ability to activate each mTAAR. Subsequently, we tested 73

amines that included known mTAAR agonists and related chemicals for the ability to activate each rTAAR. Test chemicals are listed in the Supporting Information.

Using this strategy, we identified ligands for nine additional olfactory TAARs that were previously orphan receptors (Figure 1), including mTAAR7b, mTAAR7e, rTAAR3, rTAAR5, rTAAR7b, rTAAR7d, rTAAR7h, rTAAR8c, and rTAAR9. The first ligands for mTAAR7b and mTAAR7e were identified in previous unpublished work (S. D. Liberles and L. B. Buck). Responding TAARs were functional with or without a Rho tag except for mTAAR4 and rTAAR5, which required a Rho tag, and mTAAR3 and rTAAR7b, which did not work with a Rho tag. Each of these nine TAARs was activated by volatile amines, while other chemicals (0 of 202) lacking amino groups did not activate any mTAARs. Ligand preferences were similar between mouse and rat orthologs, in cases where ligands were identified

for both receptors. Amines that activated mTAAR3, mTAAR4, and rTAAR3 were primary amines that could be derived from natural amino acids by a single decarboxylation reaction. In contrast, 10 other TAARs were activated by tertiary amines, including several *N,N*-dimethylated amines. Identified ligands elicited half-maximal TAAR responses at concentrations (EC_{50}) that ranged from 100 nM to 30 μ M (Figure 1 of the Supporting Information), comparable to the agonist sensitivity of ORs in similar assays.^{8,10} Interestingly, several TAAR ligands were natural products secreted by animals, including various amino acid derivatives and the serotonin metabolite 5-methoxy-*N,N*-dimethyltryptamine whose production patterns in urine are dynamic and vary with physiological state.^{26,27}

We noted that TAARs could be clustered into two groups based on whether they detected primary or tertiary amines. Interestingly, these two groups mapped to distinct branches of the TAAR phylogenetic tree (Figure 2a). This phylogeny was constructed by Bayesian analysis of all *Taar* nucleotide sequences in the mouse, human, and rat genomes. Unlike vomeronasal receptors, which are rapidly evolving,²⁸ TAAR orthologs are highly conserved in sequence and gene number between species, as well as in ligand binding preference when determined (Figures 1 and 2 and Figure 2 of the Supporting Information). Exceptions are lineage-specific expansions of the TAAR7 and TAAR8 subfamilies, which occurred independently in mouse and rat. Our analysis suggests that the last common ancestor of rat and mouse likely had one TAAR8 and one TAAR7.

The rapid expansion of the TAAR7 subfamily led to the evolution of highly related olfactory receptors with distinct response profiles. Based on this observation, we reasoned that the TAAR7 subfamily could provide a unique opportunity to study how evolutionary changes in receptor sequence drive changes in odor binding preference.

We identified three amines, **6** (*N,N*-dimethylphenylethylamine), **7** (5-methoxy-*N,N*-dimethyltryptamine), and **10** (*N,N*-dimethylcyclohexylamine), that activated different TAAR7 paralogs in mouse and rat. Two receptors (mTAAR7e and rTAAR7h) were activated by **6** but not **10**, while four receptors (mTAAR7b, mTAAR7f, rTAAR7b, and rTAAR7d) were activated by **10** but not **6**. We aligned the sequences of responding TAAR7s to identify amino acid variations that correlated with differences in odor responses (Figure 2b,c). These sequences were highly related (>87% identical), and most amino acids were conserved (found in >5 of 6 analyzed sequences). Of the few amino acid variations identified, only residues 132^{3,37} and 133^{3,38} varied in accordance with ligand response profiles. (Superscripts indicate the TM number and relative TM position of particular residues, as defined by the indexing method of Ballesteros and Weinstein¹⁸. This method defines the most conserved residue in each TM helix across all GPCRs as position 50, and positions of other residues are defined in relation to this position. For example, residue 132^{3,37} is 13 amino acids from the most conserved residue of TM helix III, Arg145^{3,50}.) Interestingly, residues 132^{3,37} and 133^{3,38} are immediately adjacent on TM helix III and in the proximity of Asp127^{3,32}, the conserved amine contact site of biogenic amine receptors.¹⁸ Furthermore, a key odor contact site of a eugenol-detecting OR, Ser113^{3,40}, occupies a similar position in TM helix III.¹⁰ Based on these observations, we reasoned that amino acid variations at positions 132^{3,37} and 133^{3,38} could contribute to selective TAAR responses.

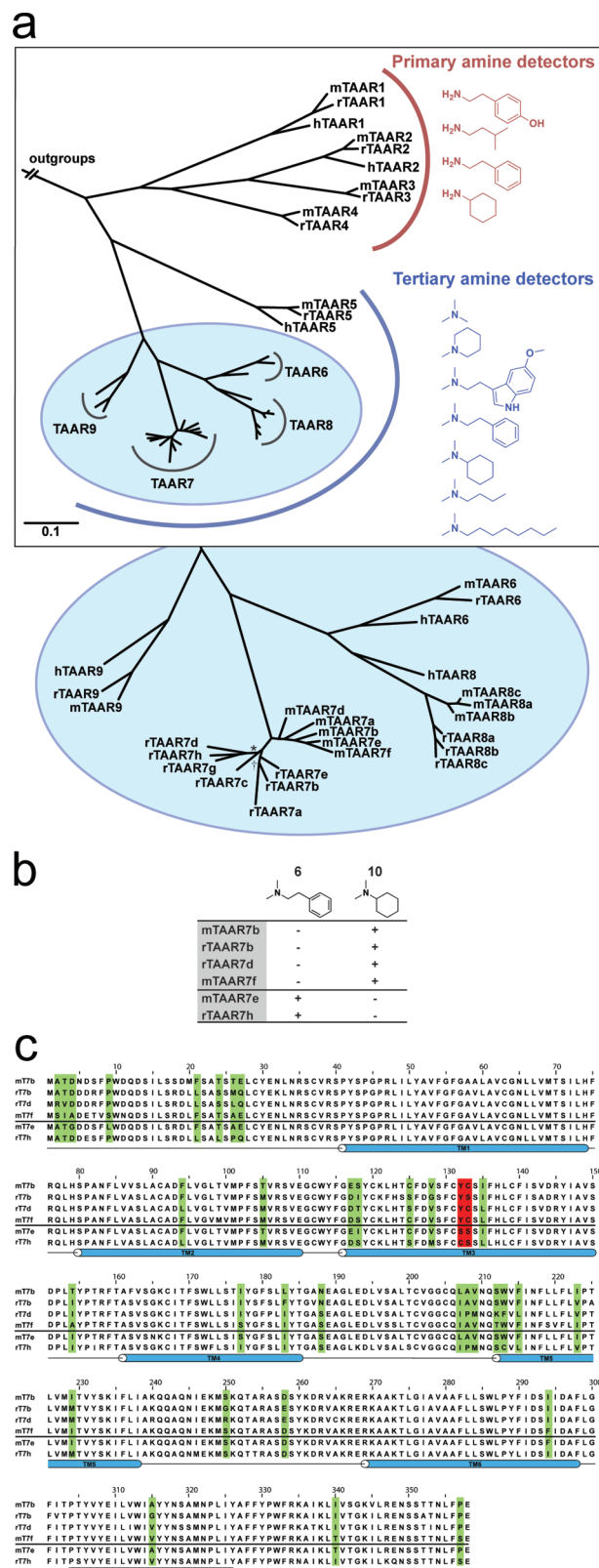


Figure 2. Functional evolution of the TAAR family. (a) TAAR phylogenetic tree constructed by Bayesian analysis of all *Taar* nucleotide sequences in the mouse, human, and rat genomes. TAARs cluster into two groups, which exhibit distinct binding preferences for primary or tertiary amines. All tree nodes have a posterior probability above 0.9, except those marked (* = 0.87, † = 0.86). (b) TAAR7s respond (+) or do not respond (−) to ligands **6** and **10** (10 μ M). (c) Alignment of the amino acid sequences of six

Figure 2. continued

TAAR7s with identified ligands. Four TAARs with sequences shown above the line (mTAAR7b, rTAAR7b, rTAAR7d, and mTAAR7f) respond to ligand **10** but not ligand **6**, whereas two TAARs with sequences shown below the line (mTAAR7e and rTAAR7h) respond to ligand **6** but not to ligand **10**. Residues that vary in two or more receptors are colored green; mutated positions (see Figure 3) are colored red, and TM segments are colored blue.

To test this hypothesis, we created mutant receptors in which amino acids at positions 132^{3,37} and 133^{3,38} of mTAAR7e were swapped into mTAAR7f and vice versa. Position 132^{3,37} is a tyrosine in mTAAR7f and the other three receptors that detect **10**, but a serine in mTAAR7e and a cysteine in rTAAR7h, the two receptors that detect **6**. Furthermore, position 133^{3,38} is a cysteine in mTAAR7f but a serine in mTAAR7e and rTAAR7h, the two receptors that detect **6**. We altered positions 132^{3,37} and 133^{3,38} by mutation of mTAAR7e (mTAAR7e^{S132Y, S133C} or “mTAAR7e-YC”) and mTAAR7f (mTAAR7f^{Y132S, C133S} or “mTAAR7f-SS”) and examined odor responses of these mutants using the cellular reporter gene assay (Figure 3).

Interestingly, this modification caused a dramatic reversal in odor responsiveness (Figure 3). mTAAR7e-YC had the same ligand selectivity profile as mTAAR7f rather than mTAAR7e. These effects were striking, as mTAAR7e-YC had >1000-fold enhanced affinity for **10** and >1000-fold decreased affinity for **6** or **7**. Furthermore, the reciprocal mutant, mTAAR7f-SS, had

mTAAR7e-like responses, displaying >1000-fold increases in affinity for both ligands **6** and **7** and ~100-fold reduced affinity for ligand **10**. These data provide strong evidence that residues 132^{3,37} and 133^{3,38} are part of the TAAR ligand binding pocket and form an important selectivity filter that imparts selective odor responses.

To gain additional insights into the structure of the odor-binding pocket in TAARs, we created homology models of mTAAR7e and mTAAR7f (Figure 4 and Figures 3 and 4 of the Supporting Information). We based our model on the X-ray crystal structure of the human β_2 adrenergic receptor (β_2 AR, Protein Data Bank entry 3P0G).²⁹ Although several other GPCR structural templates are now available,³⁰ β_2 AR was selected because it is 25% identical to mTAAR7e and mTAAR7f, binds similar amine ligands, and aligns without gaps in 6 of 7 TM helices. Based on these models, mTAAR7e and mTAAR7f have the canonical bundle of 7 α -helices followed by the intracellular helix VIII that runs parallel to the membrane axis. In addition, we observed other conserved motifs of class A GPCRs,³¹ such as a disulfide bridge between extracellular loop 2 (ECL2) and the extracellular end of helix III (Cys205 and Cys120^{3,25}), as well as the D/ERY sequence of the “ionic lock” motif at the cytoplasmic end of helix III.³¹ Surprisingly, we also observed a short α -helix in ECL2 (Glu193–Thr200) in mTAAR7e and mTAAR7f, a motif that is not common among most GPCRs, but is present in β_1 AR and β_2 AR.³¹

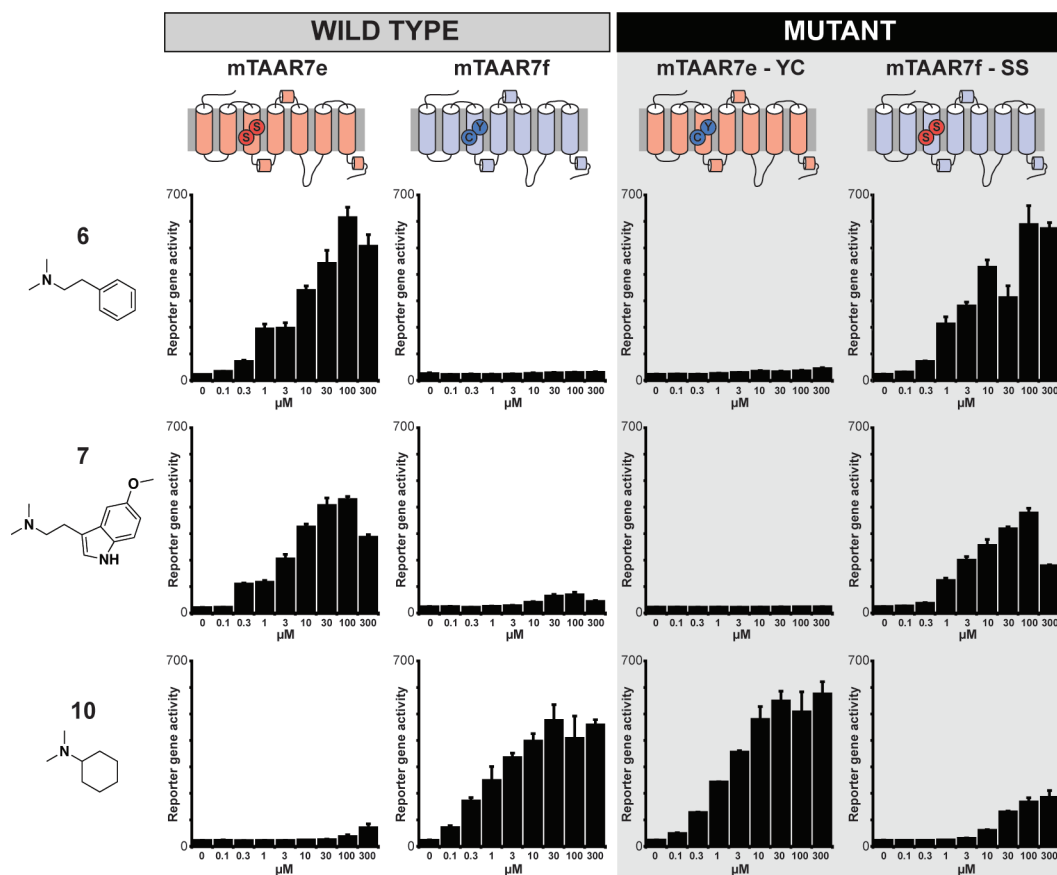


Figure 3. Altering TAAR responses by mutation of an odor selectivity filter. Sequences of mTAAR7e were swapped into mTAAR7f and vice versa by exchanging residues 132^{3,37} and 133^{3,38} of mTAAR7e (mTAAR7e-YC) and mTAAR7f (mTAAR7f-SS). Odor responses of these mutant receptors are shown using the cellular reporter gene assay for ligands **6**, **7**, and **10** at the indicated concentrations (triplicates \pm the standard error of the mean).

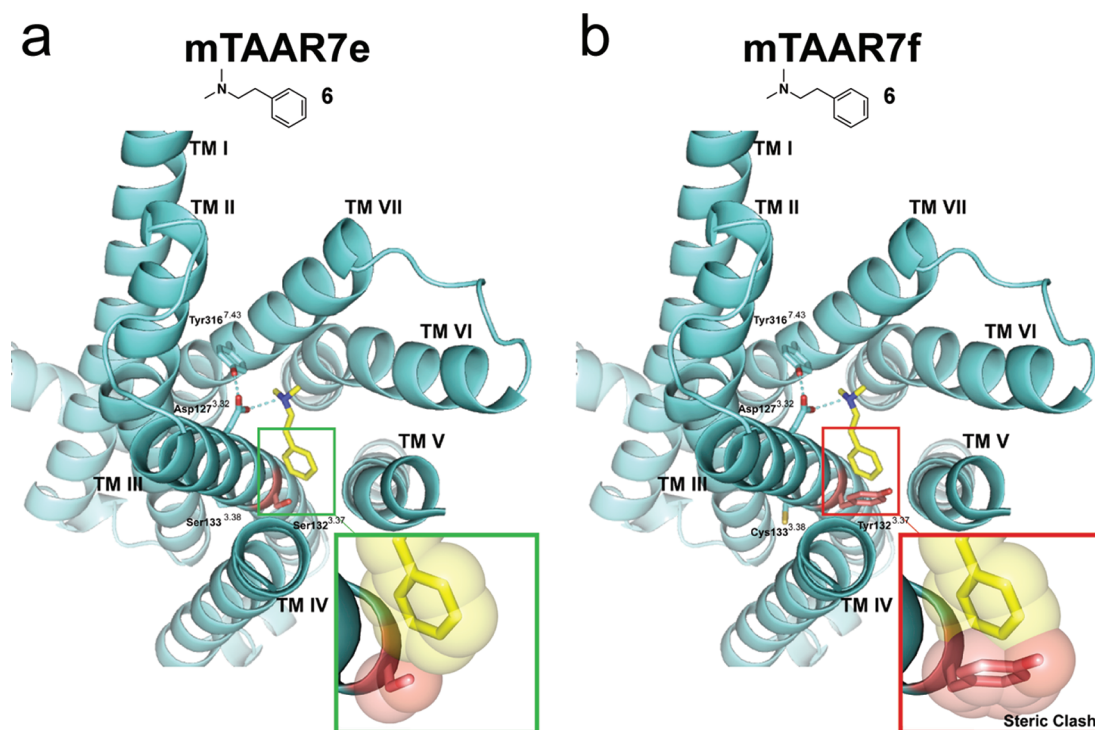


Figure 4. Homology modeling of mTAAR7e and mTAAR7f provides a molecular basis for selective odor recognition. Predicted structures (cyan) of mTAAR7e (a) and mTAAR7f (b) bound to *N,N*-dimethylphenylethylamine (**6**) (yellow). GPCR transmembrane helices are numbered from TM I to VII, and side chains of key residues that line the ligand binding site are displayed. Hydrogen bonds are shown as dotted cyan lines. Insets represent magnified views of ligand **6** interacting with residue 132^{3,37} of mTAAR7e and mTAAR7f. Van der Waals radii are shown with a transparent space-filling model and predict a steric clash of ligand **6** with residue Tyr132^{3,37} of mTAAR7f but not Ser132^{3,37} of mTAAR7e.

Next, we examined the putative ligand contact sites in the structural models of mTAAR7e and mTAAR7f. Our models suggested that the ligand amino group forms a salt bridge with Asp127^{3,32}, which itself is anchored by a hydrogen bond to the hydroxyl group of Tyr316^{7,43} (Figure 4 and Figures 3 and 4 of the Supporting Information). Asp127^{3,32} is conserved among many GPCRs, and a similar salt bridge between receptor and ligand was also found in crystal structures of β_1 AR, β_2 AR, and the H1 histamine receptor.^{31,32} The model shows that Tyr132^{3,37} of mTAAR7f extends into the ligand binding pocket where it sterically blocks both ligands **6** and **7** (Figure 4 and Figures 3 and 4 of the Supporting Information). In contrast, Ser132^{3,37} of mTAAR7e does not sterically interfere with ligand **6** and may even stabilize ligand **7** through formation of a hydrogen bond between the hydroxyl group of its side chain and the indole nitrogen of the aromatic ligand moiety (Figure 4 and Figures 3 and 4 of the Supporting Information). We did not detect any additional amino acid variations in or near the odor binding pockets of mTAAR7e and mTAAR7f, other than positions 132^{3,37} and 133^{3,38}. On the basis of these structural models, and the dramatic functional change caused by mutation of these residues (Figure 3), we conclude that these two residues are critical determinants of ligand selectivity differences between these two receptors.

Here, we show how neofunctionalization of the TAAR7 family occurred during evolution by gene duplication and subsequent mutation. The olfactory system uses such evolutionary mechanisms to generate large repertoires of sensory receptors with divergent recognition properties, and these mechanisms are enabled by the inherent flexibility of olfactory system development. Minimal requirements for incorporation of a new GPCR into olfactory circuits include (1) obtaining

proper gene regulation and (2) coupling to the correct G protein. For this reason, sensory neurons expressing foreign GPCRs, such as the β_2 AR,³³ can be readily incorporated into the system and can couple to unique neural circuits in the brain. Also for this reason, gene duplication events followed by subsequent mutation of one duplicate is a powerful mechanism for achieving receptor diversity.³⁴ Here, we observe recent expansion of the TAAR7 family in rodents, and subsequent incorporation of specific mutations that alter odor responses. Through this process, evolutionary mechanisms have sculpted the TAAR7 subfamily, leading to rapid and functional expansion of the olfactory receptor repertoire.

METHODS

Detailed methods for chemicals tested, TAAR functional assays, phylogenetic analysis, and homology modeling are provided in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

Supplementary Figures 1–4, methods, and references. This material is available free of charge via the Internet at <http://pubs.acs.org>.

AUTHOR INFORMATION

Corresponding Author

*E-mail: stephen_liberles@hms.harvard.edu. Phone: (617) 432-7283. Fax: (617) 432-7285.

Notes

R.C.S. is a founder and BOD member of Receptos, a GPCR drug discovery company.

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