

Crucial role of copper in detection of metal-coordinating odorants

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Odorant receptors (ORs) in olfactory sensory neurons (OSNs) mediate detection of volatile odorants. Divalent sulfur compounds, such as thiols and thioethers, are extremely potent odorants. We identify a mouse OR, MOR244-3, robustly responding to (methylthio)methanethiol (MeSCH₂SH; MTMT) in heterologous cells. Found specifically in male mouse urine, strong-smelling MTMT [human threshold 100 parts per billion (ppb)] is a semiochemical that attracts female mice. Nonadjacent thiol and thioether groups in MTMT suggest involvement of a chelated metal complex in MOR244-3 activation. Metal ion involvement in thiol-OR interactions was previously proposed, but whether these ions change thiol-mediated OR activation remained unknown. We show that copper ion among all metal ions tested is required for robust activation of MOR244-3 toward ppb levels of MTMT, structurally related sulfur compounds, and other metal-coordinating odorants (e.g., strong-smelling *trans*-cyclooctene) among >125 compounds tested. Copper chelator (tetraethylenepentamine, TEPA) addition abolishes the response of MOR244-3 to MTMT. Histidine 105, located in the third transmembrane domain near the extracellular side, is proposed to serve as a copper-coordinating residue mediating interaction with the MTMT-copper complex. Electrophysiological recordings of the OSNs in the septal organ, abundantly expressing MOR244-3, revealed neurons responding to MTMT. Addition of copper ion and chelator TEPA respectively enhanced and reduced the response of some MTMT-responding neurons, demonstrating the physiological relevance of copper ion in olfaction. In a behavioral context, an olfactory discrimination assay showed that mice injected with TEPA failed to discriminate MTMT. This report establishes the role of metal ions in mammalian odor detection by ORs.

Humans are extremely sensitive to the smell of thiols, for example, the natural gas odorant 2-methyl-2-propanethiol (Me₃CSH), detectable at 0.3 parts per billion (ppb) (1). Spider monkeys are yet more sensitive, detecting 0.001 ppb ethanethiol (2). Malodorous volatile thiols and amines are protein degradation products found in putrid food, so sensitive identification of these compounds is crucial to avoiding intoxication (2, 3). The molecular mechanisms underlying olfactory detection of thiols are yet to be elucidated. Strong-smelling volatiles are usually good metal-coordinating ligands: thiols are also named “mercaptans,” from *mercurium captans* (Latin: capturing mercury). Odorant receptors (ORs) mediating odor perception have been suggested to function as metalloproteins, whereby transition metal ions (e.g., Zn²⁺, Ni²⁺, Cu²⁺, and Cu⁺) may be required for activation of ORs by thiol and amine ligands (4–7). In 1977 Crabtree (4) proposed that “these substances bind chemically to a nasal receptor . . . containing a transition metal at the active site,” that Cu(I) is “the most likely candidate for a metallo-receptor site in olfaction,” and that “the Cu(I) centre would be stabilized by coordination, perhaps to a protein thiolato-group, and . . . two or three additional protein S or N neutral donor groups.” Crabtree supported his hypothesis observing that, compared with unstained olefins, strained olefins like strong-smelling *trans*-cyclooctene, “give much

more stable complexes, e.g., [Cu₂Cl₂-(*trans*-cyclooctene)₃]³⁺ (4). In 1969 Henkin and Bradley (8) suggested that the physiology of taste involved copper. In 1996 Turin (7) proposed a key role for zinc in olfaction. In 2003 Wang et al. (6) reported that the short synthetic pentapeptide HACKE corresponding to a conserved sequence in the extracellular loop of ORs could effectively bind to metal ions and may form the basis for sensitive activation of ORs by thiols. However, it has not been shown that intact OR proteins rely on interaction with metal ions for function.

(Methylthio)methanethiol (MeSCH₂SH; MTMT), a strong-smelling volatile semiochemical found specifically in male mouse urine, activates certain olfactory sensory neurons (OSNs), mediating attraction in female mice (9). Here we show that copper ion is an essential cofactor for detection of MTMT and other thiols, and *trans*-cyclooctene. Identification of an OR activated by thiols and elucidation of metal ion involvement represent an important step forward in deciphering the molecular mechanism of olfaction, especially that of sensitive detection of thiols and other metal-coordinating odorants.

Results

MOR244-3 Is Selectively Activated by MTMT. We first screened a previously defined library of 219 mouse ORs for MTMT using an HEK293T-based OR heterologous expression system (10–12). Candidate ORs were further tested, and MOR244-3 (alternatively, MOR83 or Olfr1509) robustly responded to MTMT. To determine the receptor specificity of MOR244-3, we tested for its response against a panel containing MTMT, 19 analogs, and 24 other sulfur-containing compounds (Fig. 1A and Table S1), and a panel of 89 diverse odors (Fig. 1B and Table S2). MOR244-3 also selectively responded to several analogs of MTMT, including certain thiols, disulfides, and thiocarbonyl compounds. Simple thiols (e.g., methanethiol and octanethiol) did not elicit a response.

Copper Ion Enhances the Activation of MOR244-3 in Response to MTMT. We examined the effects of metal ions on MOR244-3 activation. First, we supplemented the stimulation medium used for odorant dilution with various metal ions. The addition of Cu²⁺

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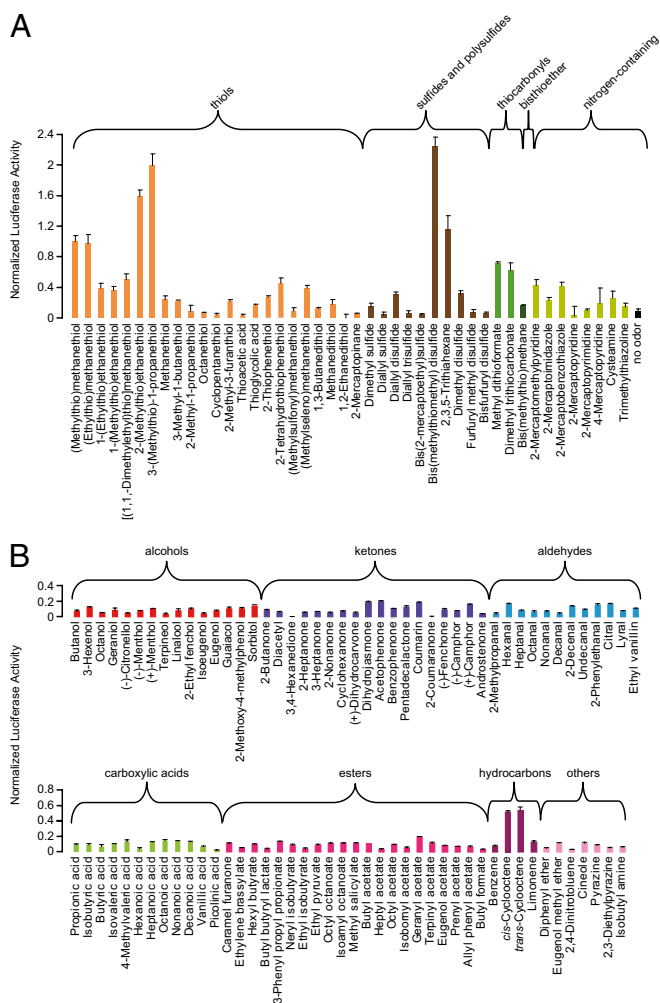


Fig. 1. MOR244-3 is selectively activated by MTMT and other sulfur-containing odors. Responses of MOR244-3 to (A) MTMT and a sulfurous odor panel containing 19 MTMT analogs and 24 other sulfur-containing compounds and (B) a diverse odor panel containing 89 compounds. The concentration of all odors was 30 μM , except when cell toxicity was observed, the maximally tolerated concentrations were used, including 0.3 μM for 2-(methylthio)ethanethiol and 10 μM for 2-mercaptobenzothiazole and 2-mercaptopyridine. All responses are normalized to the response to MTMT. For all figures, normalized luciferase responses are shown as mean \pm SEM ($n = 3$). Odorants are color-coded by structural features and functional groups. For odorants with multiple functional groups, the functional group with the highest oxidation state is used for categorization.

induced a stronger response of MOR244-3 to 30 μM MTMT (Fig. 2A). This effect intensified with increasing Cu^{2+} concentrations up to 30 μM . The total copper concentration in mouse nasal mucus measured by inductively coupled plasma-mass spectroscopy is also in the micromolar range [$41.7 \pm 2.8 \mu\text{M}$ (mean \pm SD, $n = 2$)], similar to that of the cell culture system. Although we introduce extracellular copper as Cu^{2+} , owing to the reducing environment of most cells, *intracellular* copper may exist mainly as Cu^{1+} , presumably the form in which it reduces S–S bonds and forms odorant complexes (13). Other metal ions tested (e.g., Ni^{2+} , Co^{2+} , Fe^{3+} , Mg^{2+} , and Zn^{2+}) did not affect receptor activation (Fig. 2A). When the Cu^{2+} concentration was fixed at 30 μM , the response of MOR244-3 against increasing concentrations of MTMT had a >20-fold decrease in EC_{50} values and higher saturation responses compared with the negative control with basal level of copper [$\approx 0.6 \mu\text{M}$ (Table S3)], indicating higher potency and efficacy of MOR244-3 in the presence of Cu^{2+} (Fig. 2B). In contrast, adding

30 μM Ni^{2+} , Co^{2+} , Fe^{3+} , Mg^{2+} , Zn^{2+} , Au^{3+} , 10 μM Pt^{2+} , or 5 μM Ag^{+} did not enhance receptor activation compared with the control. Although Au^{3+} , Pt^{2+} , and Ag^{+} do not occur naturally, they are found in pharmaceuticals such as the chemotherapeutic agent cisplatin (Pt^{2+}).

We used the GloSensor assay to measure real-time changes in cAMP levels caused by receptor activation upon ligand addition. MOR244-3 was robustly activated only in the presence of *both* Cu^{2+} and MTMT. MTMT alone elicited a much diminished response, and adding Cu^{2+} alone did not elicit a response (Fig. 2C). This effectively excluded the possibility of MOR244-3 acting as a copper receptor. Finally, we directly measured the change in levels of cAMP after the activation of the receptor by MTMT. We observed an increase in cAMP concentration only in the presence of 30 μM Cu^{2+} (Fig. S1A).

To further validate the effect of copper in MOR244-3's response to MTMT, we next determined whether copper deficiency would reduce receptor activation. We supplemented the stimulation medium with the membrane-permeable, high-affinity copper chelator tetraethylenepentamine [($\text{H}_2\text{NCH}_2\text{CH}_2\text{NHCH}_2\text{CH}_2$) $_2\text{NH}$; TEPA]. When the concentration of Cu^{2+} was fixed at 10 μM , increasing TEPA decreased the response of MOR244-3 to 10 μM MTMT (Fig. 2D). Notably, adding 10 μM TEPA to the medium without exogenous Cu^{2+} entirely abolished the receptor's response to 30 μM MTMT, indicating that basal levels of Cu^{2+} in the stimulation medium are required for MOR244-3 activation by MTMT. Furthermore, when the TEPA concentration was fixed at 10 μM , receptor activity was rescued by increasing Cu^{2+} concentration. This suggests that excess Cu^{2+} reversed TEPA activity, reinforcing the role of copper ion in the enhancement of receptor activity (Fig. 2D). The effect of TEPA was also confirmed in the more direct measurement of cAMP (Fig. S1B).

Specificity and Selectivity of the Copper Ion Enhancement Effect. We investigated the structural requirement for the copper effect by profiling MOR244-3's response against a panel of 19 MTMT analogs (Fig. 3 and Tables S1 and S4). A previous study showed that conformers and isomers selectively activate an OR (14). Because of the simplicity of MTMT (1; Fig. 3A), a limited number of structurally related compounds are possible; for example, isomeric with MTMT or differing (*i*) by addition of one or two carbon atoms and associated hydrogens, or of two oxygens (giving a sulfone), (*ii*) by deletion of two hydrogen atoms, or (*iii*) by replacing the thioether sulfur with selenium. Isomerization or atom addition, deletion, or substitution could alter the number of thiol and thioether groups, change the steric crowding at the sulfur atoms and the bulk of resulting copper complexes, modify the ligand "bite angle," alter the S–H acidity, change the availability of thioether electron pairs, and affect the compound lipophilicity, in turn, potentially modifying the corresponding copper complex and its protein docking ability.

Replacement of the MTMT methyl group by ethyl in 2 has no effect on activity (modest steric effect), whereas addition of a methyl group on the carbon between the sulfur atoms (17), addition of both the ethyl in 2 and methyl in 17 (5), or replacing ethyl with *tert*-butyl (6) diminishes the activity (greater steric effects, more lipophilic). Interestingly, methanethiol (16) shows a significant copper effect (Fig. 3B), albeit at much higher concentrations than 1, whereas octanethiol remains unreactive with or without copper. Although copper(I) thiolates derived from unhindered thiols such as 16 are polymeric or form polynuclear clusters (15, 16) [e.g., $(\text{MeSCu})_n$], displacement of methylthio groups on copper by available OR cysteine thiol groups (the small size of MeS should favor such known rapid ligand exchange) (17) would allow the resultant copper methanethiolate to be accommodated in the OR, although not as comfortably as the MTMT complex. The confined space within the OR active site, where Cu^{1+} is ligated by OR amino acids, disfavors conversion of OR-bound $(\text{MeS})_2\text{L}_2\text{Cu}$ to $(\text{MeSCu})_n$. Both of the above processes would fail with the larger octanethiolate complex: octanethiolate would be less easily

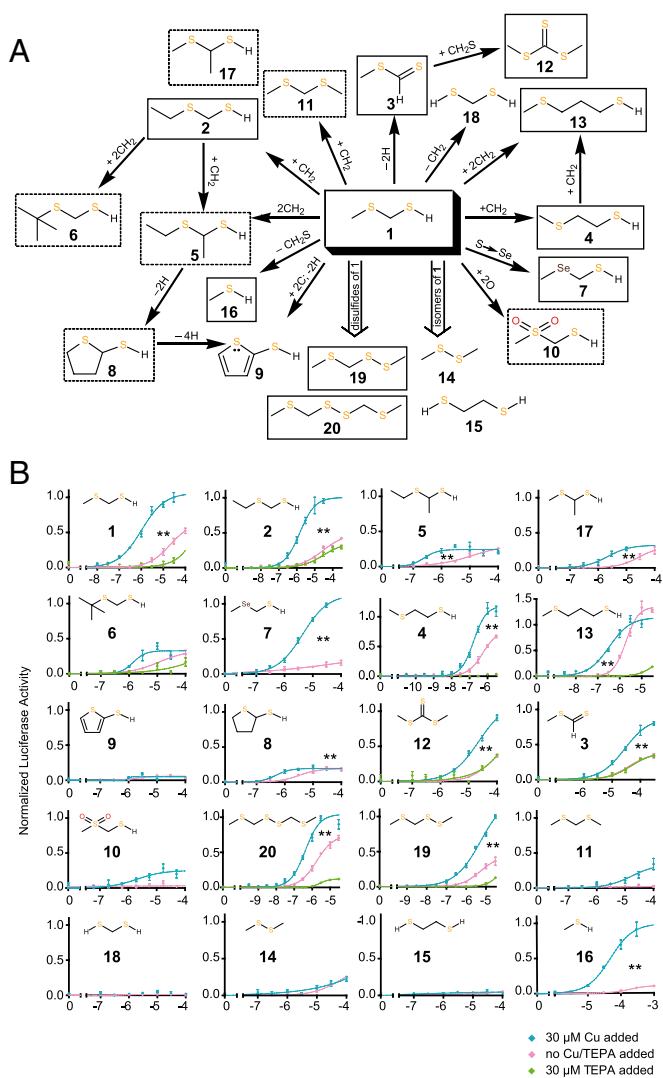


Fig. 3. Structural requirements of the copper ion enhancement effect of MOR244-3. (A) Structural relationship between MTMT and its analogs. Odors boxed with solid lines are those with prominent responses in the presence of 30 μM Cu^{2+} , and odors boxed with dashed lines are those with less prominent responses, as defined by a more than 50% reduction in efficacy compared with MTMT. Unboxed odors did not elicit MOR244-3 response. (B) Dose-response curves of MOR244-3 to representative sulfur-containing compounds with and without 30 μM exogenous Cu^{2+} addition. For odors with a significant response in the absence of exogenous Cu^{2+} , as defined arbitrarily by a top value greater than 0.32, dose-response curves with 30 μM of TEPA are also shown. The chemical structures of the respective compounds are shown in the upper left-hand corners. *F* tests were used to compare the pairs of dose-response curves with or without Cu^{2+} . Asterisks represent significance of *P* values after Bonferroni corrections. $**P < 0.01$ ($n = 3$).

did not significantly affect the Cu^{2+} -induced enhancement when stimulated by three concentrations of MTMT, excluding these sites as copper- and/or ligand-binding, whereas some of the sites reduced the response of MOR244-3 both with and without Cu^{2+} . The rest of the sites, including C97, H105, H155, C169, C179, and H243, abolished responses to MTMT completely when mutated, regardless of Cu^{2+} (Fig. S3B). Of these, we found that mutants C97S, H155R, C169S, C179S, and H243R, but not H105K, have little or no cell-surface expression (Fig. S3C), suggesting that these mutants lost their functions as a result of defects in receptor folding/trafficking. The H105K loss-of-function mutation may either disrupt copper/ligand binding or lead to general receptor defects, by affecting downstream signaling.

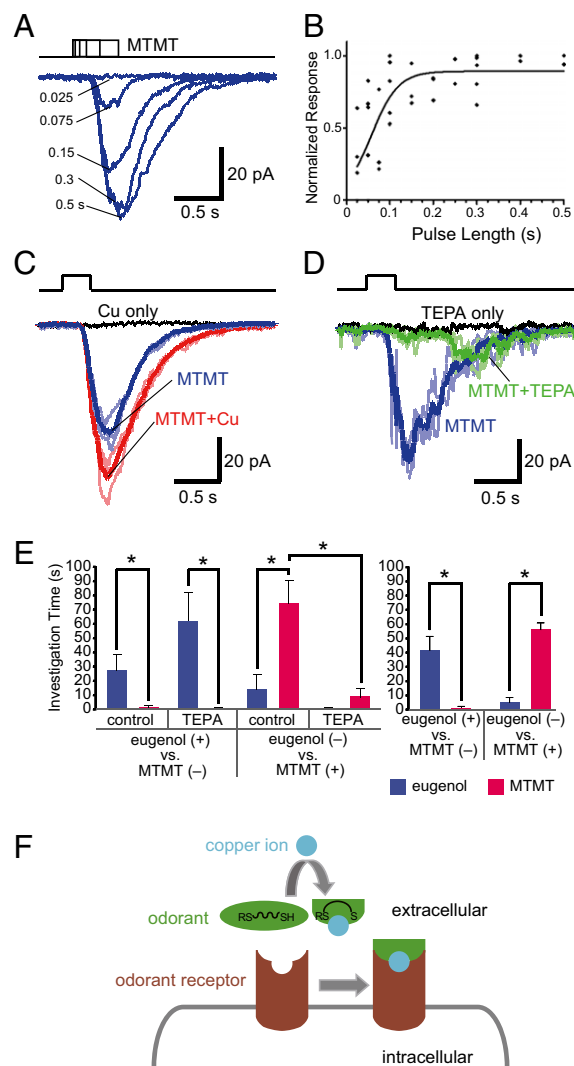


Fig. 4. Physiological relevance of copper in the detection of MTMT. (A–D) MTMT responsive OSNs are present in the SO. (A) In a single non-SR1 cell, inward currents were elicited by MTMT pulses with varying length from 0.025 to 0.5 s. (B) The dose–response curve was fitted by grouping the data from six cells tested by MTMT pulses with different lengths. For each cell, the peak current induced by a pulse was normalized to the maximum response in that cell. (C) In some cells, the MTMT (10 μM) response was facilitated by adding 30 μM Cu^{2+} . (D) In some cells, the MTMT response (10 μM) was reduced by adding 30 μM TEPA. In C and D, the thick trace was averaged from three trials under that condition (thin lines). All recordings were performed under voltage clamp mode with a holding potential of -60 mV. (E) Mice were trained to associate either eugenol or MTMT with sugar reward. On the test day, mice were injected with dH_2O or TEPA and then tested for the ability to discriminate the two odors. *Left*: TEPA injection specifically abolishes olfactory detection of MTMT. *Right*: Recovery of olfactory discrimination ability for eugenol and MTMT 2 d after TEPA injection. Two days after the initial testing, mice were retested for the recovery of the ability to discriminate the two odors. The y axis represents time spent investigating each odorant during the 9-min test period, shown as mean \pm SEM. Paired *t* test was used to compare the investigation times between groups. $*P < 0.05$ ($n = 4$). (F) A model for OR activation involving copper ion as a cofactor with the copper ion binding to the ligand for subsequent binding of the copper ion–odor complex to the OR.

Notably, this mutant retains the ability to respond to some of MOR244-3's nonsulfurous ligands (e.g., cineole) and to ligands with no copper effect (e.g., dimethyl sulfide), indicating that the mutant receptor is intact and that this residue might be at the interface of MTMT and/or copper binding (Fig. S44).

MOR244-3 was modeled on the basis of the closest-matching structure turkey $\beta 1$ adrenergic receptor [Protein Data Bank entry 2VT4, resolution 2.7 Å] (26). We searched for candidate ligand pockets as implemented in POCASA (<http://altair.sci.hokudai.ac.jp/g6/service/pocasa/>), which predicted a binding pocket formed by the loops and transmembrane domains facing the extracellular side of the receptor (Fig. S4B), consistent with the location of a possible ligand-binding pocket in ORs (27–30). Notably, H105 is found between the second to fifth transmembrane domains close to the extracellular side, representing a plausible copper coordinating residue. Thus, MTMT and copper ion may share a binding site in this region. Alternatively, the pocket could harbor an allosteric copper-binding site, important for ligand binding.

MTMT-Responsive OSNs Are Abundantly Present in the Septal Organ (SO). To confirm that in situ OSNs can respond to MTMT, we performed perforated patch clamp recordings in the SO, which expresses SR1 and MOR244-3 in high abundance (31, 32) (Fig. S5A). Because copper is present in the olfactory mucosa, we expected to observe MTMT responses among the non-SR1 cells. In fact, of 132 cells recorded, 76 cells or 58% responded to MTMT in a dose-dependent manner (Fig. 4A and B). This percentage is higher than that of MOR244-3 cells (27%) in the non-SR1 cells of the SO, suggesting that additional ORs in the SO are responsive to MTMT. We used in vitro screening to test this possibility and found that another OR, MOR256-17, showed robust responses to MTMT (Fig. S5B). Interestingly, the MTMT responses of MOR256-17 were not modulated by copper addition. These results indicate that MTMT-responsive cells in the SO are heterogeneous in their OR choice and in their modulation by copper. Consequently, we expect to observe different modulations of MTMT responses by copper. From 32 cells tested by adding copper together with MTMT, the MTMT responses were enhanced in 12 cells (38%) (Fig. 4C), unchanged in 15 cells (47%), and, curiously, decreased in five cells (16%). Similarly, MTMT responses of the SO cells showed different modulation by TEPA; TEPA reduced the MTMT responses in 36% of the cells ($n = 50$) (Fig. 4D) but had no effect in other cells (64%). The patch clamp data indicate that many cells in the SO are capable of responding to MTMT in the absence of exogenous copper, suggesting that mice can readily detect these compounds in the urine. Depending on the OR identity and the endogenous copper concentration in the olfactory epithelium, these cells show heterogeneous modulation by copper and its chelator.

In Vivo Injection of TEPA Abolishes Olfactory Recognition of MTMT. To assess the role of copper in the perception of MTMT in a behavioral context, we performed an olfactory discrimination assay using food-restricted mice that were trained to associate either eugenol or MTMT with sugar reward. On the test day, we first performed bilateral injection of the copper chelator TEPA into the nasal cavities of the mice. The mice were then exposed to both odorants in a testing cage, and the time spent investigating the odorants was scored. Mice trained to associate MTMT with sugar reward spent significantly less time investigating the odor after TEPA injection, suggesting that they had lost the ability to recognize the odor (Fig. 4E, Left). The group of mice trained for the nonsulfurous odorant eugenol could always discriminate the odor with or without TEPA injection, indicating that the negative effect of TEPA on olfactory discrimination is specific to MTMT (Fig. 4E, Left). Consistent with metabolic clearance of TEPA, the group trained to recognize MTMT regained olfactory discrimination ability 2 d after TEPA injection (Fig. 4E, Right). The results from the behavioral experiment indicate that copper is required for the olfactory detection of MTMT.

Discussion

Humans have an exquisitely sensitive sense of smell toward low valent sulfur compounds. The possible involvement of metal ions

in olfaction has long been postulated (4, 6). However, the definitive role of metal ions in odor-evoked activation of the ORs is unknown. In this study, to evaluate the hypothesis, we used MTMT, a thiol and also a semiochemical in male mice urine that attracts female mice and activates mitral cells in the main olfactory bulb (9). By screening a panel of mouse ORs, we identified a highly potent OR for MTMT; importantly, we found that the function of the MTMT OR was greatly enhanced in the presence of Cu^{2+} , allowing ppb detection of MTMT. We thus hypothesized that a copper–ligand complex would present more stable interaction with the ORs than the ligands without copper.

Past studies have shown the involvement of metal ions at the interface of protein–ligand interaction, such as the *Arabidopsis* ethylene receptor ETR1 that has a copper ion bound to its transmembrane domain (33). Certain metal ions, such as Zn^{2+} , can also modulate G protein-coupled receptor (GPCR) activation, including chemokine receptor CXCR4, the μ -opioid receptors, and the melanocortin receptors (34–37). How does copper ion enhance MOR244-3 receptor activation? Our data are congruent with the model that the overall thiolate–copper complex is more conformationally constrained, or smaller and more spherical in overall extension, than the free “odor” structure. A wide range of structure types are known for complexes of Cu^{1+} and Cu^{2+} with thiol, thiocarbonyl, thioether, selenoether, and disulfide groups, alone or in various combinations. In the case of MOR244-3, Cu^{1+} could simultaneously bind to ligands 1–20 and OR protein’s nitrogen, sulfur, or oxygen functionalities affording a variety of structures. As implied by Fig. 4F, the ligand–copper complex would have to be of an appropriate size to fit into a receptor pocket. Furthermore, the ligand should not bind to copper so tightly so as to preclude complexation by the receptor protein, as is the case with TEPA, and presumably 1,2- and 1,3-dithiols.

By way of background on copper complexes of thiolates, the following is known. (i) Thiolates can act as monodentate or as bridging ligands between two copper centers; very bulky thiolates favor mononuclear copper thiolates. (ii) Thiolates can act as reducing agents, forming disulfides, hampering synthesis of thiolate complexes of Cu^{2+} ; however, copper complexes of disulfides are also known, as are those of thioethers or selenoethers, all of which are relatively poor ligands. (iii) Cu^{1+} can reduce disulfides to thiolates when the disulfide contains additional chelating groups (38). (iv) Cu^{2+} uses coordination numbers 4 (square planar), 5 (trigonal bipyramid or square pyramid), or 6 (octahedron), whereas Cu^{1+} prefers coordination numbers 2, 3, 4 (tetrahedral), and occasionally 5 (square pyramid). Representative examples of various types of Cu^{1+} and Cu^{2+} complexes involving divalent sulfur ligands are described in Table S5. Future crystallization studies may elucidate the structure of the copper–MTMT complex. Our proposed model of copper simultaneously binding to OR protein residues and thiol/thiolate as well as thioether or selenoether groups in odorants $\text{MeX}(\text{CH}_2)_n\text{SH}$ may be analogous to the binding that occurs in blue copper proteins, which involves simultaneous binding to cysteine and methionine (or selenomethionine) (39–41). Sophisticated molecular dynamics simulation studies may be required to resolve the precise mode of interaction among the receptor–ligand–metal trio associated with odorant docking.

Materials and Methods

Chemicals. All odorants, except for those described in *SI Materials and Methods*, as well as CuCl_2 , NiCl_2 , and ZnSO_4 , were purchased from Sigma-Aldrich, Contech Inc. (TMT), Sinopharm (CoCl_2 , AgNO_3), or Aladdin Reagent Database Inc. (FeCl_3 , AuCl_3 , and PtCl_2). The chemicals were dissolved in DMSO or ethanol and diluted further into working concentrations before experiments.

Dual-Glo Luciferase, GloSensor, and cAMP-Glo Assays. HEK293T and its derived cell line Hana3A and that containing ATP7A/B RNAi constructs were grown in minimum essential medium (HyClone) containing 10% FBS at 37 °C with 5% CO_2 . Lipofectamine 2000 (Invitrogen) was used for transfection. Luciferase assays were performed as previously described (11). An *F* test was used to compare the best-fit values of EC_{50} , top, and Hill slope between the pairs of dose–response curves with or without copper ion. Bonferroni corrections

were applied to account for multiple comparisons. For GloSensor and cAMP-Glo assays, Hana3A cells were plated onto 96-well plates (Biocoat; Becton Dickinson Biosciences). After 18–24 h, OR and mRTP1S (and a GloSensor plasmid in the case of GloSensor assay) were transfected into cells. Twenty-four hours after transfection, the cells were stimulated with odorants plus various concentrations of metal ions dissolved in CD293 (Invitrogen). We used the GloSensor (Promega) and cAMP-Glo (Promega) kits and followed the manufacturer's protocol for measuring chemiluminescence. Additional materials and experimental procedures are given in *SI Materials and Methods*.

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