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The role of metals in mammalian olfaction of low molecular weight organosulfur compounds

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Abstract

While suggestions concerning the possible role of metals in olfaction and taste date back 50 years, only recently has it been possible to confirm these proposals with experiments involving individual olfactory receptors (ORs). A detailed discussion of recent experimental results demonstrating the key role of metals in enhancing the response of human and other vertebrate ORs to specific odorants is presented against the backdrop of our knowledge of how the sense of smell functions both at the molecular and whole animal levels. This review emphasizes the role of metals in the detection of low molecular weight thiols, sulfides, and other organosulfur compounds, including those found in strong-smelling animal excretions and plant volatiles, and those used in gas odorization. Alternative theories of olfaction are described, with evidence favoring the modified "shape" theory. The use of quantum mechanical/molecular modeling (QM/MM), site-directed mutagenesis and saturation-transfer-difference (STD) NMR is discussed, providing support for biological studies of mouse and human receptors, MOR244-3 and OR OR2T11, respectively. Copper is bound at the active site of MOR244-3 by cysteine and histidine, while cysteine, histidine and methionine are involved with OR2T11. The binding pockets of these two receptors are found in different locations in the three-dimensional seven transmembrane models. Another recently deorphaned human olfactory receptor, OR2M3, highly selective for a thiol from onions, and a broadly-tuned thiol receptor, OR1A1, are also discussed. Other topics covered include the effects of nanoparticles and heavy metal toxicants on vertebrate and fish ORs, intranasal zinc products and the loss of smell (anosmia).

1 Introduction: odorants and the olfactory system

> I counted two and seventy stenches, All well-defined, and several stinks!
>
> Cologne by Samuel Taylor Coleridge

> The olfactory system did not evolve to decode the catalogue of Sigma-Aldrich; it evolved to decode the world around us.

Lawrence C. Katz, 1956-2005

The vast variety of odorants[1] -- small, volatile molecules of variable sizes, charges, and functional groups -- that humans can smell[2] range from the sublime to the repugnant: from the enticing aromas of food and beverages, floral fragrances and the odor of a sexual partner, to the malodors of halitosis, spoiled food, leaking natural gas, smoke and airborne pollutants. For animals, by signalling the existence of predators and prey, conspecifics and food, odorants provide critical information for their survival. Some animals, such as the skunk[3] and marine sponge *Ircinia felix*,[4a] survive through use of malodorous repellents such as 3-methyl-1-butanethiol and methyl isonitrile, respectively. Certain flowering plants, such as the titan arum (corpse flower; *Amorphophallus titanium*)[4b] and dead-horse arum (*Helicodiceros muscivorus*),[4c] emit foul-smelling dimethyl trisulfide to attract carrion-feeding pollinators. In addition to the immense number of individual chemical compounds as well as complex mixtures of compounds that the olfactory systems of all creatures can detect, special note should be made of the enormous range of concentrations detectable. However, while the nose can distinguish many different types of odorants, it is not equally sensitive to them. Indeed, as shown for the family of odorants with a single methyl group (Fig. 1), olfactory response to methanethiol is over 1 million times stronger than to methanol, and 100 million times stronger than to ethane.[5a] Emil Fischer, in 1887, wrote that concentrations of ethanethiol as low as 0.05 parts per billion (ppb) are “clearly perceptible to the sense of smell”;[5b] chiral 3-methyl-3-sulfanyl-hexan-1-ol, identified in armpit odor, can be perceived at levels as low as 0.000001 ppb.[5c] For 2-propenethiol (in garlic breath), 6×10^7 molecules/mL air can be detected, compared to ethanol which requires 2×10^{15} molecules/mL of air![5d] Such differences in odor between simple alcohols and thiols are all the more surprising given the similarities in structure (Fig. 2). Just how olfaction -- the sense of smell -- works at the molecular level in humans and other animals is the subject of this Review, with a special emphasis on the role of metals and strong-smelling, volatile, natural and unnat-ural low molecular weight organosulfur compounds in this process.

The sense of smell may be defined as “the central nervous system's (CNS) perception of a stimulus that activates the olfactory receptors (ORs).”[6a] Our modern understanding of how the sense of smell works owes much to the pioneering studies by Nobel Laureates Richard Axel (2004), Linda Buck (2004), Brian Kobilka (2012) and Robert Lefkowitz (2012) for their research on olfaction (Axel[6b] and Buck[6c]) and G-protein-coupled receptors (GPCRs; Kobilka[6d] and Lefkowitz[6e]), respectively. Olfaction in animals is mediated by specialized sensory cells in the main olfactory epithelium (OE) in the nasal cavity. In these cells, ORs are embedded in the 20–30 hair-like cilia attached to each of 6–10 million olfactory sensory neurons (OSNs; Fig. 3). These very fine cilia are bathed in a thin layer of nasal mucus in which the odorants dissolve. The OSNs also project neurons -- the *first cranial nerve* (CN I) -- that synapse with second order neurons in the olfactory bulb (OB). The primary role of the OSNs is to detect environ-mental information (e.g., odorants) and to convey this information to the OB.[7a]

OSNs are regenerated and replaced approximately monthly. ORs are members of the superfamily of GPCRs, which are heptahelical transmembrane proteins, so-called because their a-helical coils pass seven times through the plasma membrane. A typical GPCR,

containing more than 300 amino acids, consists of an extracellular portion (the N-terminus), an intracellular portion (the C-terminus), and a middle segment containing seven trans-membrane domains, with the odorant-binding site on the outer, periplasmic domain and a G protein-binding site on the inner, cytoplasmic domain. These proteins sense exogenous odorants (chemical ligands or agonists possessing an affinity for the receptor) within an ice cream cone-like cavity formed by the seven transmembrane domains, triggering a conformational change in the seven-transmembrane region. This, in turn, activates signal trans-duction pathways which transform the chemical energy of binding into a neural signal, ultimately delivering a message to the OB in the forebrain.[7b] Specifically, the conformational change triggers an increase in the intraciliary cAMP concentration by activation of the Golf olfactory G protein and the type III adenylyl cyclase. The increased cAMP level opens a cyclic nucleotide-gated ion channel, allowing an influx of Na^{+} and Ca^{+2} ions, which ultimately contributes to OSN depolarization, resulting in action potentials conducted along the axons toward the olfactory bulb (Fig. 4).[7c]

ORs constitute nearly 50% of the approximately 826 GPCRs in humans that are known to be important in neurotransmission, photoreception (rhodopsin is a GPCR) and other cellular processes, as well as receptors for numerous pharmaceuticals.[8] Interestingly, ORs have also been found in a large variety of nonolfactory tissues as well, including human colon tissue, sperm, blood cells, skin tissue, brain, smooth muscles, and melanocytes.[7a] Furthermore, their expression is up-regulated in different types of cancer cells.[9,10] It has been suggested that understanding the function of the non-olfactory ORs may pave the way for use of the OR family as novel targets for therapeutic agents.[7a] The fact that there are 1,000 genes for ORs in the mammalian genome make it the largest gene family in the entire genome. At the same time, 60% of the human (or primate) OR genes appear to be pseudogenes, non-functional DNA sequences that arise through nonsense or frame-shift mutations of protein-coding genes, reflecting low selection pressures on loci no longer relevant to the fitness of a species. The approximately 400 unique, active human ORs (ca. 1100 in mice) are extremely diverse in their amino acid sequences, consistent with their ability to recognize a very large variety of odorants. Despite the smaller number of functional ORs when compared to other species, the human sense of smell is very accurate and sophisticated.[11a,b] It does need to be kept in mind that OR genes are highly polymorphic in mammals, such that single base changes can lead to different odorant-OR interactions and altered odorant perception.[11b] There are, on average, five common variants for a given OR in the population.[11c]

As already indicated, when activated by odorants, the ORs undergo a conformational change followed by a cascade of events, termed *signal transduction*, transforming the chemical energy of odorant binding ultimately causing olfactory neuron depolarization and signaling to the brain, leading to odorant recognition, termed *perception*. Each OSN expresses only one of the OR genes. All OSNs expressing the same OR send their *axons* (the long, slender projections of nerve cells, or neurons, which conduct electrical impulses) to targets in the olfactory bulb called *glomeruli*. The axons from all cells expressing a particular OR converge onto two or a few glomeruli in the OB in the brain.[7b]

Many mammals also have a second, accessory olfactory system, called the vomeronasal organ or VNO, separate from the main olfactory epithelium (OE). The VNO, which can

recognize non-volatile chemical cues, is associated with the detection of pheromones, scents which act as chemical communication signals from other individuals of the same species, implicated in courtship, mating, suckling, aggressive behavior, etc. It should be noted, however, that recent work suggests that the main olfactory system also detects a range of volatile pheromones to facilitate mate recognition.[11d] Thus, (methylthio)methanethiol (MTMT), discussed below, which is suggested to be a pheromone, can also be detected by the receptors in the OE.[12] The VNO, thought to be nonfunctional in adult humans, will not be considered here.[13a] In addition to expressing ORs, OSNs also express two other types of olfactory receptors known as trace amine-associated receptors (TAARs), which respond to volatile amines at nanomolar ranges, and receptors that signal via guanylyl cyclase, which respond to both carbon dioxide and carbon disulfide, the latter at submicromolar levels.[7a] Non-GPCR chemosensors and oxygen chemosensors are also known.[13b,c] In insect olfaction, not considered here, ORs are mainly located on the antennae and have significantly different structures than their vertebrate counterparts.

As already noted, humans and other vertebrates have many millions of OSNs. Thus, numerous OSN replicates express each unique OR. In vertebrates, an odorant will dissolve in the nasal mucus of the olfactory epithelium (where chemical transformations may occur, as discussed below) and then bind to an OR. Since the vast number of perceived odors greatly exceeds the number of ORs, it is proposed that mammalian olfactory systems use a combinatorial receptor coding scheme in the identification and discrimination of odors.[1,14a,b] That is, rather than binding specific odorant-ligands, ORs display affinity for a range of odorants, and, conversely, a single odorant molecule may bind to several ORs with varying affinities. This difference in affinities causes differences in activation patterns, combinations, and permutations, all of which result in unique profiles for a very large number of odorant molecules. While some ORs may display affinity for a range of odorants, acting as broadly-tuned “generalist receptors,” others may act as narrowly-tuned “specialist receptors,” responding to a limited number of odorants.[1] It has been noted that some odors change their perceived quality depending on the intensity of the stimulus. Thus, 1-*p*-menthene-8-thiol (grapefruit mercaptan), which is repugnant at high concentrations, has a sweet citrus aroma at lower concentrations. Other examples of thiols which are pleasant smelling at low concentrations include furfuryl mecaptan (from coffee) and *p*-menthane-8-thiol-3-one (from blackcurrant and hops).[2] It has been proposed that as the concentration of an odor is increased, additional glomeruli are recruited, suggesting that new receptors are being activated as concentrations increase.[15a]

Until recently most ORs remained “orphan receptors” with unknown chemical ligands, reflecting difficulties with functional assays. For example, to date only about 13% (ca. 53) of the 400 unique human ORs have been “deorphanized” by being matched with their odor ligands.[11b] Crystal structures of ORs are not yet available due to difficulties expressing them in sufficient quantities and in crystallizing them.[7b,15b] Presently, structural information on ORs is obtained by site-directed mutagenesis experiments combined with computational modeling techniques, using the human M2 muscarinic receptor as a homology model template. These studies led to identification of specific amino acid residues that are involved in ligand binding or in the conformational changes of the ORs which lead to signaling.[7c] Understanding structure/function relationships responsible for ligand-binding and activation

of GPCRs remains an outstanding challenge of broad research interest. ORs are members of the class A rhodopsin-like family of GPCRs. Homology structural models of ORs can therefore be based on crystallographic structures for rhodopsin-like receptors. The main challenge, however, is to provide experimental support for the proposed models for ligand-receptor interactions by site-directed mutagenesis and comparative analysis of ligand binding by biochemical measurements of the receptor activation. In families of ORs there is a range of similarity in amino acid sequence ranging from 40-90% identity. At the same time there is a region of hypervariability, where the amino acid sequences particularly diverge, in the third, fourth and fifth transmembrane regions.[7b] In a three-dimensional GPCR model these three barrel-like α-helical coils presumably face each other, forming a pocket about one-third of the way into the membrane, which is the probable binding site for ligands.[16] To screen ORs with a diverse library of ligands it is first necessary to express ORs in heterologous systems suitable for high-throughput screening or site-directed mutational analysis. Several cell lines derived from human embryonic kidney (HEK) cells have been developed for the overexpression of various mammalian GPCRs including ORs.[7] Once an OR is expressed on the plasma membrane, its response to a panel of odorants can be measured using the GloSensor cAMP assay (Promega), a sensitive real-time luminescent method for measuring GPCR activation by detecting the levels of the intracellular second messenger cAMP.[17]

The perception of odor,[18,19] and the memory and associations of that odor perception, are a fascinating aspect of the subject of olfaction, eloquently captured by Marcel Proust in Remembrance of Things Past: *"… But when from a long distant past nothing subsists, after the people are dead, after the things are broken and scattered, still alone, more fragile but with more vitality, more unsubstantial, more persistent, more faithful, the smell and taste of things remain, poised a long time, like souls ready to remind us, waiting and hoping for their moment, amid the ruins of all the rest; and bear unfaltering in the tiny and impalpable drop of their essence, the vast structure of recollection."*[20] The strong connection between olfaction and memory recall is likely related to the proximity of neural regions important for smell and cortical areas implicated in higher order functions of emotional memory. This relationship is relevant to the understanding of neurodegenerative disease, e.g., given that changes in the sense of smell can be an early indicator for Alzheimer's disease.[21a,b]

2 Volatile sulfur compounds (VSCs) and nociceptive (pain) neurons TRPA1 and TRPV1

A 2010 review indicates that 554 naturally occurring volatile sulfur compounds (VSCs) are found in terrestrial plants.[21c] Among all VSCs, dimethyl sulfide commands particular attention, accounting for 75% of the global sulfur cycle: about 40 million metric tons annually is released from the ocean, with an insignificant contribution from terrestrial plants.[21c] Phytoplankton are a major source of oceanic dimethyl sulfide. Harbour seals (Phoca vitulina), foraging phytoplankton, possess an extraordinarily high olfactory sensitivity to dimethyl sulfide (.0000045 ppb), several orders of magnitude higher than humans.[21d] Specific types of VSCs are often associated with certain terrestrial plant families, e.g. isothiocyanates in Brassicaceae, thiosulfinates in Alliaceae (Amaryllidaceae),

and thiophenes in Asteracea. Some VSCs, such as allyl isothiocyanate [CH_2=$CHCH_2N$=C=S] from wasabi and horseradish, allicin [CH_2=$CHCH_2S$(O)SCH_2CH=CH_2] from garlic, and propanethial *S*-oxide [CH_3CH_2CH=S=O], the lachrymatory factor from onion, cause a painful, burning sensation upon inhalation or contact with skin, cornea and mucosa. Nociceptors (noci-, from the Latin for "hurt") are neurons that respond to painful stimuli, as from the above irritating chemicals. While the first cranial nerve (CN I), or olfactory nerve, transmits nerve impulses about smell to the olfactory bulb (OB), the fifth cranial nerve (CN V), or trigeminal nerve, also responds to sensory information from nociceptors in the face and oral cavity, such as the pungency of food. It has extensive innervations throughout the mouth, nose and facial areas, including the nasal passageways, where it is also found mixed in with the olfactory receptors, and is sometimes confused with the sense of smell, even though CN V is separate from CN I. Trigeminal innervation on the cornea of the eye is responsible for tearing on exposure to the lachrymatory factor released when peeling an onion or cutting horseradish, and to irritation from ammonia and other irritants. Chemical nociceptors employ transient receptor potential (TRP) ion-channel proteins such as TRPA1 (transient receptor potential ankyrin 1) and TRPV1 (transient receptor potential vanilloid 1), which can be activated by a variety of compounds including VSCs such as diverse isothiocyanates[21e,f,g], diethyl trisulfide,[21h,i] diallyl trisulfide,[21g] allicin[21j] and ajoene.[21k] Activated TRPs mediate the flow of calcium ions into the endings of specialized neurons, analogous to activation of ORs, but resulting in local inflammation and pain. In contrast to the readily reversible binding of odorants to ORs, electrophilic TRP activators, e.g., from wasabi and alliums, are thought to covalently bind to the abundant TRP cysteine residues (Scheme 1). Channel protein such as TRPA1 are well conserved across the animal kingdom, with likely orthologs from human to nematode, suggesting an ancestral role, probably in sensation. Pungency of plant VSCs could be reasonably associated with a defense mechanism to protect them from herbivorous predators. Detailed discussion of nociceptor-activating VSCs, as well as the large number of VSCs not yet matched with their ORs, is beyond the scope of this Review.

3 The role of mucus in odorant recognition: Perireceptor effects

In some cases, OR sensitivity and ligand specificity obtained from OB activation patterns differ from that in isolated OSNs or cell-based heterologous expression systems.[1,22a] These differences could be attributed to events occurring prior to OR-ligand interaction, e.g., *perireceptor events*.[22b] Perireceptor events are defined as those biophysical and biochemical events involving odorant molecules taking place before the odorants reach the ORs in the extracellular space in the proximity of OSNs, for example in the 5–30 mm thick, continuously flowing nasal mucus in vertebrates in which the OSN cilia are immersed.[22c,d] These interactions involve both odorant-binding proteins (OBPs) as well as odorant-degrading enzymes (ODEs) present in nasal mucus[22].

The OBPs, which include high molecular weight mucins or large glycoproteins, are collectively known as lipocalins, abundant in nasal mucus (100 mM to 1 mM), and rapidly replenished by synthesis. Lipocalins are carrier proteins in aqueous media for hydrophobic ligands, including odorants, and may also have a role in clearance of odorants.[1,22c,d] The ODE, also abundant in nasal mucus, protect the OE, lungs and brain against potentially

dangerous inhaled xenobiotics. Enzymes involved in biotransformation of xenobiotics include cytochrome P450 monooxygen-ases, aldehyde dehydrogenases, aldehyde oxidases, aldehyde reductases, carbonic anhydrases, carboxyl esterases, and various transferases and transporters, with activities comparable if not higher than that measured in the liver.[1]

In studies involving mice, Nagashima and Touhara[22e] demonstrated, using both pharmacological and behavioral tests, that aldehyde and ester odorants are targets of metabolic enzymes secreted into the mouse nasal mucus, resulting in their oxidative and hydrolytic conversions to the corresponding acids and alcohols, respectively. As a consequence, the ultimate perception is of a mixture of the original odorant and its metabolic derivatives, rather than of a single species of odorant molecule. When an odorant molecule is modified by enzymatic action, even before the odorants reach the ORs, the quality of the olfactory message and perception can be altered. Furthermore, odorant metabolites may be more soluble than the parent odorants, and may reach high concentrations in the mucus bathing the ORNs.[22f] Nasal mucus constitutes a barrier odorants need to cross to reach the ORs. In addition to action by OBPs and ODEs, this aqueous protein solution can modify the characteristics of the olfactory message through selective concentration and partitioning of components of mixtures.[22c,d] In the context of this review, and as will be discussed below, it is significant that nasal mucus of mice has been found to contain 40 μM levels of copper ions. In humans, levels of Zn, Cu, Mg and Ca in nasal mucus are 14, 16, 1554 and 5303 μg/dL, respectively, and 92, 106, 2150 and 9820 μg/L in human serum, respectively.[23d] Clearly, it is important to consider the role of the proteinaceous content of the nasal mucus, and as we shall see, metal ions present, in determining the exact sequence and nature of transportation, concentration and modification of odorants, activation of ORs, as well as their clearance.[1]

4 Theories of olfaction

More than 2000 years ago, the Roman philosopher Lucretius speculated that different odors are attributed to different shapes and sizes of “atoms” (odorants in today's parlance) that stimulate the sense of smell. “Smells are differentially better suited to different animals because [the smells have] unlike shapes. That is the reason why bees are attracted over enormous distances by the scent of honey, or vultures, by that of corpses…different animals endowed with their different [senses of] smells are attracted each to its own food, and, compelled to reject what is a foul poison…Although this very smell which excites the nose has varying capacities for distance of movement through [space], never is any smell carried so far as sound, or voices, not to mention those things which strike the eyeballs and excite vision…There are also those particles [molecules] which cannot legitimately be considered either smooth or hooked [and equipped] with bent edges; but [they consist], instead of small angles that jut out just a bit in such a way that the particles [molecules] can rather excite the senses that harm them, as for example in the case of wine lees or the flavor of endive.”[24] Lucretius' theory of olfaction is surprisingly similar in concept to molecular recognition theories of olfaction discussed below, broadly, but imprecisely referred to as the “shape theory of olfaction.”

A very different proposal regarding the sense of smell was made in 1870 by the British physician William Ogle, who wrote, foreseeing the so-called vibration theory of olfaction: "As in the eye and the ear the sensory impression is known to result not from the contact of material particles given off by the object seen or heard, but from waves or undulations of the ether or the air, one cannot but suspect that the same may be true in the remaining sense, and that the undulatory theory of smell… [may be] the true one."[25] The "shape theory of olfaction" is today the most widely accepted theory. Both the shape and vibration theories will be discussed in more detail below.

4.1 Vibration theory of olfaction

The vibration theory of olfaction (VTO), initially championed by Dyson[26a,b] and Wright,[26c-e] holding that olfaction involves detection of vibrational frequencies of odorant molecules, remains speculative since it has not been experimentally validated with isolated ORs.

Luca Turin,[27a-c] beginning in 1996–1997, elaborated on the VTO, suggesting that a mechanism analogous to inelastic electron tunneling spectroscopy may be involved, where inelastic tunneling electrons within ORs probe the vibrational frequencies of odorant molecules. His observations that acetophenone and acetophenone-d_8[27a] as well as non-deuterated and deuterated musks (with 15–18 carbons and 28 or more hydrogens)[27d] smell different despite being identical in structure, and that dimethyl sulfide has an odor that is "repulsive, sharp, green, cabbage-like" while dimethyl sulfide-d_6 has an odor that is "cleaner, more truffle-like without the gassy cabbage-like note of the parent compound"[27c] support the VTO theory.[27a] He reasoned that olfaction like vision is a "spectral sense," that the difference in odor of alcohols and thiols reflects the differing vibrational frequencies of the O–H and S–H groups (3200-3550 versus 2550 cm^{-1}, respectively), which are otherwise similar in shape,[27a,b] that a musk receptor "detects vibrations in the 1380- to 1550-cm^{-1} range…[and that a musk] has intense bands in that region."[27d] He argued that the existence of a congenital specific anosmia to mercaptans [see Section 6.10] is consistent with the VTO given that "it is difficult to understand how a mutation would wipe out a large number of receptors while only affecting a single odour class."[27a] Turin proposed a test for the VTO involving a molecule containing a sterically hindered S–H group (a "buried functional group"), for example a trimethylsilyl-substituted thiophenol. Since the S–H vibration should not be effected by steric shielding but the shape would be very different, the VTO would predict little change in the thiol odor. [27c]

4.2 Receptor ligand-docking theory of olfaction (modified "shape" theory)

Since to date there is no experimental evidence based on vertebrate ORs supporting the vibration theory of olfaction, and since there are several questionable assumptions made in this theory of olfaction, we next consider the so-called "shape"-theory. Suslick argues that the kind of molecular recognition required for detecting a vast number of different odorants and odorant mixtures over an equally vast range of concentrations cannot be simply based on "the usual model of biospecificity, i.e., the lock-and-key mechan-ism of enzyme–substrate interaction [e.g., the "shape" theory]. The olfactory receptors represent the exact opposite of that kind of specificity and show highly cross-reactive, nonspecific interactions

with odorants. Molecular recognition instead occurs through the pattern of response from hundreds of different types of olfactory receptor epithelia cells…" This pattern of response is, in turn, based on a range of intermolecular interactions connecting the odorant with the receptor over a continuum from the stronger ligand coordination involving metals, to electrostatic ion–ion and proton acid–base interactions, hydrogen bonding, halogen bonding, charge-transfer and π–π molecular complexation, dipolar and multipolar interactions, and weaker van der Waals interactions.[28a] While there is also a dynamic aspect to odorant-OR interaction (the odorants must rapidly separate from the ORs to allow real-time sensing of changes in odor nature and concentration), stronger interactions favor increased sensitivity and greater chemical specificity. Recent work should be noted here by Kobilka and coworkers on GPCR dynamic processes for signaling by β_2-adrenergic and μ-opiod receptors (β_2AR and μORs, respective-ly),[28b-d] given that there should be similarities in behavior of olfactory and non-olfactory GPCRs in view of their extensive morphological similarities, as well as the already noted[7a] occur-rence of ORs in non-olfactory tissues. Indeed, it has been asserted that ORs are model systems for GPCRs within neurobiology.[28e]

Given the above concerns, as well as results presented below, the "shape" theory might be better named the "receptor ligand-docking" theory of olfaction to more fully describe the range of molecular interactions which could occur when an odorant interacts with a receptor. Indeed, as early as 1940, Pauling and Delbrück noted that interacting biomolecules "must have complementary surfaces, like die and coin, and also a complementary distribution of active groups."[28f]

To evaluate the plausibility of the VTO, several of the key assumptions and claims have been examined and tested, as have assumptions of the alternative receptor ligand-docking theory. In addition, the distinction between *perception* and *reception* of an odor needs to be addressed.

We first consider the reported difference in odor of acetophe-none and acetophenone-d_8.[27a,c] This claim has more recently been shown by Turin himself,[27d] as well as Keller & Vosshall,[29] to be untrue. A 2013 paper indicates that, in a blinded behavioral study, smell panelists distinguished between deuterated and nondeuterated isotopologues of cyclopentadecanone and other musk odorants.[27d] However, it was shown that the human musk-sensitive receptor OR5AN1, expressed in HEK293T cells derived from human embryonic kidney, and identified through screening of 330 similarly expressed human ORs as the sole bona fide receptor for cyclopentadecanone, fails to distinguish a series of deuterated and nondeuterated isotopologues of cyclopentadecanone and other musk odorants. Furthermore, pairs of deuterated and nondeuterated acetophenone, benzaldehyde, and several other deuterated and nondeuterated odorants, and pairs of ^{13}C and normal isotopologues of both acetophenone and benzaldehyde failed to show differences when tested against their respective, responsive ORs.[30] In addition, contrary to the claim that a musk receptor "detects vibrations in the 1380- to 1550-cm^{-1} range" where musks are said to have intense bands,[27d] it was found that fully deuterated muscone, which strongly activates OR5AN1 equally as well as the undeuterated muscone, is devoid of 1380- to 1550-cm^{-1} absorption.[30]

The response found with OR5AN1 is consistent with a report[31] identifying OR5AN1 as a human muscone OR, a second report on OR5AN1 as the only functional human homolog of mouse muscone ORs in vivo,[32] and two reports indicating that only a small number of receptors are thought to be involved in sensing musk odor.[33] While experiments with OR5AN1 are done with cells in a dish rather than within whole organisms, scientists have successfully used HEK cells to study rhodopsin and adrenaline receptors and such systems are considered the standard in studying ORs.[35] Examples of discrimination of isotopologues by insects have been reported,[36] some of which are at the behavioral and not at the receptor level.[35,37] Recent electroantennogram insect studies show that several, but not all, deuterium isotopologues can be distinguished in *Drosophila* antennae.[38a] A second recent study reports limited differential odor coding of isotopologues in the honeybee brain, with a significant isotope sensitivity found in 20% of the glomerula tested.[38b] It is not known in these two cases whether similar discrimination would occur at the OR level given the possible involvement of perireceptor effects. Furthermore, it should be emphasized that the protein receptors identified in insect OSNs are ion channels, and are not related to mammalian G-protein coupled receptors.[38b] Thus, results with insects should not be predictive of the ability of humans to distinguish isotopologues. Finally, it should be noted that deuteration has more effects than just changing the vibrational spectrum, including changing intermolecular interactions due to lowering of the zero point energy of bonds to D compared to H. The acidity of D_2O and H_2O are different, hydrogen bonding of O–H and O–D are different, boiling points and freezing points are different. Notably, the gas chromatographic retention times of isotopologues studied were found to be significantly different.[30a] The lack of isotope effects seen with the deuterated musks with OR5AN1 reflects the fact that C–H/C–D bonds are unlikely to be broken during the docking process.

Theoretical analysis of the VTO indicates that quantum effects of nonodorant molecular vibrational mode could easily suppress the proposed electron transfer mechanism of the vibrational frequencies of odorants.[30] A theoretically-grounded kinetic model for the activation of mammalian ORs has been reported in which the odor activity depends on equilibrium dissociation and rate constants.[39] It is argued that to date there is no evidence for the existence of electron transfer (ET) processes in ORs, nor is it clear whether plausible electron donor and acceptor sites can be found in ORs.[39] Computations based on quantum mechanics/molecular mechanics (QM/MM) hybrid methods can also be used to analyze isotopologue-OR interactions, such as interactions of CH_3SCH_2SH (MTMT) and CD_3SCD_2SH (MTMT-d_5) with the mouse OR MOR244-3 in the presence of copper ion, as discussed in more detail below.[40] These calculations reveal that both MTMT and MTMT-d_5 have similar binding affinities, and that no difference in response is predicted to occur upon deuteration, consistent with the experimental observations.[30a]

Proponents of the VTO have misrepresented shape-related features claiming that most enantiomers have identical odors, which is inconsistent with the chiral nature of ORs.[27d, 36a] These assertions are at odds with the highly developed ability of mammals to discriminate numerous nonpheromonal chiral odorant enantiomeric pairs,[41] and, in particular, with the highly selective response of the musk-sensitive mouse receptor, MOR215-1, to (R)-muscone (“l-muscone”) compared with (S)-muscone (“d-muscone”).[31]

Finally, when examined the theories of olfaction it is important to keep in mind the distinction between how different smells are perceived – the perception of an odor – and how odorants are recognized by olfactory receptors at the molecular level – the recognition of an odor.[30a] It should be noted that isotope effects in odorant response at the behavioral/organismal level do not necessarily support the VTO. As discussed above, volatile odorants enter the nasal passage where they dissolve in the nasal mucus and are transported by the mucus to ORs on the cilia of OSNs. In the nasal mucus odorants are subject to a variety of perireceptor effects by the complex mixture of enzymes, including the OBPs and ODEs, mucopolysaccharides, antibodies, and salts, including metal ions. For example, it has been proposed that the nasopharyngeal mucus "behaves like a polar chromatographic column,"[42a] with differential diffusion rates, air/mucus partition coefficients[42b] and solubility toward dissolved odorants,[42c] which could lead to isotopologue separation. As noted above, the musk isotopologues show significant differences in gas chromatographic retention times. Thus, when isotopologues are involved as odorants, isotope effects could come into play, even if no such effects occur at the ORs.

4.3 The effect of steric hindrance on odor

As noted above, the absence of a lutidine-like odor in 2,6-di-*tert*-butylpyridine (Fig. 5) has been suggested to be due to "steric blocking of coordination to relatively large electron-pair acceptors in the tissues affected."[43] Thiophenols as well as isonitriles, both known to possess highly offensive odors, can also be rendered practically odorless when access to the reactive group is hindered, as in the case of 2-(trimethylsilyl)thiophenol[44a] and the hindered benzeneisonitrile.[44b] These observations argue against the VTO since the mercapto and isonitrile vibrational bands would still be present in the two compounds shown. Steric interference represents a potentially useful tool to improve models for receptor binding.

4.4 The effect of impurities on odor

Caution must be used when evaluating the comparative smell of divalent sulfur compounds, e.g., commercial dimethyl sulfide and dimethyl sulfide-d_6, (as discussed above as evidence for the VTO). Morton points out that sulfur "has a rich oxidation chemistry in the presence of air, which produces impurities that have a characteristic stench. The reader can confirm the effect of these impurities by contrasting commercial dimethyl sulfide with a sample that has been freshly washed with saturated aqueous mercuric chloride to remove di- and polysulfides and mercaptans!"[46] It is probable that dimethyl sulfide-d_6 was more highly purified than the normal istopologue and thus could have a different odor; this possibility was not considered when the odors were compared.[27c]

5. Early work on metals and olfaction

A rule of thumb for inorganic chemists is that if a volatile molecule is a good ligand for coordination to metal ions, it probably smells strongly. Thus, the human olfactory system is extremely sensitive to thiols, amines, and isonitriles (see Fig. 1), which are all good ligands for metal ions, but not to alcohols, which are only weak ligands. Since malodorous volatile thiols and amines are protein degradation products found in putrid food, sensitive identification of these compounds is crucial to avoiding food-poisoning.[26a,b] Because it is

necessary for odorants to bind to ORs to initiate the sequence of events that lead to smell, it is hard to account for the million–fold lower threshold level for methanethiol, and hundred thousand–fold lower threshold level for methylamine compared to methanol based just on differences in hydrogen bonding or van der Waals interactions.

It is the above consideration that led Robert Crabtree to prophetically speculate in 1978 that some ORs might be Cu^{1+}–containing metalloproteins, given the strong amine–, thiol– and phosphine–binding properties of copper.[47] Crabtree commented: “Of the biologically important metals, copper(I), particularly when coordinated to a ‘soft’ anionic center such as I or SR, has a high affinity for all the malodorous substances mentioned above [thiols, selenols, tellurols, amines, phosphines, arsines, isonitriles] and a low affinity for CO and seems to be the most likely candidate for a metallo-receptor site in olfaction…The Cu(I) centre would be stabilized by coordination, perhaps to a protein thiolato-group, and, most probably also to two or three additional protein S or N neutral donor groups. In the former case, a ligating odorant could coordinate directly to the metal, in the latter, it would have to displace a labile ligand already present. In either case, considerable geometrical changes would occur at the receptor site, leading to the activation of the appropriate neuron.” [47] As we shall see, Crabtree is quite correct in what he proposed.

Also in 1978 Jack Day, discussing the absence of a lutidine-like odor in the sterically hindered 2,6-di-*tert*-butylpyridine, suggested that this might be due to “steric blocking of coordination to relatively large electron-pair acceptors in the tissues affected. Possibly a transition metal serves in the olfaction of certain functional groups.”[43] It is notable that both Crabtree and Day in 1978 independently arrived at the same conclusion that transition metals may have a role in olfaction! Regarding steric blocking of coordination, olfactory receptors show unusual differences in their shape selectivity for alcohols compared with thiols, consistent with the metalloprotein hypothesis. Thus, substitution at the α-carbon of alcohols *increases* the olfactory threshold for alcohols, consistent with the expectation that greater steric bulk leads to weaker binding, as expected for sterically restricted binding sites. On the other hand, α-substitution of thiols typically *decreases* the olfactory threshold for thiols, consistent with the expectation for OR binding sites containing a coordinately accessible metal ion, since α-substitution increases the Lewis basicity of the thiol and, hence, its strength of binding to metal ions, as long as the increased steric hindrance is not too great.[43]

Several papers have discussed the role of metal ions in GPCR activation: certain divalent cations modulate ligand-binding of opioid receptor subtypes (Mg^{+2}, Mn^{+2})[48a], increase the affinity of a ligand to CXCR4 chemokine receptors (Cu^{+2}, Zn^{+2}, Ni^{+2})[48b], and bind and activate melanocortin MC1 and MC4 receptors (Zn^{+2}).[48c] Turin suggested a role for zinc ions in conjunction with his vibrational theory of olfaction,[27a] namely that zinc ions coordinate to receptor proteins in binding both compounds with lone pair electrons as well as π-bonds.[27c] In 1999 it was reported that the ETR1 receptor from *Arabidopsis* binds the gaseous hormone ethylene employing a copper(I) ion in the ethylene-binding domain. Cysteine65 and histidine69 are postulated to serve as ligands for the Cu(I) ion; silver ions can also occupy the binding site and interact with ethylene.[48d] The possibility that some olfactory receptors could be metalloproteins was further explored by Kenneth Suslick and

coworkers.[49,50] Suslick presented a hypothesis that there is a metal-binding site in the loop between the fourth and fifth helices (4–5 loop) of ORs and proposed that Cu^{2+} or Zn^{2+} binding is required for OR's high sensitivity and selectivity to amines and thiols. They prepared a pentapeptide that contains this putative OR metal binding site and found that it not only had a high affinity for Cu^{2+} and Zn^{2+} ions, but that it also undergoes a transition to an α-helical structure upon metal ion binding. Based on these findings, they proposed a "shuttlecock" mechanism for the structural change in ORs upon odorant binding. This mechanism (Fig. 6) involves membrane penetration of the 4–5 loop after residue charge neutralization by metal ion binding.[49,50] This mechanism could be adapted to convert ORs into artificial metalloprotein sensors through genetic code expansion, allowing direct visualization of analytes. It was suggested that biomacro-molecules such as the proposed metalloprotein ORs "have many more degrees of freedom than an inorganic complex and can therefore use the protein scaffold supporting the catalytic metal centers to tune reaction specificities and reaction rates."[50]

In studies beginning in the late 1960's, predating Crabtree's suggestion, a connection was made between body levels of copper and/or zinc and disorders of the senses of taste (hypogeusia; decreased taste acuity) and smell (hyposmia; decreased smell acuity). Thus, Robert Henkin reported that treatment of humans as well as rats with the potent copper-chelator D-penicillamine produced a decrease in taste (but not olfactory) sensitivity, which could be restored by withdrawal of the D-penicillamine or by administration of oral copper or zinc. It was noted that a patient with multiple myeloma suffered from loss of taste, which was postulated to be associated with elevated levels of thiols in the patient's plasma, due to the multiple myeloma. The patient's taste could be temporarily restored to normal through administration of copper or zinc; cessation of copper or zinc therapy led to a return of the hypogeusia. It was proposed that the biological thiols chelate or reduce the copper.[51,52] It should be noted that the levels of zinc and copper in human cerebrospinal fluid has been determined to be 31.5 and 7.5 mg/L[53] and that excess zinc leads to copper deficiency.[54] It is known that zinc is a trace element with multiple roles in biological systems including structural and cofactor functions for proteins. Although most zinc in the central nervous system (CNS) is bound to proteins, the CNS contains a pool of mobile zinc found within neurons. While mobile zinc occurs in the hippocampus, hypothalamus, and cortex in the brain, the olfactory bulb contains one of the highest zinc concentrations in the entire CNS, where it is concentrated in the glomerular and granule cell layers.[55a] A 2001 paper reported that when tested on the voltage-gated K^+ channel of rat olfactory neurons, both zinc and odorants inhibited K^+ currents; in addition, the effect of zinc was dramatically diminished in the presence of odorants, indicating that they share a common inhibitory binding site on the external surface of the voltage-gated K^+ channel.[55b] The authors conclude that analysis of the odorant binding properties of metal binding sites with known structure may also reveal clues as to the structural basis of odorant detection and discrimination.

Patients in a double-blinded placebo-controlled randomized clinical trial who were undergoing chemotherapy and who reported alterations in taste and/or smell were given the equivalent of 50 mg elemental zinc (as $ZnSO_4$) or a placebo daily for three months. At the end of the trial it was reported "there was no statistically significant improvement in loss or distortion of taste or smell with the addition of zinc."[56a,b] At elevated concentrations, zinc

sulfate is highly toxic to the MOE, and in fact is used to destroy the MOE in studies of olfaction in animals (see Section 8.1). A recent paper reports that dietary nickel deprivation negatively impacts olfaction and taste in rats.[56c]

6 Demonstration of the activation of olfactory receptors by metals

6.1 In mice: characterization of a mouse thiol semiochemical

In 2005, a collaboration involving graduate student Dayu Lin and (the late) Professor Lawrence C. Katz at Duke University together with Eric Block and postdoctoral fellow Shaozhong Zhang at the University at Albany led to the discovery that male mouse urine contains (methylthio)methanethiol (MTMT; $MeSCH_2SH$), a semiochemical (signaling compound) with a powerful garlic-like odor, which is highly attractive to female mice.[57] Significantly, this work identified MTMT is a novel sex-specific chemical cue, able to initiate a defined innate behavior and that acts through the main olfactory system rather than through the vomeronasal organ. MTMT was identified by a process employing solid phase micro-extraction (SPME) to collect mouse urinary volatiles, which were separated by gas chromatography (GC; Fig. 7). The GC effluent was split, with one stream going either to a flame-ionization or mass-selective detector and the other stream directed at the mouse's nose. GC peaks were correlated with their ability to induce an electrophysiological neural response in the mouse, recording electrically from the main olfactory bulb mitral cells, which in turn received direct excitatory inputs from OSNs. When urine-responsive OSNs were tested with individual, separated urine components, 33% were activated by a single compound, present in male but not in female mouse nor castrated male mouse urine.[57] Based on the MS fragmentation pattern and comparison of retention times and fragmentation patterns with that of an authentic sample, the male-specific compound was identified as MTMT. Significantly, mouse OSNs are highly, and specifically sensitive to MTMT, responding at a threshold of 10 ppb, yet not responding to any of the >100 other mouse urine volatiles. A more recent study showed that MTMT can be discriminated at a concentration as low as 10 nM when added to a solution of gonadectomized (castrated) male urine diluted in water.[12] Furthermore, MTMT elicited a specific behavioral response in female mice: females were more interested in urine produced by intact rather than castrated males. Finally, addition of synthetic MTMT to urine from castrated males increased the attractiveness of the urine to female mice.[57] The electrophysiological work demonstrates that MTMT induced clear responses in MOB mitral cells of female mice, indicating that the main olfactory system mediates the attraction of females to this pheromone.[12]

6.2 In mice: demonstration of the role of copper in detection of (methylthio)methanethiol by mouse olfactory receptor MOR244-3 and the effect of added copper chelator on *in vitro* and *in vivo* receptor response

How do mice detect the very low concentrations of the thioether-thiol MTMT present in male mouse urine? To answer this question, Hiroaki Matsunami and Hanyi Zhuang, at Duke University isolated the specific mouse olfactory receptor (MOR) responsive to MTMT, termed MOR244-3 (Olfr1509), using the HEK293T-based OR heterologous expression system.[59-61] With Eric Block, they explored the possibility that Cu or Zn ions might be involved in the detection of MTMT by MOR244-3. They found that physiological levels (30

μM) of Cu ions, but not Zn ions or other common transition metal ions, robustly activated MOR244-3 toward MTMT (Fig. 8).[23a] They used the luciferase assay to measure the luminescence resulting from receptor activation by odorant solutions of known concentrations.[60] It was separately established that epithelial mucus taken from the mouse and analyzed by inductively coupled plasma mass spectrometry (ICP-MS) showed the presence of levels of inorganic copper similar to those used in testing MOR244-3 in vitro (ca. 40 μM). [23a]

The above experiments were performed with and without the membrane-permeable, high-affinity copper chelator tetraethylene-pentamine [$(H_2NCH_2CH_2NHCH_2CH_2)_2NH$; TEPA] to determine whether copper deficiency would reduce receptor activation, since trace levels of copper were present in the commercial stimulation medium used. Notably, it was found that adding 10 μM TEPA to the medium without exogenous Cu^{2+} entirely abolished the receptor's response to 30 μM MTMT, confirming that basal levels of Cu^{2+} in the stimulation medium are required for activation of MOR244-3 by MTMT.[23a] With a fixed 10 μM concentration of Cu^{2+}, increasing TEPA concentration decreased the response of MOR244-3 to 10 μM MTMT; with a fixed 10 μM concentration of TEPA, receptor activity was rescued by increasing Cu^{2+} concentration (Fig. 9). Our results suggest that excess Cu^{2+} reversed TEPA activity, reinforcing the role of copper ion in the enhancement of receptor activity. The effect of TEPA was also confirmed using the GloSensor assay, which is a more direct measurement of cAMP.[23a]

TEPA was also used to study behavioral effects in mice, namely olfactory recognition of MTMT, in the presence or absence of copper. Thus, mice were trained to associate either eugenol or MTMT with sugar reward. On the test day, the mice were given bilateral nasal cavity injections of TEPA. After the TEPA injections, it was found that mice trained to associate MTMT with sugar reward spent significantly less time investigating the odor, whereas time spent investigating the nonsulfurous odorant was unaltered. Two days later, after metabolic clearance of TEPA, the mouse group trained to recognize MTMT regained olfactory discrimination ability (Fig. 10). The results from the behavioral experiment clearly establish that copper is required for olfactory detection of MTMT by mice.[23a]

The response of a panel of organosulfur compounds structurally related to MTMT toward MOR244-3 in the presence and absence of copper was also determined by measuring dose-response curves under in vitro conditions (Fig. 11, 12). Among the compounds which were found to show highest activity (solid boxes in Fig. 11) were (methylseleno)methanethiol ($MeSeCH_2SH$), disulfides $MeSCH_2SSMe$ and $MeSCH_2SSCH_2SMe$, and methyl dithioformate (MeSCH=S). The compounds tested were isomeric with MTMT, or differed by the addition of one or two carbon atoms with associated hydrogens or two oxygens (a sulfone), by the deletion of two hydrogen atoms (dithioformate), or by substitution of the thioether sulfur by selenium. These structural changes could alter the number of thiol and thioether groups, alter the steric crowding at the sulfur atoms, modify the ligand “bite angle,” alter the S–H acidity, and change the availability of thioether electron pairs, in turn, potentially modifying the coordinating ability of the copper complex with the functional groups of the ORs. Testing of the analogs in Fig. 11 was performed with addition of 30 μM Cu (as $CuSO_4$), with no added copper, and with 30 μM of copper chelator TEPA to eliminate

background levels of Cu in the MOR244-3 culture medium. The results for MTMT (Fig. 8), show the dramatic difference between the response with added 30 μM Cu (top, blue trace, with a limiting detection threshold of 10^{-8} M and an EC_{50}, of 10^{-6} M), with no added Cu (middle, magenta trace, with a limiting detection threshold of 10^{-6} M and EC_{50} of 10^{-5} M), and with 30 μM TEPA (bottom, green trace, with a limiting detection threshold of 10^{-5} M and an EC_{50} of 5×10^{-4} M).[23a]

Replacing the thioether methyl group in MTMT by ethyl ($EtSCH_2SH$) has no effect on activity (modest steric effect), whereas addition of a methyl group on the carbon between the sulfur atoms (MeSCHMeSH), addition of both the ethyl and methyl groups (EtSCHMeSH), or replacing the ethyl with a *tert*-butyl group (*t*-$BuSCH_2SH$) diminishes the activity (more significant steric effects). Cyclization to 2-mercaptothiolane results in modest loss of activity (shape change). Further removal of four hydrogens from the latter compound giving 2-thiophenethiol results in almost complete loss of OR activity. This is presumably due to the very poorly nucleophilic character of thiophene lone pairs. Similarly, oxidation of MTMT to $MeSO_2CH_2SH$ results in partial loss of OR activity due to the inability of the sulfone group to coordinate to copper, although the acidity of the thiol group is increased. Activity is retained with the selenium analog of MTMT ($MeSeCH_2SH$) and in methyl dithioformate MeSCH=S and trithiocarbonate MeSC(S)SMe. Enhanced OR activity is shown by both $MeS(CH_2)_2SH$ and $MeS(CH_2)_3SH$, where 5- and 6-membered ring copper chelates can form. OR activity is lost upon methylation of the thiol group of MTMT in $CH_2(SMe)_2$, loss of the thioether methyl group in $CH_2(SH)_2$ or isomerization to MeSSMe or $HSCH_2CH_2SH$. 2,3,5-Trithiahexane ($MeSCH_2SSMe$), found in male mouse urine, and $MeSCH_2SSCH_2SMe$, an oxidation product of MTMT, elicited strong responses by the receptor and displayed a dramatic reduction in the response with added 30 μM TEPA. These observations are consistent with the report that a neighboring electron donor in disulfides, when ligated to Cu(I), “enhances the electron transfer from Cu(I) to the disulfide leading to S–S bond scission,”[62a] for example, the methylthio groups in 2,3,5-trithiahexane and 2,4,5,7-tetrathiaoctane, since dimethyl disulfide is apparently not reduced under these same conditions.[23a]

Both Cu(I) and Cu(II) species should be available to ORs, consistent with the known reducing environment within cells.[62b-e] In fact, the coordination geometries of Cu in the QM/MM models of ORs are typically intermediate between the expected square planar configuration of Cu(II) and the tetragonal configuration of Cu(I). So, Cu(I)/Cu(II) oxidation state transitions between the two oxidation states should be facilitated by the coordination environment.[63a]

Various studies have shown that thiols readily react with Cu(II) giving disulfides and Cu(I) (Fig. 13), for example as established using EXAFS and EPR.[63] Formation of Cu(II)-thiolates is in fact quite challenging due to the facile redox process leading to disulfides, involving rapid electron transfer. Reduction of Cu(II) by thiols is second order with respect to the S-bonded intermediates leading to concerted two-electron transfer and formation of an S–S bond.[62e] Soft donors such as thiolates cause the reduction potential of copper to be positive, stabilizing Cu(I) complexes. Cytoplasm is rich in thiolates such as glutathione (GSH), the single most abundant thiol in eukaryotic cells. GSH binds Cu(I) tightly, with the

consequence that copper compounds can only be present as Cu(I) in cytoplasm.[62h] If it enters the cytoplasm Cu(II) is reduced by acquiring one electron. Dynamic copper sites can exchange with GSH and other small-molecule ligands. In addition, Cu(I) is extensively trafficked by a system of intracellular proteins called chaperones which selectively bind and escort Cu(I) preventing free ionic copper from appearing in the cytoplasm where it could generate radicals and reactive oxygen.[62c,f,g,h]

Most of our in vitro OR experiments in HEK293T cells were done using Cu(II) under aerobic conditions. Under the same conditions, addition of Cu(I), kept reduced by ascorbic acid prior to cell culture addition, gave similar results compared to Cu(II). Our results suggest that the most active complexes involve sulfur compounds of type $RX(CH_2)_nSH$ (X = S or Se; n = 1–3; R = Me or Et), with one terminal thiolate (C–S–), or RXCH=S, with a thiocarbonyl (C=S) sulfur.

In addition to the main olfactory epithelium (OE) and the vomeronasal organ (VNO), the mammalian nose also contains the septal organ, a small patch of olfactory neuroepithelium at the ventral base of the nasal septum found in many mammals that expresses SR1 (also known as MOR256-3), MOR244-3, and a few other ORs in high abundance.[64a] Perforated patch-clamp recordings were performed on dissected septal organs from SR1-IRES-tauGFP mice, and MTMT responses among the non-SR1 cells were examined. Of 132 cells recorded, 76 cells (58%) responded to MTMT in a dose-dependent manner.[23a] This percentage is higher than that of MOR244-3 cells (27%) in the non-SR1 cells of the septal organ, suggesting that additional ORs in the septal organ are responsive to MTMT. In vitro screening was used to test this possibility and it was found that another OR, MOR256-17, showed robust responses to MTMT. Interestingly, the MTMT responses of MOR256-17 were not modulated by copper addition. While modeling results are not yet available to explain how MTMT and other thiols might bind to ORs such as MOR256-17 in the absence of a metal, it is relevant that thiols have been found to engage favorable with aromatic rings in S–H/π-interactions within proteins. These interactions were reported to be driven significantly by favorable molecular orbital interactions between an aromatic π donor orbital and the S–H σ*acceptor orbital, a π®σ* interaction.[64b]

6.3 In mice: Mutational and molecular modeling studies on MOR244-3

Because histidine, cysteine, and methionine frequently coordinate copper in cuproenzymes, and because amino acids remote from each other in the primary structure may be closely interacting in actual spatial arrangement, in order to understand how the respective mutations affect MOR244-3 activation by MTMT in the luciferase assay, a series of single-site mutants were constructed in MOR244-3, changing all methionine residues to alanines; all histidines to arginines, lysines, tyrosines, leucines, valines, phenylalanines, asparagine, and/or alanines; and all cysteines to serines, valines, and/or phenylalanines.[23a]

Some of the mutations 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, completely abolished responses to MTMT when mutated, regardless of the presence of

Cu^{2+}. Figure 14 shows a serpentine model of MOR244-3 color-coded for the methionine (M, green), histidine (H, red), and cysteine (C, yellow) residues subjected to mutagenesis analysis. Residues circled in blue are those that exhibited complete loss-of-function phenotypes in the luciferase assay. Of these, mutants C97S, H155R, C169S, C179S, and H243R (S = serine, R = arginine), but not H105K (K = lysine), have little or no cell-surface expression, suggesting that these first five mutants may have lost their functions due to defects in receptor folding/trafficking. Notably, the H105K mutant retains the ability to respond to some of MOR244-3's nonsulfurous ligands, such as cineole, and to ligands with no copper effect, such as dimethyl sulfide, indicating that the mutant receptor is intact. Presumably the H105K loss-of-function mutation disrupts copper/ligand binding, making H105–C109 the most likely location of the MTMT-copper binding active site. [23a]

The most widely accepted view of the mechanism of action of ORs is that signal transduction depends on the shape and concentration of odorants in the nasal mucus, and the response kinetics determined by specific odorant-OR interactions at the binding site.[65] Due to the lack of crystallographic or NMR structures of ORs, computational structural models are particularly valuable and can be tested against the above mutagenesis experiments and activation profiles. In this way an MOR244-3 model was built using density functional theory (DFT) in conjunction with quantum mechanics/molecular mechanics (QM/MM) hybrid methods and homology modeling based on the x-ray structure of the M2 human muscarinic receptor as a template (Fig. 15).[40,66a] The homology model was felt to be appropriate because of the high sequence identity between the MOR244-3 and M2 receptor TM regions. A common structural feature of both MOR244-3 and the M2 receptor is an internal aqueous channel (Fig. 15a, *green*) extending from the extracellular surface through the ligand-binding pocket to a depth of ca. 30 Å.

A hydrophobic cap (Fig. 15a *blue*) formed by L66 and L114 interrupts the internal aqueous channel. These residues form an analogous hydrophobic cap separating the extracellular and cytoplasmic parts of the water channel in M2. The computed ligand-binding site (Fig. 15b) consists of Cu^{+} coordinated to the heteroatoms N, S, and O of amino acid residues H105, C109 (in thiolate form), and N202, respectively, in the aqueous channel. In the absence of a ligand, Cu^{+} adopts a linear coordination with NH105 and SC109 and a weak interaction with ON202 (Fig. 15c). Upon binding to MTMT, the SMe-MTMT group exchanges with the ON202 ligand and induces Cu^{+} to adopt a trigonal planar coordination with NH105, SC109, and SMe-MTMT (Fig. 15d). An H-bond cage linking the amino acid residues H105, D180, K269, Y258, T259, F256, I255, C254, and S207, and water molecules encloses the binding site, forming a lid over the organosulfur ligand (Fig. 15b).[45] The coordination computed for Cu^{+} in MOR244-3 to the heteroatoms of histidine and cysteine residues is consistent with that seen in azurin and other metalloproteins; coordination to thiolate, RS^{-}, is in accord with the preference of Cu^{+} for soft anionic centers.[47]

The proposed coordination of Cu^{+} to H105 and C109 is consistent with mutagenesis studies. Thus, H105 mutants show loss-of-function in receptor functional assays. The C109V mutant (e.g., cysteine 109 replaced by valine), in which thiolate is replaced by the larger isopropyl (Me_2CH–) group, results in loss of coordination of Cu^{+} to site 109 even in the presence of bound MTMT (Fig. 16a). On the other hand, the C109M mutant (e.g., cysteine 109 replaced

by methionine) is activated in the presence of Cu^+ (Fig. 16b), consistent with the inhibition of activity by chelator TEPA. Here, the methionine CH_3S ligand replaces the cysteine RS^- ligand.

6.4 Low molecular weight sulfur-containing scents from flowers and truffles: an MTMT-responsive bat OR orthologous to MOR244-3 with a copper effect

Just as male mice use MTMT and related low molecular weight sulfur compounds to attract a mate, plants use surprisingly similar compounds for their own reproductive purposes. For example, sapromyiophilous flowers attract pollinators that breed or feed on dung or carrion by mimicking these foul-smelling substrates with carrion-like, sulfur-containing floral odors. Sulfur compounds are unusual in floral scent,[66b] occurring almost exclusively in sapromyiophilous and bat-pollinated (chiropterophilous) flowers. We will consider three notable examples here: the dead-horse arum and other sapromyiophilous flowers, truffles as an example of use of sulfur compounds by fungi, and bat-pollinated flowers.

6.4.1 The dead-horse arum—Perhaps the most famous example of "deceit by resource mimicry" occurs in the aptly named dead-horse arum (*Helicodiceros muscivorus*), which fools flies into pollinating it by emitting a dead animal-like odor.[4c,66c] Pollinators of this flowering plant are primarily two blowfly (Calliphoridae) species, which are also carrion visitors. When blowfly antennae are separately stimulated with GC-separated volatiles from the plant and from a rotting carcass, identical electroantennograms are observed. The major component of both volatiles is dimethyl trisulfide; dimethyl sulfide and disulfide are also present.[4c] Curiously, however, the authors[4c] state that "the trisulphide derivative was present as two structural conformers in both the arum and carcass volatiles." The alleged "conformers" are well separated by GC. However, since dimethyl trisulfide can only exist in one form, the alleged "structural conformer" is most likely a $C_2H_6S_3$ isomer, possibly the disulfide analogue of MTMT, CH_3SSCH_2SH [(methyldithio)methanethiol]; alternative structures such as CH_3CH_2SSSH, CH_3SCH_2SSH and $HSCH_2SCH_2SH$ cannot be excluded. Other plants which are reported to use a mixture of dimethyl disulfide and trisulfide as foul-scented baits in attracting flies as pollinators include the titan arum (*Amorphophallus titanium*),[4b] notable for having the largest inflorescence in the world, *Jaborosa rotacea* (Solanaceae),[66d] *Eucomis* (Hyacinthaceae),[66e] *H. gordonii*, *H. keniensis*, *O. variegate* and *P. cubiformis*[66f] as well as *B. ungulata, C. ghiesbreghtiana, C. scandens, C. cujete, P. alata* and *V. gladiolifora.*[66g] While both dimethyl disulfide and trisulfide are often found together in plant-derived VSCs, blowflies and other Challiphoridae species respond exclusively to dimethyl trisulfide rather than dimethyl disulfide.[66h] It is interesting that dimethyl trisulfide is known to be the main source of malodor of fungating cancer wounds in human.[66i] While the focus of this review is on mammalian olfaction, it would be of interest to investigate the possible role of copper in insect olfaction involving these low molecular weight sulfur compounds.

6.4.2. Truffles and other non-floral sources of low molecular weight organosulfur compounds—Truffles, members of the genus *Tuber*, are the fruiting bodies of a subterranean fungus. These mushrooms, highly prized as food because of their unique and characteristic aroma, grow in symbiosis with certain trees, especially oaks. They

remain underground during their entire biological cycle. They characteristically produce dimethyl sulfide and other low molecular weight sulfur compounds, which attract truffle-eating (hydnophagous) animals and insects, such as the truffle fly (*Suillia pallida*), which in turn act as agents of spore dispersal. Throughout their life cycle they use volatile signals to regulate their interaction with other organisms.[67a] While truffles have been traditionally located using pigs, dogs are preferred because they can detect truffles by their smell from 30 to 50 meters and have little appetite for mushrooms (unlike pigs).[67b] Analysis of the volatiles from six truffle species showed the presence of dimethyl sulfide and disulfide, methyl propyl sulfide and 2,4-dithiapropane.[67c] Recent work has shown that the white truffle (*Tuber borchii*) emits 2-methyl and 3-methyl-4,5-dihydrothiophene, both of which are derived from the bacterial community inhabiting truffle fruiting bodies. The latter dihyrothiophene is described as having an “onion, savory, roast meat, truffle, garlic and buttery” aroma.[67d]

It is noteworthy that the tyrosinase enzyme from truffles is reversibly inhibited by both dimethyl sulfide and 2,4-dithiapropane (syn. bis(methylthio)methane).[67e,f] Tyrosinase, which occurs in prokaryotic and eukaryotic organisms, contains a binuclear, type 3 copper center within its active site, where two copper atoms are each coordinated with three histidine residues. Tyrosinase catalyzes the production of melanin and other pigments from tyrosine by oxidation, as in the blackening of a sliced potato exposed to air. Tyrosinase is involved in truffle development and differentiation including the hardening of cell walls. Dimethyl sulfide is thought to be one of the physiological factors that in vivo keeps tyrosinase inhibited, thus avoiding the premature phenolic oxidation that would damage the plant tissues.[67f] It has also been found that mushroom tyrosinase catalyzes asymmetric sulfoxidetion of organic sulfides in a manner similar to monooxygenases.[67g] In view of the effect of low molecular weight sulfur compounds on the truffle tyrosinase enzyme, it would be of interest to determine if there is a copper effect for pig and dog sulfur-responsive ORs.

Shiitake mushrooms (*Lentinula edodes*) are another example of a fungi producing VSCs. Their unique odor is due to the antibiotic compound lenthionine (1,2,3,5,6-pentathiepane).[67h,i] Bark, roots, bulbs, and leaves can also serve as a source of VSCs. The bark of four trees found in the Gabonese rain forest have a strong garlic- or onion-like odor. These so-called “wild onion trees” or “wild garlic trees” include *Afrostyrax kamerunensis*, *Afrostyrax lepidophylleus*, *Scorodophloeus zenkeri* and *Hua gabonii*.[67j] Bark volatiles of these trees include dimethyl disulfide, 2,3,5-trithiahexane, 2,4,6-trithiaheptane, 2,4-dithipentane, 1,2,4-trithiolane and 1,3,5-trithiacyclohexane. The mechanosensitive roots of *Mimosa pudica*, a perennial shrub endemic to Brazil, respond to touch by emitting thioformaldehyde, carbon disulfide, S-propyl propanethiosulfinate, methanesulfinic acid, ethanesulfinic acid, propanesulfenic acid, and 2-aminothiophenol.[67k] Similarly, *Petiveria alliacea*, a perennial shrub widely distributed in South and Central America and used in folk medicine, releases a powerful lachrymatory principle, (Z)-thiobenzaldehyde S-oxide, upon disruption of the root tissue.[67l,m] The type of chemistry seen with these non-floral sources of low molecular weight organosulfur compounds has been extensively investigated in the case of genus *Allium* and *Brassica* plants.[67n,o,p,q]

6.4.3 Bat-pollinated flowers—More than 750 species of flowering plants are chiropterophilous – pollinated by bats.[68a,b] These plants have characteristics that are attractive to bats, often including an odor unpleasant to the human nose, which is associated with sulfur compounds in their abundant nectar.[68c] Flower-visiting bats are described as night-active, with a well-developed sense of smell for remote location, and good night vision for close range orientation.[68a] They are among the largest flower-visiting animals and observed to cling to or hover in front of flowers, lapping nectar or pollen. Chemical analysis of the floral scents from the New World plants *Parmentiera alata*, *Crescentia cujete*, *Cleome anomala* and *Pilosocereus tweedyanus* showed the presence of dimethyl disulfide, trisulfide and tetrasulfide, 2,4-dithiapentane, 2,3,5-trithiahexane, 2,3,4,6-tetrathiaheptane, 2,3,5,7-tetrathiaoctane, 2,3,5,6,8-pentathianonane as well as isopropyl isothiocyanate and *s*-butyl isothiocyanate.[68a] The African plant Adansonia digitate is the only bat pollinated plant from Africa found to have significant quantities of sulfur compound, dominated by dimethyl disulfide, with smaller amounts of dimethyl trisulfide.[68b] The floral scent of a bat-pollinated flower of *Sonneratia alba* from Japan was reported to contain 2,4-dithiapentane (bis[methylthio]methane).[68c]

Bat-pollinated nocturnal cacti (Cactaceae) flowers, which are open during only one night, have floral scents described as reminiscent of leek, garlic, or rotten cabbage.[68d] The scent composition of *Epostoa blossfeldiorum*, *Pilosocereus arrabidae*, *P. catingicola* and *P. pachycladus* include methyl thioacetate, 2,3,5-trithiahexane, 2,4,5,7-tetrathiaoctane, dimethyl thiosulfonate, and dimethyl disulfide, trisulfide, and tetrasulfide;[68d] some of these compounds are suggested to be analysis artefacts. It is postulated that the sulfur compounds may be key substances in the co-evolution of bats and bat-pollinated flowers, since these compounds are rarely found in scents of flowers with other pollination syndromes.[68d]

The bat-pollinated, night-blooming Costa Rican flowering plant *Calyptrogyne ghiesbreghtiana* releases a foul-smell containing a mixture of dimethyl disulfide, trisulfide, and tetrasulfide, along with MTMT, dimethyl trithiocarbonate, and 2,4-dithiapentane.[68e] While genomic DNAs of the New World flower-visiting bats was not tested, it was possible to clone the MOR244-3 and MOR180-1 orthologs from the Old World bat species, *Myotis lucifugus* (the little brown bat). It was found that both orthologs responded to MTMT and showed a copper effect (Fig. 17; unpublished results, H. Zhuang).

6.5 In humans: OR2T11 thiol olfactory receptors

6.5.1 Gas odorization and demonstration of the role of copper and silver in detection of low molecular weight alkanethiols by hOR2T11—Ethanethiol and 2-methyl-2-propanethiol (*tert*-butyl mercaptan; TBM), with odor thresholds in the range 0.03–0.05 ppb, are odorizers in liquified petroleum gas (LPG) and natural gas, respectively. The earliest mention of gas odorization involved the use of ethanethiol in Germany in 1880 to give water gas (a fuel gas consisting of a mixture of carbon monoxide and hydrogen) a distinguishable smell. Efforts to standardize and regulate gas odorization of gas were galvanized following a deadly explosion in New London, Texas in 1937 involving non-odorized natural gas that killed 239 people (Fig. 18). Gas odorization guidelines were codified in the U.S. in the Code of Federal Regulations [49 CFR 192.625, odorization of

gas]. Many other countries have adopted similar rules. Based on these regulations, with country-by-country variation, gas must be detectable whenever concentration in air reaches 0.1-1.0%.

Due to their very low odor thresholds, other low molecular weight thiols have an important sensory impact as trace aroma components in wine,[70a-c] beer,[70d,e] cheese,[70f] onions,[70g] grapefruit,[70h] durian,[70i] roasted coffee,[70j] and sesame seeds,[70k] among other foodstuffs. The approach employed involved first screening of 99 mouse and 18 human known receptor–ligand pairs for thiols, amines and carboxylic acid using 30 μM $CuCl_2$, $NiCl_2$ and $ZnSO_4$. No metal effects were found with ligands concentration of 10–100 μM. Then 330 human ORs (representing more than 80% of the total active human OR genes) were screened for response to 2-methyl-2-propanethiol in the presence of copper ions.

After follow-up confirmation experiments with an extended concentration range, and exclusion of false-positive results, it was found that OR2T11 was the one and only human receptor for TBM with a strong Cu effect that could be counteracted with addition of the copper chelator TEPA. The specificity of OR2T11 toward TBM and other thiols was assessed using both the luciferase reporter gene system and the real-time GloSensor systems, with the latter being more sensitive (Fig. 19, 20).[71] OR2T11 was found to respond, showing a copper effect, to low molecular weight monothiols methanethiol, ethanethiol, 1-propanethiol, prop-2-ene-1-thiol, 2-propanethiol, 1-butanethiol, all branched-chain four carbon thiols (2-methyl-2-propanethiol, 2-methyl-1-propanethiol, and 2-butanethiol), and selected branched-chain or cyclic five-carbon thiols (3-methyl-2-butanethiol, 2-pentanethiol, and cyclopentanethiol) and short, straight-chain dithiols as well as MTMT, 2,3,5trithiahexane, bis(methylthiomethyl) disulfide, (ethylthio)methanethiol, 1-(methylthio)ethanethiol and 2-thiolanethiol. OR2T11 did not respond to hydrogen sulfide (in the form of NaSH at pH 6), nor to 5- to 10-carbon straight-chain thiols (Fig. 19-21). OR2T11 also responded to 2-methyl-2-propanethiol in the presence AgAc and $AgNO_3$, as well as colloidal silver; it did not respond in the presence of $AuCl_3$, $ZnSO_4$, $NiCl_2$, $FeCl_3$, $CoCl_2$, $MgCl_2$ or $PtCl_2$ (Fig. 22). Interestingly, OR2T11 responded to $AgNO_3$ but not $CuCl_2$ in the absence of added thiol. Notably, the alcohol counterparts to the responsive monothiols were also nonresponsive (Fig. 23). The response of OR2T11 and other ORs to silver is consistent with similarities in ligand coordinating properties of Ag(I) and Cu(I).[62g]

6.6.2. Homology modeling and QM/MM studies—By using the X-ray crystal structure of the human muscarinic receptor[66] as a template, as described in Section 5.3 for MOR244-3, it was possible to obtain QM/MM structural models of OR2T11 (Fig. 24 and 25) as well as MOR244-2 (Fig. 25), a mouse receptor responding to copper and silver but not to thiols. These models, which elucidate the odorant binding sites, share common features such as a highly conserved disulfide S–S bond, likely to be critical for structural stability.[71] In OR2T11, the two Cu(I) binding sites identified by modeling (Site 1 and Site 2) are supported by site directed mutagenesis and activation profiles. Thus, mutation of the key amino acid residues responsible for Cu binding leads to loss of response to thiols (Fig. 26). Importantly, while other explanations are possible for loss of function on mutagenesis, control experiments where ten other residues are mutated in different regions of the OR showed that the copper effect response remained intact (Fig. 27).[71] Site 1 involves M115 of

TM3 (transmembrane 3) together with C238 and H241 of TM6 (Fig. 26A); site 2 involves M56 of TM2 and M133, R135, and C138 of TM4 (Fig. 26B).

As shown in Table I, the calculated binding energies of a series of thiols toward OR2T11 are in the order (using absolute values) *t*-BuSH> *i*-PrSH> *n*-PrSH> EtSH> MeSH> AllSH> H_2S> *i*-BuSH> 3-methylbutane-2-thiol, consistent with the enhancing effect of α- alkyl substituents on electron density at sulfur, as well as the fall-off in activity for some larger thiols.

6.6.3 Demonstration of the role of copper and silver in detection of cyclic and acyclic sulfides by OR2T11 and MOR244-3—OR2T11 was found to respond with a copper effect to the four- and five-membered-ring sulfides, thietane and thiolane, as well as with a silver effect to thiolane (Fig. 28, 29).[67b,71] QM/MM modelling results for thietane are shown in Fig. 30. These results are significant for several reasons. These are the first examples of simple thioethers as OR ligands with enhancement in response by metals. Thiolane is a five-membered ring cyclic sulfide used as a gas odorant while 2-propylthietane, found in anal gland secretions of *Mustela* (weasel) species, as well as the parent four-membered ring thietane, are mouse alarm pheromones.[72] The two four-membered ring compounds induce fear in mice by signaling the presence of predating carnivores. Interestingly, and unlike 2- propylthietane, structurally similar 2,2-dimethylthietane did not elicit an avoidance response in mice.[72a] 2-Propylthietane is detectable by human subjects at levels as low as 0.006 parts per trillion.[72] It would be of great interest to see if the well-known very high sensitivity of seabirds and seals for dimethyl sulfide, as an indicator of the presence of algae as a food source in foraging areas,[73] is also associated with copper-requiring ORs. Dimethyl sulfide (DMS) is derived from breakdown of dimethylsulfoniopropionate (DMSP).[73]

6.6.3 Use of saturation-transfer difference (STD) NMR to study protein receptor-ligand interactions in OR2T11—Saturation-transfer difference (STD) NMR is a spectroscopic technique to study the interactions, in solution, between a large molecule (receptor) and a medium-small sized molecule (ligand), based on the nuclear Overhauser effect and the observation and analysis of the resonances of the ligand protons (Fig. 30).[74] Saturation transfer difference nuclear magnetic resonance spectroscopy demonstrated enhanced binding of 2-methylpropane-2-thiol (*tert*-butyl mercaptan) to MOR244-3 in the presence of copper;[75] a similar enhancement was also found for OR2T11 in the presence of copper as well as silver ions (Fig. 31).[71]

6.7 Other human thiol olfactory receptors

6.7.1 Human olfactory receptors OR2W1 and OR2C1 (and mouse receptor MOR244-3) respond to thiols without a metal effect—The human olfactory receptors OR2W1 and OR2C1 were also found to respond to thiols, but with five to eight carbon atoms, rather than one to four carbon atoms (Fig. 32). Furthermore, neither receptor

showed a copper effect. In addition, mouse receptor MOR244-3 was found to respond to thiols with one to four carbon atoms without a copper effect (Fig. 33). It should also be noted that human OR2T11 and mouse MOR244-3 are not orthologous (Fig 34). As noted above (Section 6.2), it would be of interest to determine if S–H/π-interactions with receptor aromatic rings[64b] play a role in docking of thiols with these ORs.

6.7.2 Key food odorants (KFOs) and the response of human olfactory receptors OR2M3 and OR1A1 to larger thiols—It has been proposed that although the olfactory system responds to a wide variety of chemicals, the best ligands will be those with behavioral and evolutionary significance. The identification of the best ligands can be vastly simplified by identifying key food odors (KFOs) that are the main components of food flavor and have very low odor thresholds. It has been reported that of the large number of volatile molecules present in food, only 230 are necessary and sufficient to reconstitute the perception of most foods and bever–ages.[76] By screening 3-mercapto-2-methylpentan-1-ol, a KFO found in onions having a characteristic onion odor, at either 30 or 300 μmol/L against 391 human ORs, Noe and coworkers found that OR2M3 was the only responder.[77] Furthermore, of 190 KFOs tested with OR2M3, 3-mercapto-2-methylpentan-1-ol was the only active ligand found. It is quite unusual for an odorant to activate only a single OR and for an OR to be so narrowly tuned in its response! Noe et al. also tested the Denisovan and mammalian orthologs as well as human paralogs of OR2M3 and found that only the *Homo sapiens* variant was active toward the compound. They then established the pharmacology (dose-response curves) for this receptor, which was compared against odor thresholds and odor qualities that were obtained by GC-O (gas chromatography-olfactometry).

3-Mercapto-2-methyl-pentan-1-ol is the member of the homologous series of 3-mercapto-2-methylalkan-1-ols with the lowest odor threshold. Compounds related to 3-mercapto-2-methylpentan-1-ol, including 3-mercapto-2-methylbutan-1-ol and 3-mercapto-2-methylpropan-1-ol occur in beer, wine and hops.[77] It had been previously reported that both 1-octanethiol and 1-nonanethiol activate human ORs OR2C1, OR2W1, OR1A1, while 1-pentanethiol activates ORs OR2T2 and OR2T8.[59,78] Other ORs were found to be more broadly tuned in their response to thiols and KFOs.[23a,71,79a] When a family of 3-mercapto-2-methylalkan-1-ols were tested against OR2M3 using a cAMP luminescence assay, it was found that three substances with chain lengths C4–C6 activated OR2M3 in a specific and concentration-dependent manner. Homologues with C-atom chain lengths less than four and greater than six did not activate OR2M3. Although 3-mercapto-2-methylalkan-1-ols with three-, seven- and eight-carbon chain lengths failed to activate OR2M3, other ORs are thought to be involved in their recognition. OR2M3 showed a much weaker response to the structurally related, but non-KFO compounds 3-mercapto-2-methylbutan-1-ol and 3-mercapto-2-methylhexan-1-ol. In the case of the 348-amino acid-long OR2M3, there is a CSSHL sequence in TM6 with C291 and H294; in OR2T11 the CSSHL sequence involves C238 and H241. While it has yet to be determined whether the response of OR2M3 to 3-mercapto-2-methylpentan-1-ol or homologues is enhanced by addition of copper, a copper effect might be expected given that OR2M3 shares the “CSSHL” motif with OR2T11.

6.8 Human anosmia to thiols

Olfactory dysfunction including anosmia[6a] – the inability to detect odors -- is associated with other serious problems, including inability to smell warning odors (gas, fire) and impaired ability to taste food.[21b] Anosmics may still be able to "smell" ammonia, since their CN V may still be functioning, although in some cases with decreased sensitivity.[79b]

Here we discuss anosmia specific to thiols. A 1945 paper describes a screening using a dilute solution of *n*-butanethiol of 4,030 individuals and their families, which revealed four individuals who could not smell the thiol even at elevated levels but had no difficulty smelling a variety of other odorants (vanilla extract, oil of cloves, eucalyptus, orange, hydrogen sulfide).[80] A more recent paper describes more widespread anosmia to the odor found in urine after consumption of asparagus. The odor is assumed to be due to methanethiol and *S*-methyl thioesters. It was found that 58.0% of men (n = 1449/2500) and 61.5% of women (n = 2712/4409) had anosmia to the "asparagus pee" odor and that 871 single nucleotide polymorphisms reached genome wide significance for asparagus anosmia, all in a region on chromosome 1 (1q44: 248139851-248595299) containing multiple genes in the olfactory receptor 2 (OR2) family, particularly OR2M7 and OR2L3.[81]

Proponents of the VTO claim that the existence of a congenital specific anosmia to mercaptans supports this theory (Section 3.1), arguing that if shape determines odor, it is difficult to understand how a mutation would wipe out a large number of receptors while only affecting a single odour class.[27a] However in screening of 330 human ORs for response to low molecular weight thiols (mercaptans), it was found that only OR2T11 responded robustly, and did so with a copper effect.[71] Recently a second human receptor, OR2M3, has been found, which responds strongly to thiols of slightly larger size than OR2T11.[77] These findings argue against there being a "large number of receptors" for thiols.

7 The effect of nanoparticles on vertebrate olfactory receptors

It has been reported that metal nanoparticles in picomolar concentrations enhance olfaction and that zinc nanoparticles act at the receptor site and are involved in the initial events of olfaction.[82] The studies involved exposing rat olfactory epithelium to 1- to 2-nm metallic particles of zinc, containing 40-300 zinc metal atoms (Zn^0), and measuring the odorant response by electroolfactogram and whole-cell patch clamp methods. It was found that a small amount of zinc nanoparticles added to an odorant or an extracellular/intracellular particle perfusion strongly increases the odorant response in a dose-dependent manner. Zinc nanoparticles alone produced no odor effects while copper, gold, or silver nanoparticles did not produce effects similar to those of zinc. A reduction of the OR neuron odorant response was found if zinc nanoparticles were replaced by Zn^{+2} ions in the same concentration range. Based on these observations, it was hypothesized that zinc nanoparticles are located close "to the interface between the guanine nucleotide-binding protein and the receptor proteins and are involved in transferring signals in the initial events of olfaction" and that "zinc metal nanoparticles can be used to enhance and sustain the initial olfactory events."[83] It was previously suggested that one metal nanoparticle binds two receptor molecules to create a dimer, which is consistent with the evidence that many G-protein-coupled receptors form dimers or larger oligomers.[84-86]

8 Toxic effects of metals on the olfactory system

8.1 Intranasal zinc, manganese and cadmium

Metal ions are readily transported from the OE to the brain, and it is well known that low sub-lethal levels of metal ions can adversely affect the sense of smell.[87-88] Among the metals of particular environmental concern are zinc, manganese, cadmium, chromium, nickel, and aluminum.[87-88] Intranasal application of $ZnSO_4$ to mice is known to produce a brief but essentially total disruption of functional connections from the OE to the main OB and a corresponding transient anosmia, raising concerns about the use of intranasal zinc preparations in humans for prevention of colds.[89] Commercial preparations of intranasal zinc gluconate gel were marketed as a remedy for the common cold. The safety of this treatment came into question when a number of individuals sniffing deeply when applying the gel were subsequently diagnosed with zinc-induced anosmia or hyposmia. After application they reported an immediate sensation of burning lasting minutes to hours. Loss of sense of smell was then perceived within 48 h. It was concluded that intranasal zinc gluconate (or other inhalational forms of zinc) therapy causes hyposmia and anosmia.[90-94] On June 16, 2009, the U.S. Food and Drug Administration (FDA) warned consumers to stop using and discard three zinc-containing intranasal products, indicating that the products may cause a loss of sense of smell.[95]

It has been suggested[96-98] that zinc and other divalent cations including copper but not magnesium can block the ion channels that facilitate transduction of odors into electrical signals in the olfactory epithelium. In an animal study, mice who underwent a buried food-pellet test gauging olfactory function were nasally irrigated with one of three divalent cationic compounds. When these mice were tested after treatment, mice irrigated with zinc and copper gluconate showed a significant increase in food-finding time. This was interpreted as indicating that they had lost their ability to smell a hidden food source compared to control mice who were irrigated with saline. Based on these observations, the authors conclude that zinc gluconate as well as other divalent cations can cause anosmia and negatively impact olfaction. It was noted that many patients who experienced anosmia after using a zinc gluconate nasal spray reported that they ha sniffed the product in deeply, forcing the solution onto the OE. It was further observed that magnesium gluconate did not produce anosmia in any of the subjects, a result which can probably be explained by the small molecular mass of magnesium. Ions that cause anosmia or block cation channels on the OE such as zinc, barium and copper all have molecular masses larger than that of calcium and are more likely to physically block calcium channels than are the smaller magnesium ions. The authors conclude that “divalent cations larger than Ca^{2+} can negatively affect olfaction.”[96]

It has been reported that intranasal exposure to manganese disrupts neurotransmitter release from glutamatergic synapses in the central nervous system in vivo. In a mouse study, acute Mn exposure via intranasal instillation of 2– 200 μg $MnCl_2$ solution caused a dose-dependent reduction in odorant-evoked neurotransmitter release, with significant effects at as little as 2 μg $MnCl_2$.[97] Intranasal exposure to cadmium has been related to olfactory dysfunction in humans as well as to nasal epithelial damage and altered odorant-guided

behavior in mouse models. McGann and coauthors note that intranasal $CdCl_2$ instillations in mice reduced olfactory sensory activity, monitored in vivo in mouse brain olfactory bulbs, by up to 91% in a dose-dependent manner.[98] These authors note that “The olfactory system is the only point in the mammalian nervous system in which neurons are physically exposed to the organism's external environment. This position makes the olfactory epithelium uniquely vulnerable to environmental neurotoxicants. The olfactory nerve can also serve as a vector for neurotoxicants to be transported into the central nervous system, bypassing the blood-brain barrier.”[98]

8.2 Effect of metal ions and metal nanoparticles on fish

Given the critical role that olfaction plays in the survival of fish, allowing them to detect waterborne chemical cues, mediating their ability to migrate, find a mate, recognize conspecifics, find food, and avoid predation, it is appropriate to consider how exposure to heavy metals can affect this sense.[99] It is well known that fish are at risk of exposure to heavy metals introduced into the aquatic environment by human activity. In particular, low concentrations of copper can have a harmful effect on olfaction in fish, although there are variations in copper sensitivity among different species of fish, as well as among different types of olfactory sensory neurons (OSNs). Thus, at 30 mg/L Cu^{2+}, the cilitated OSNs (cOSNs) are more sensitive than the microvillous OSNs (mOSNs); the underlying processes leading to these differences are not fully understood.[99-101]

Short-term, environmentally realistic concentrations of Cu have been reported to not only bind to the olfactory epithelium (OE) of fathead minnows but also to impair their olfactory sensitivity and behavioral responses to olfactory stimuli. Waterborne Ca^{2+} reduces Cu-OE binding but does not protect against olfactory impairment.[102] At these low levels of Cu, over time there was at least a partial recovery of olfactory function, despite the continuous Cu exposure.[103] It has been proposed that the biochemical mechanism of disruption by copper is that it acts by blocking the cyclic nucleotide gated channel of olfactory receptor cells, halting the movement of calcium ions into the cell, thereby inhibiting the transmission of appropriate signals between ORs and the brain, resulting in either hyposmia, the reduction of olfaction, or anosmia, the complete loss of olfaction.[104] It has been found that olfactory mediated behaviors of fish are potentially more sensitive to Cu nanoparticles than $CuSO_4$. Furthermore, nanoparticles elicited effects by a mechanism distinct from that of the metal salt.[105]

A study by Graham George and coworkers of the effects of mercury exposure in the olfactory pits of zebrafish larvae using a combination of X-ray fluorescence imaging and immunohisto-chemistry showed that mercury accumulates in the sensory cells of the olfactory pits and also that it may also damage primary neurons, such as those that innervate olfactory pits.[106] Similarly, a study of the effect of exposure of salmon to environmentally relevant concentrations of waterborne cadmium revealed injury to the OSNs along with behavioral dysfunction, which was associated with significant Cd bioaccumulation within the OE. The authors observed that low-level Cd exposures from polluted waterways can induce “differential and persistent olfactory dysfunction in juvenile coho salmon.”[107] The effect of silver nanoparticles on zebrafish embryos and larvae has also been studied with the

results indicating that responding tissues included olfactory bulbs with the potential for effects on olfaction.[108] Silver nanoparticle olfactory toxicity is believed to be due to a combination of silver particles and released silver ions.[109]

9 Device-based chemical sensors modeled after olfactory receptors

Suslick observed that “many of the most toxic and certainly the most odiferous compounds … are excellent ligands for metal ions” and that many of the olfactory receptor proteins are likely to contain metal ions at their active site. He suggested that in the design of a colorimetric array detector for vapor-phase ligands (an “optoelectronic nose”), “metalloporphyrins are a natural choice for the detection of metal ligating vapors because of their open coordination sites for axial ligation, their large spectral shifts upon ligand binding, and their intense coloration.”[110] Recently, a chip with gold nanoparticles in a paper sensor was constructed to detect glutathione, the principle thiol in human skin, at levels of 11.6–47.5 mM.[111] More detailed discussion of sensors is beyond the scope of this review.

10 Conclusion

Only a few mammalian ORs have thus far emerged whose response to odorants is significantly enhanced by ionic and nanoparticulate metals. However, it is reasonable to assume that additional examples will be identified during the OR deorphanization process. Researchers are encouraged to include individual metal ions and metal nanoparticles, as well as mixtures of different metals, in their screening protocols, and expand the screening to diverse species. The inclusion during screening of a metal chelator, such as TEPA, would be useful to identify enhancement of response due to trace levels of metal ions present in the culture medium. It would also be worthwhile to screen various non-sulfur-containing, strong-smelling compounds, which are excellent metal ligands. Finally, researchers seeking to crystallize ORs might well consider selecting a metal-responsive OR with a metal ion occupying the active site.

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Abbreviations

ACIII	adenylyl cyclase type III
cAMP	cyclic adenosine monophosphate
CN	cranial nerve
CNG	cyclic nucleotide-gated
CNS	central nervous system
EXAFS	extended X-ray absorption fine structure
DFT	density functional theory
GC	gas chromtography
GPCR	G-protein-coupled receptor
HEK	human embryonic kidney
KFO	key food odor
LPG	liquified petroleum gas
M2	M2 human muscarinic receptor
MOE	main olfactory epithelium
MTMT	(methylthio)methanethiol
OB	olfactory bulb
OBP	odorant-binding protein
ODE	odorant-degrading enzyme
OE	olfactory epithelium
OMP	olfactory marker protein
ONIOM	our own n-layered integrated molecular orbital and molecular mechanics
OR	olfactory receptor
OSN	olfactory sensory neuron
cOSN	cilitated olfactory sensory neuron
mOSN	microvillous olfactory sensory neuron
PPB	parts per billion
QM/MM	quantum mechanics/molecular mechanics
SEM	standard error of the mean

SPME	solid phase microextraction
STD-NMR	saturation transfer difference nuclear magnetic resonance
TAAR	trace amine-associated receptors
TBM	*tert*-butyl mercaptan (2-methyl-2-propanethiol)
TEPA	tetraethylenepentamine
TM	transmembrane
TRPA1	transient receptor potential A1
TRPV1	transient receptor potential V1
VNO	vomeronasal organ
VSC	volatile sulfur compounds
VTO	vibration theory of olfaction

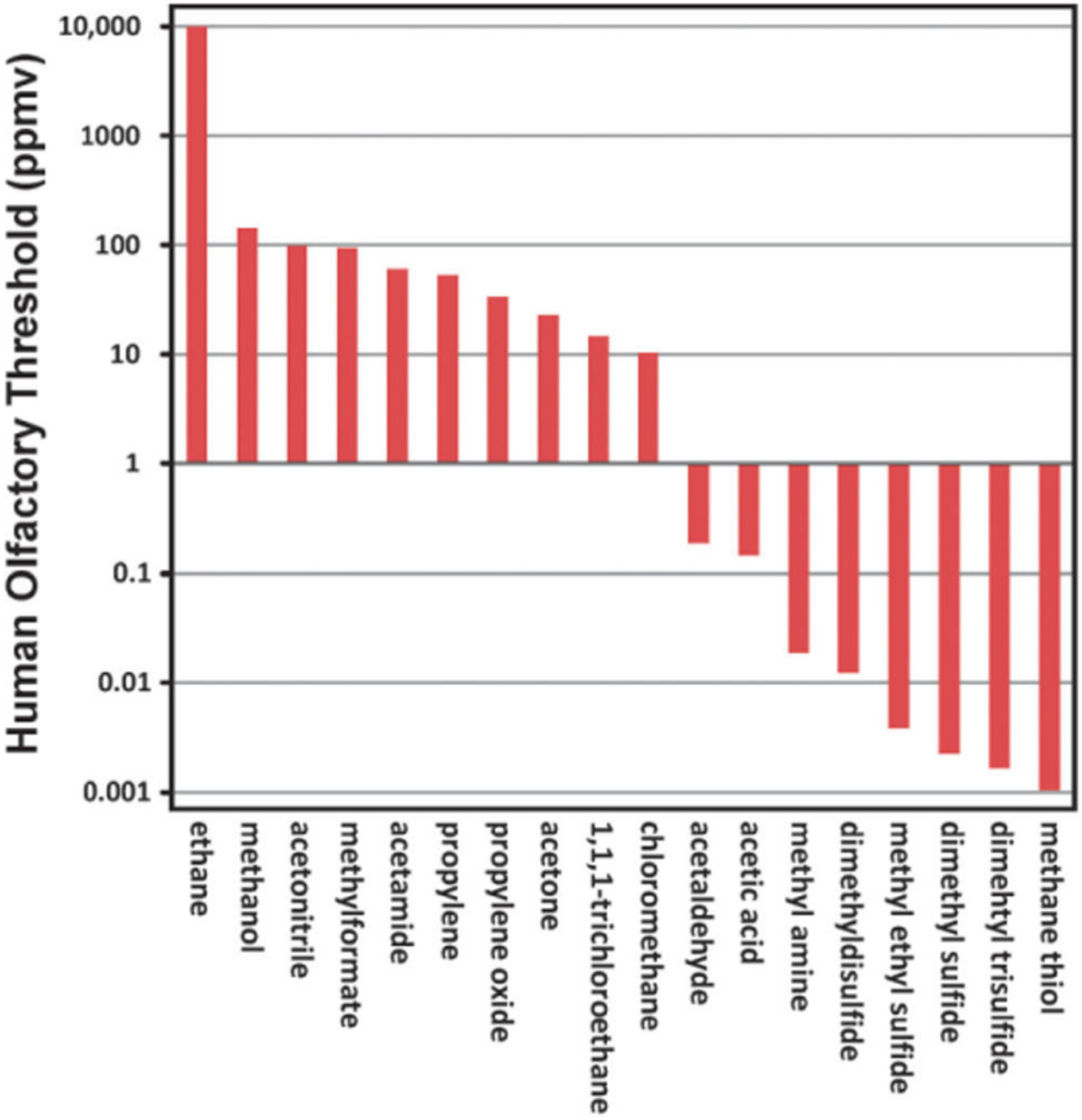


Fig. 1.

Human olfactory thresholds for detection of a series of comparable molecules with the structure CH_3–X.[5a]

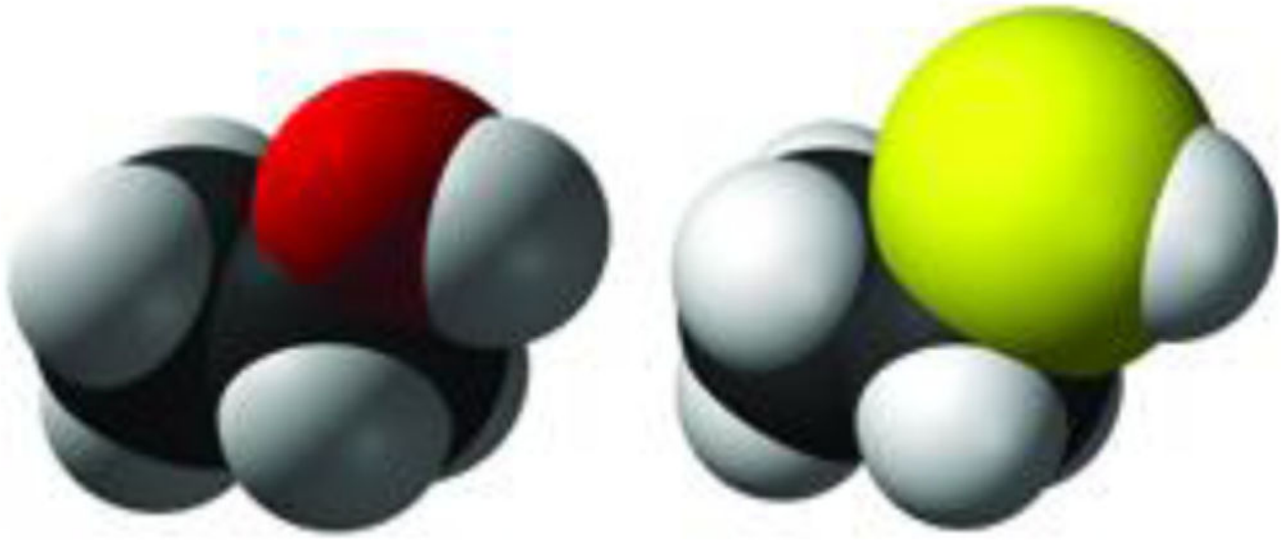


Fig. 2.
Space-filling models of ethanol (left) and ethanethiol (right) (from Wikipedia).

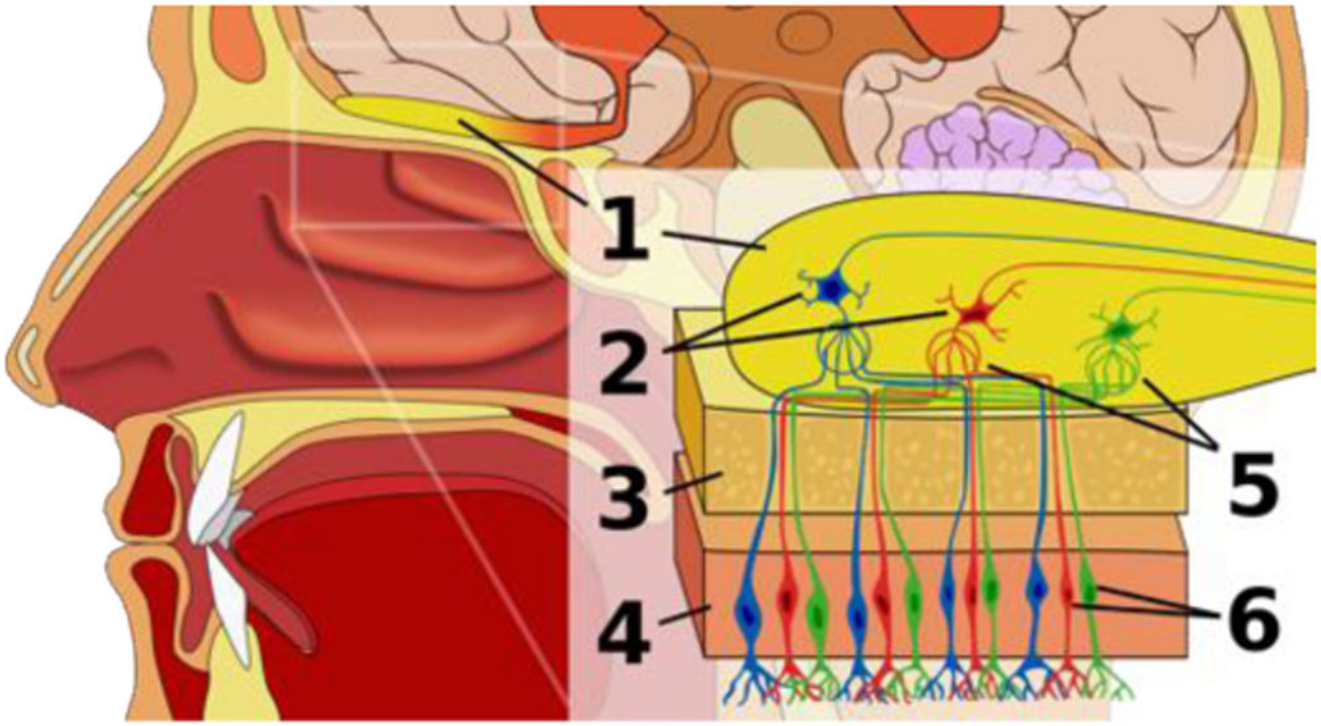


Fig. 3.

Human olfactory system. 1: Olfactory bulb 2: Mitral cells 3: Bone 4: Nasal epithelium 5: Glomerulus 6: Olfactory sensory neurons (OSNs) terminating in olfactory receptors (ORs) on cilia, bathed in nasal mucus [by Patrick J. Lynch, medical illustrator; from Wikipedia: Olfaction].

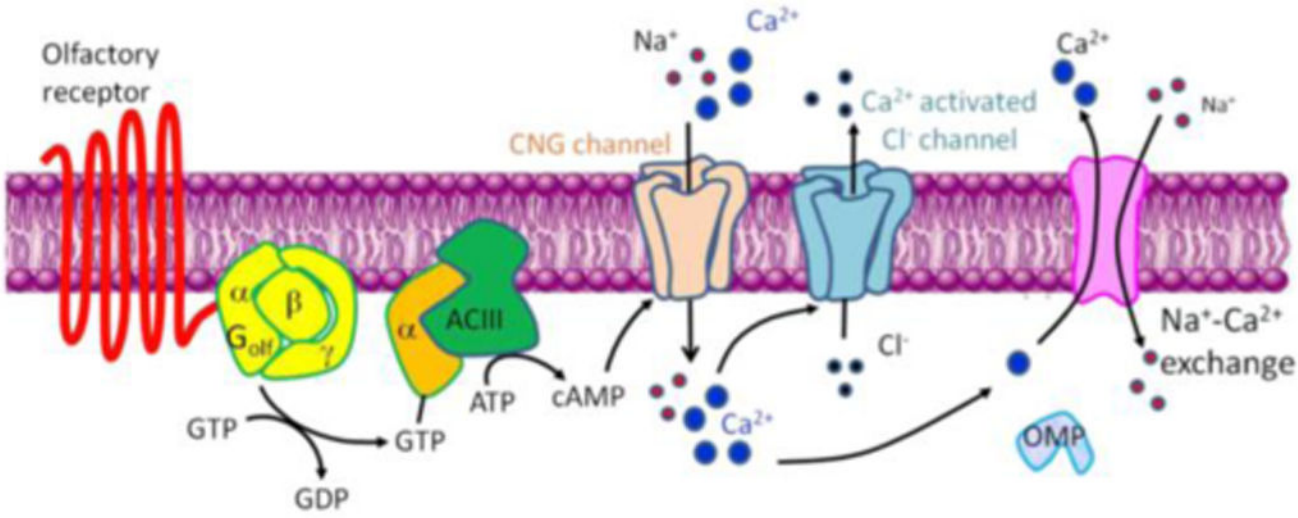


Fig. 4.

A schematic diagram of olfactory signal transduction. Olfactory signal transduction begins with the activation of an olfactory receptor (OR) in the ciliary membrane; this leads to an increase in cyclic AMP (cAMP) synthesis through the activation of adenylyl cyclase type III (ACIII) enzyme via a G protein (G_{olf})-coupled cascade. The increase in cAMP concentration causes cyclic nucleotide-gated (CNG) ion channels to open, leading to an increase in intracellular Ca^{2+} concentration and depolarization of the cell membrane by the Ca^{2+}-activated Cl^{-} channel. Among several molecules of the olfactory signal transduction, OR, olfactory marker protein (OMP), G_{olf} protein α-subunit ($G\alpha_{olf}$), and ACIII have known to be olfactory specific molecules. [Reproduced from Kang, 2012][9]

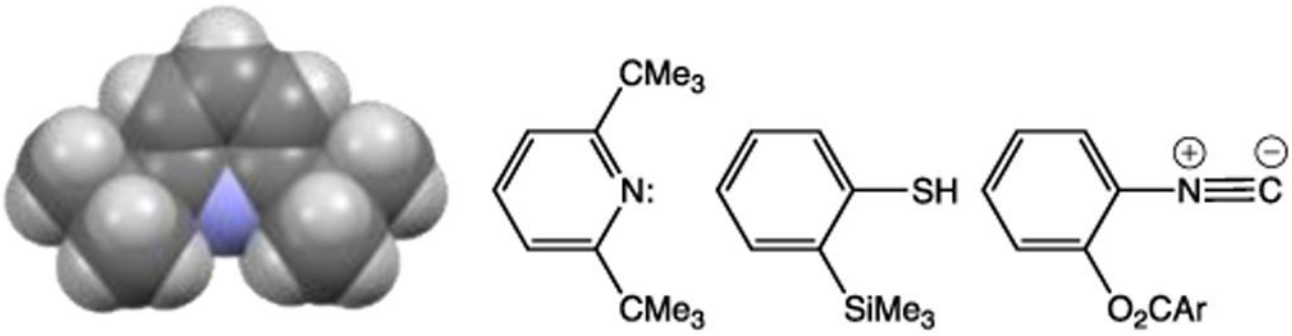


Fig. 5.

Steric hindrance can eliminate odor: 2,6-di-*tert*-butyl-pyridine (with space-filling drawing [Ben Mills, *Wikipedia*], 2-(trimethylsilyl)thiophenol and *ortho*-substituted benzeneisonitrile.

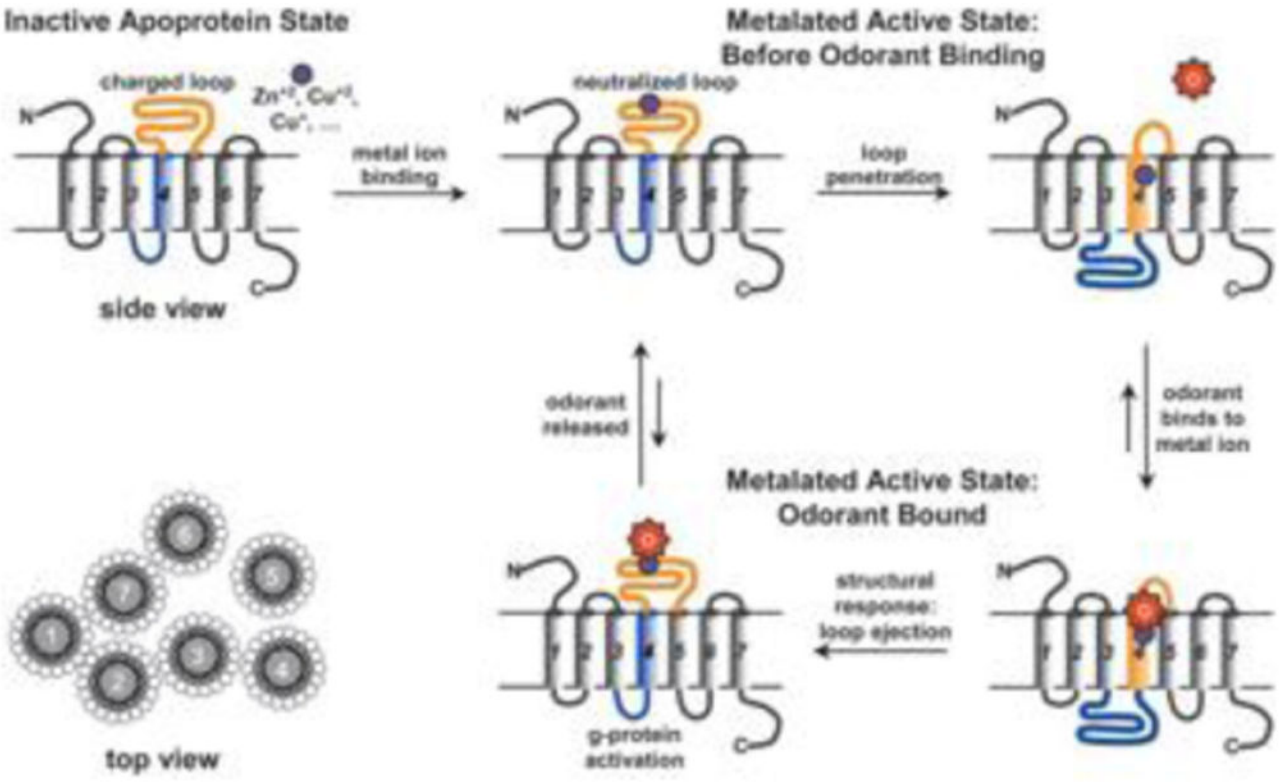


Fig. 6.

The proposed mechanism of olfactory receptor response via a shuttlecock mechanism. In the absence of an odorant, the metal binding site is in a helical conformation. Upon odorant binding, the primary response is helix ejection (lower center).[50] Copyright (2003), National Academy of Sciences.

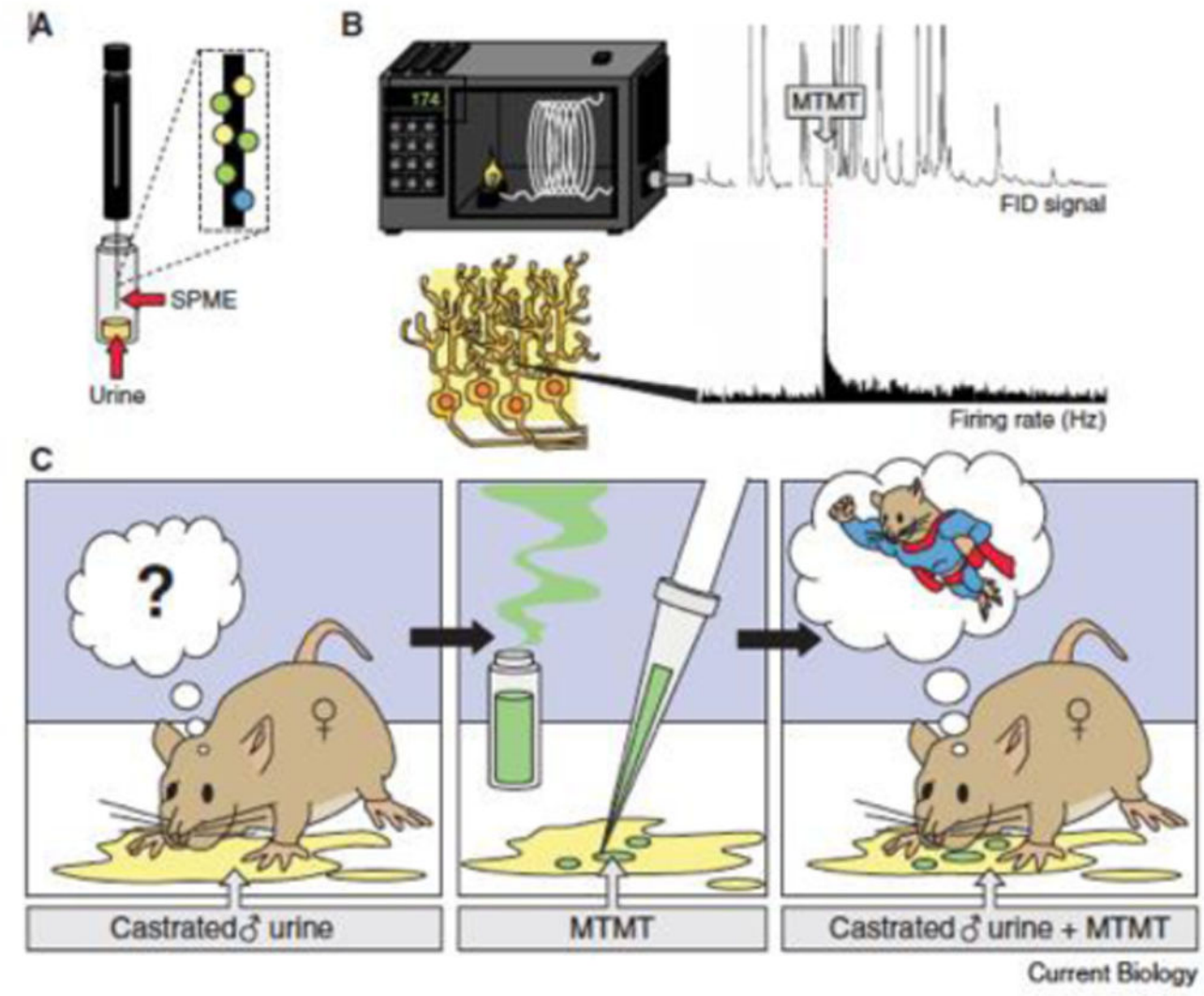


Fig. 7.

Cartoon of the experimental approach used to identify MTMT as a mouse semiochemical[58]

[From Current Biology, with permission: *Current Biology*, **2005**, *15*(7), R255-257.]

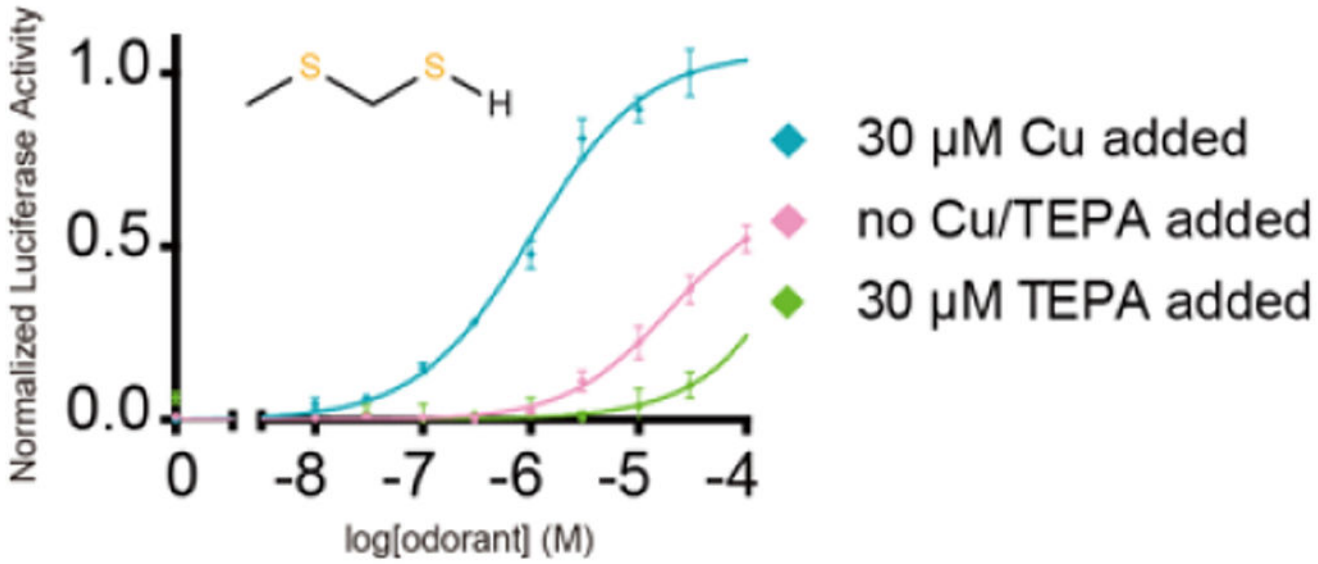


Fig. 8.

Dose-response curves of MOR244-3 to MTMT with and without 30 μM exogenous copper ion. The horizontal scale shows the exponent of the odorant molar concentration (e.g., −6 = 10^{-6} M) while the vertical axis shows the normalized luciferase activity, an indirect measure of the response of the receptor to substrates. A dose–response curve with 30 μM of TEPA is also shown. An F-test was used to compare the dose–response curves with or without copper ion, with the results showing significant p-values after Bonferroni corrections. Adapted with permission;[23a] Copyright 2012, PNAS.

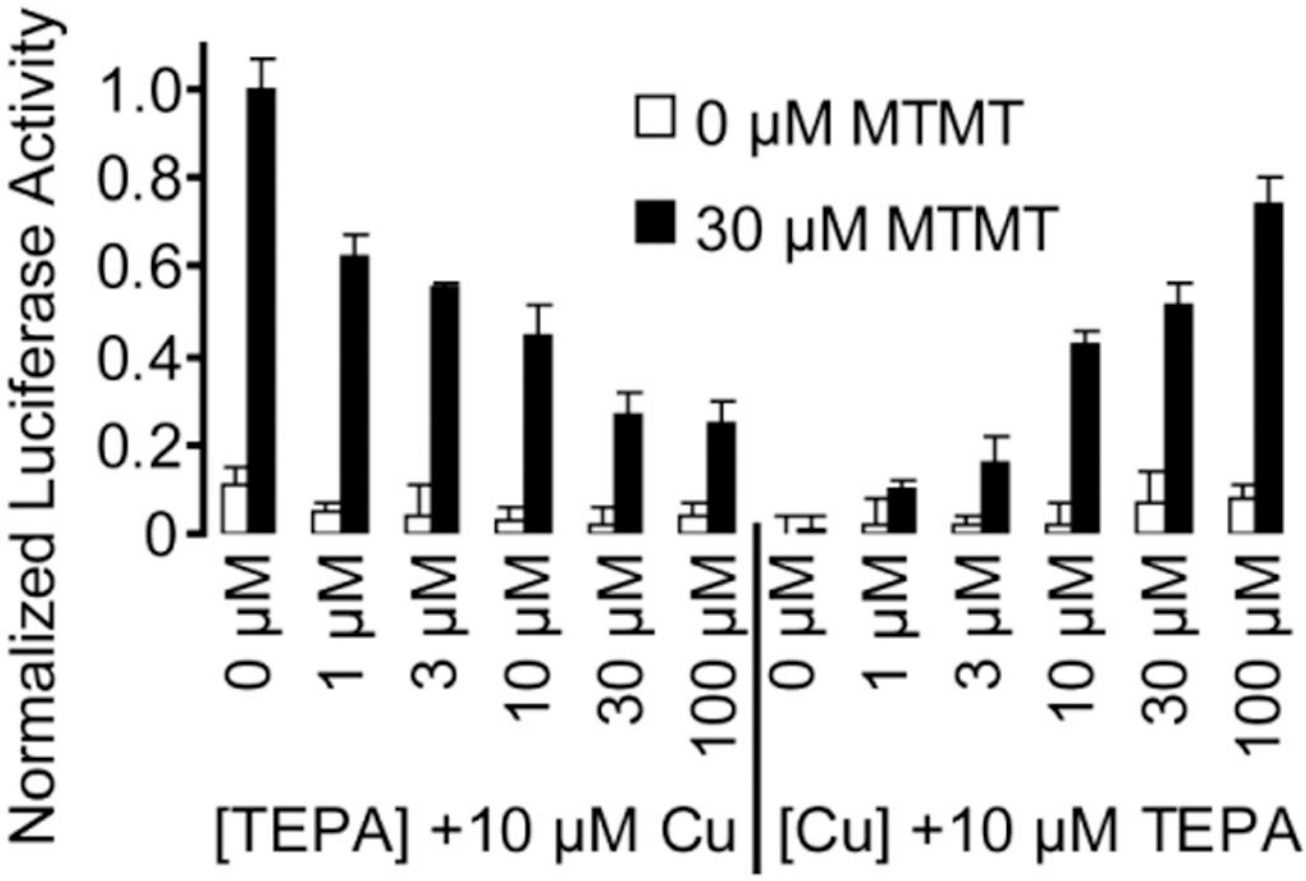


Fig. 9.

Responses of MOR244-3 to 0 and 30 μM MTMT with 10 μM Cu^{2+} or TEPA and increasing the amount of TEPA or Cu^{2+}, respectively. Responses are normalized to the highest response to MTMT. Adapted with permission;[23a] Copyright 2012, PNAS.

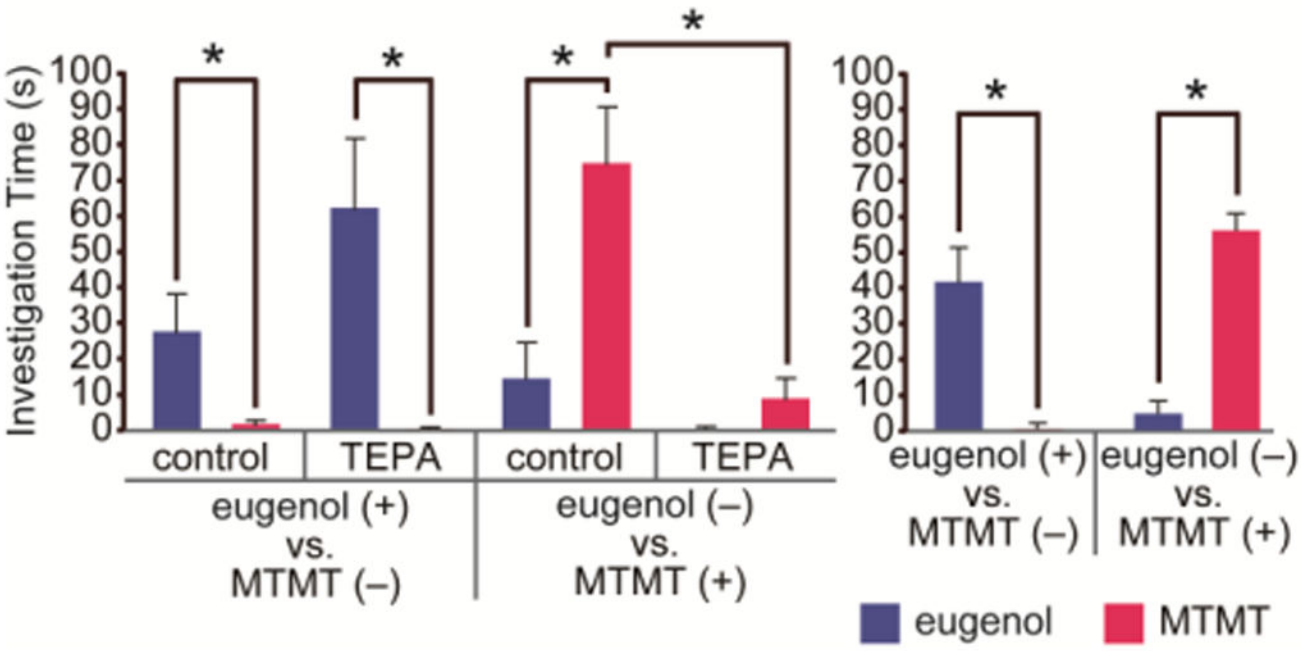


Fig. 10.

Mice were trained to associate either eugenol or MTMT with sugar reward. On the test day, mice were injected with distilled water 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 two days 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 ± SEM. Paired t test was used to compare the investigation times between groups. *$P < 0.05$ ($n = 4$). Adapted with permission;[23a] Copyright 2012, PNAS.

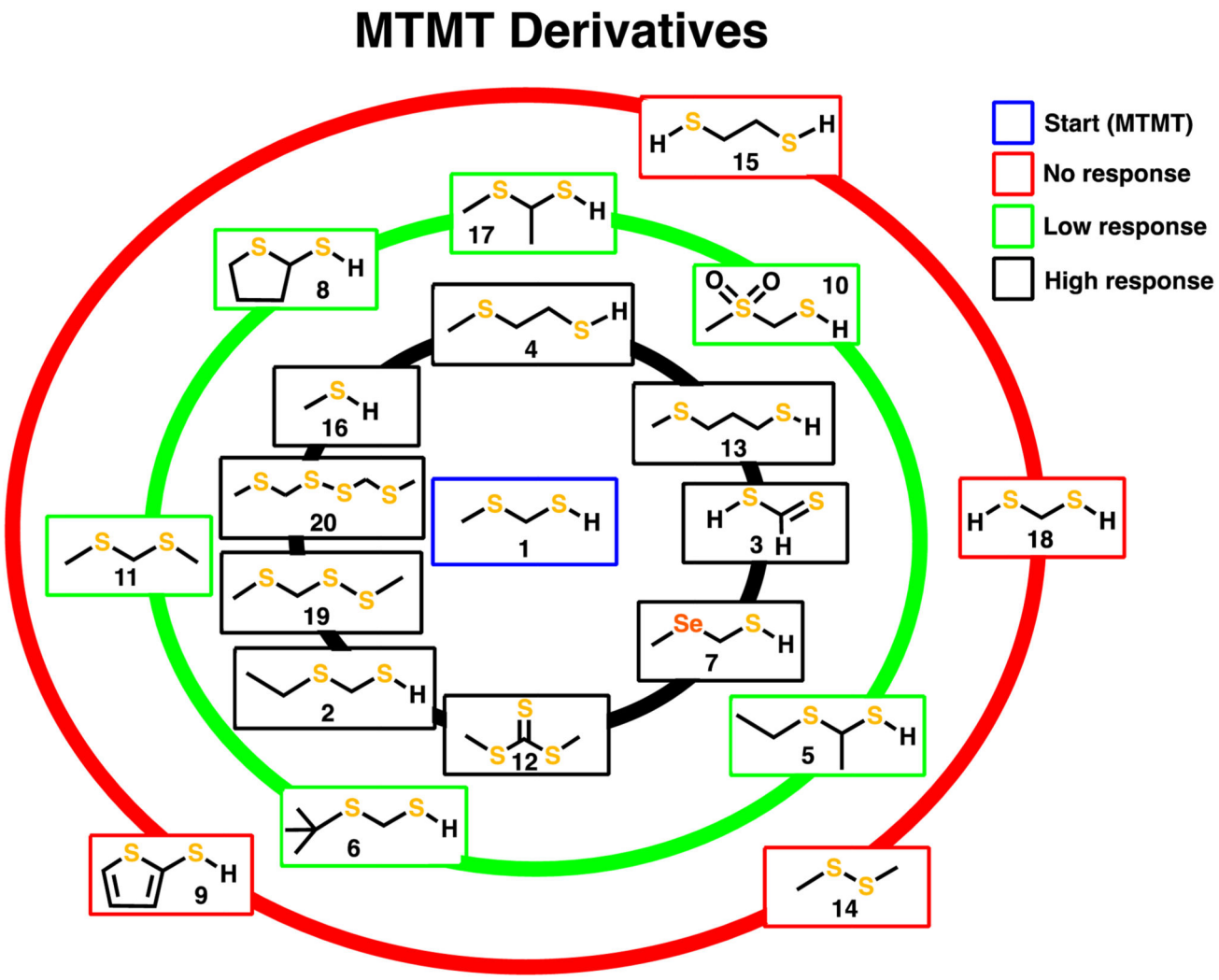


Fig. 11.
Schematic representation of response profiles of the MTMT derivatives.

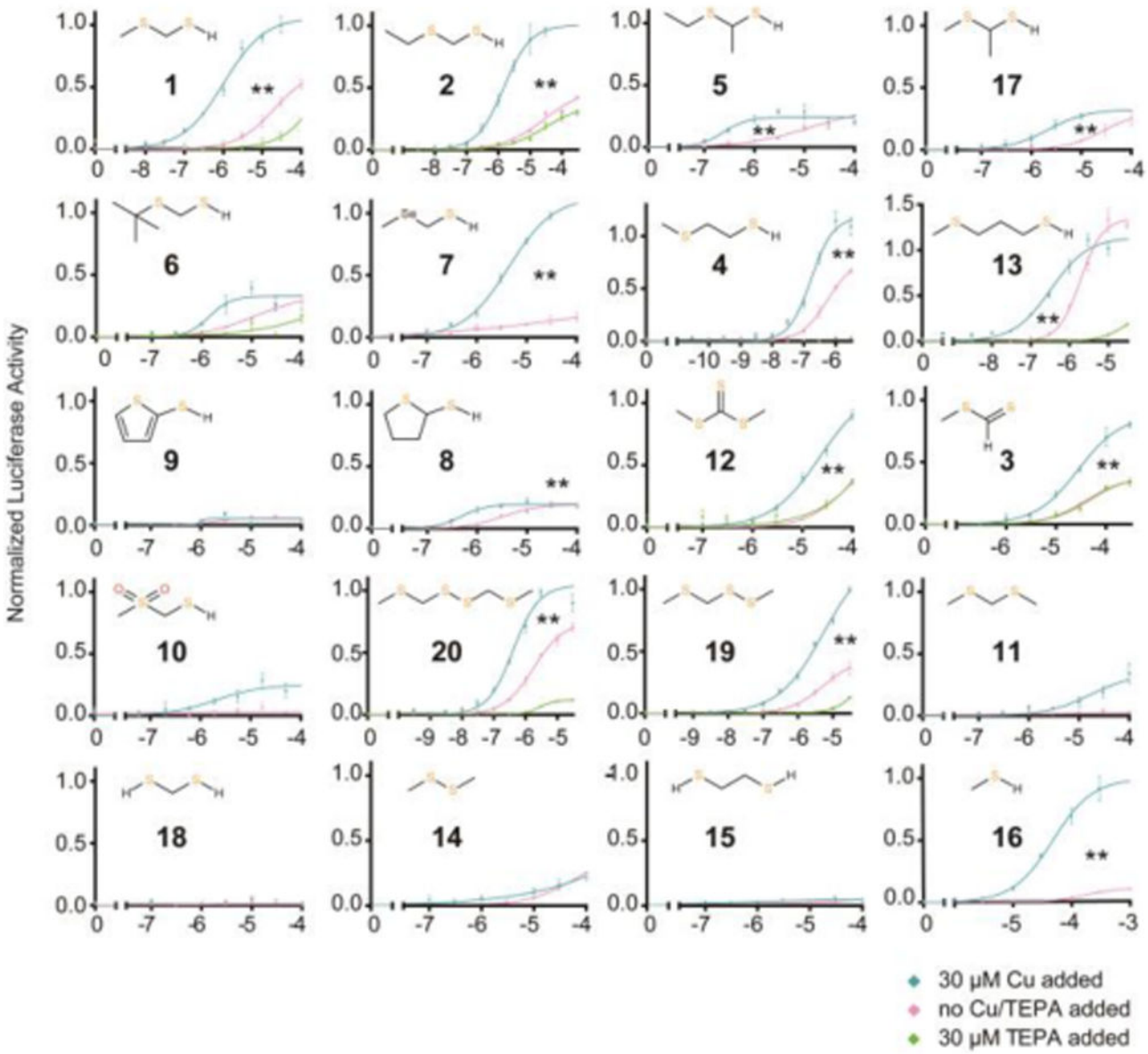


Fig. 12.

Dose–response curves of MOR244-3 to selected 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. F tests were used to compare the pairs of dose–response curves with or without Cu^{2+}. Asterisks represent significance of P values after Bonferonni corrections. **P < 0.01 (n = 3). Adapted with permission;[23a] Copyright 2012, PNAS.

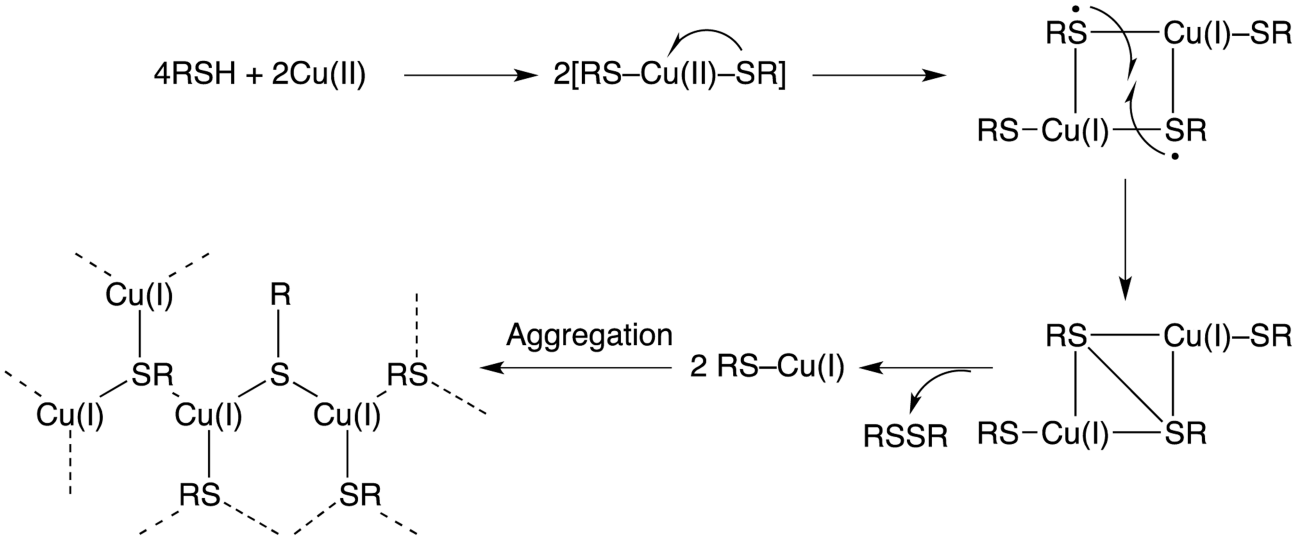


Fig. 13.

Proposed mechanism for the initial reaction of thiols with Cu(II) and Cu(I)–thiol complex formation. Only the thiol ligands are shown.[63b] Adapted from H. Yi, C. Song, Y. Li, C. W. Pao, J. F. Lee and A. Lei, *Chemistry: A European Journal*, 2016, **22**(51), 18331–18334. Copyright John Wiley and Sons, 2016.

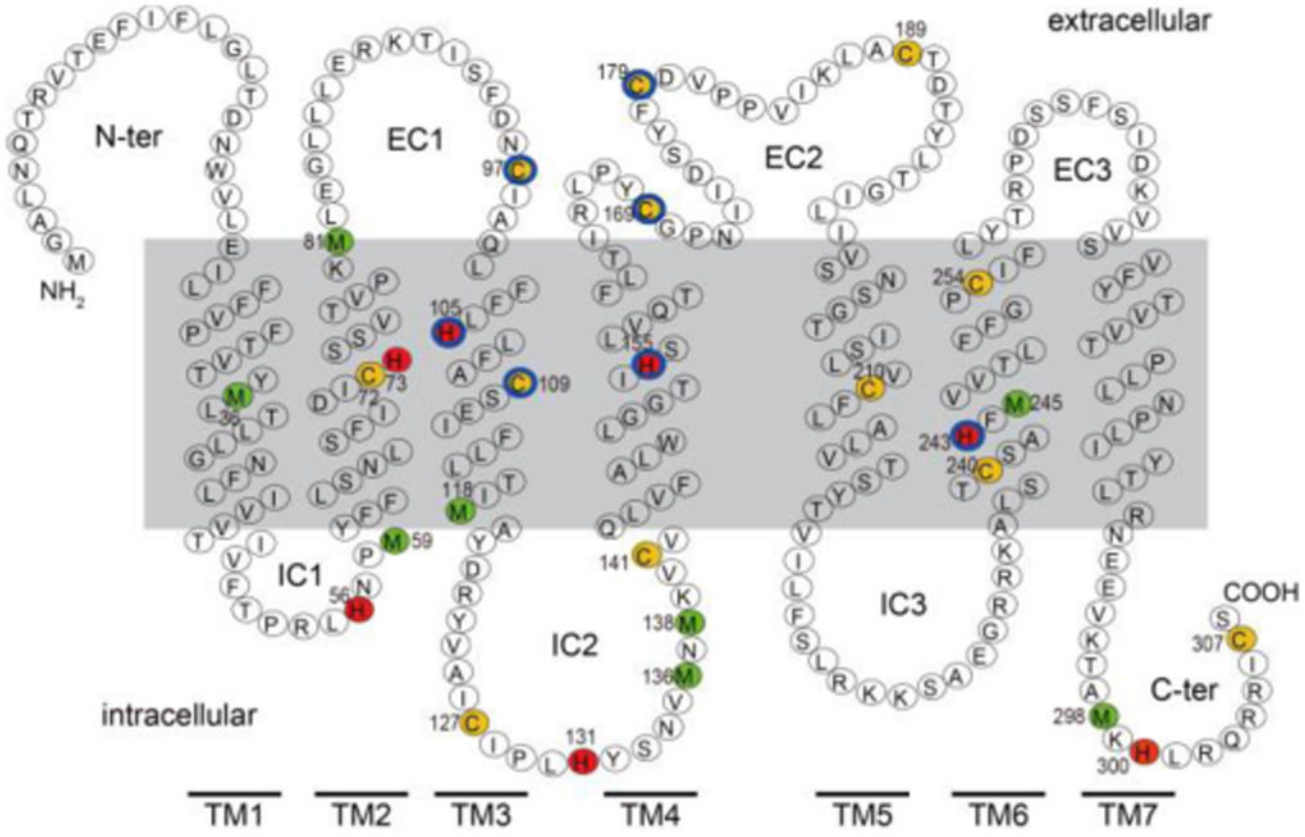


Fig. 14.

Candidate copper-binding sites in MOR244-3 as shown by single point mutations; H = histidine (red), C = cysteine (yellow), M = methionine (green). The grey rectangle represents the lipid bilayer. Not shown is a glycosylation site near the extracellular N-terminal region and putative disulfide bridges between the cysteines in the extracellular loops. Residues circled in blue are those that exhibited complete loss-of-function phenotypes in the luciferase assay. [23a] Adapted with permission;[23a] Copyright 2012, PNAS.

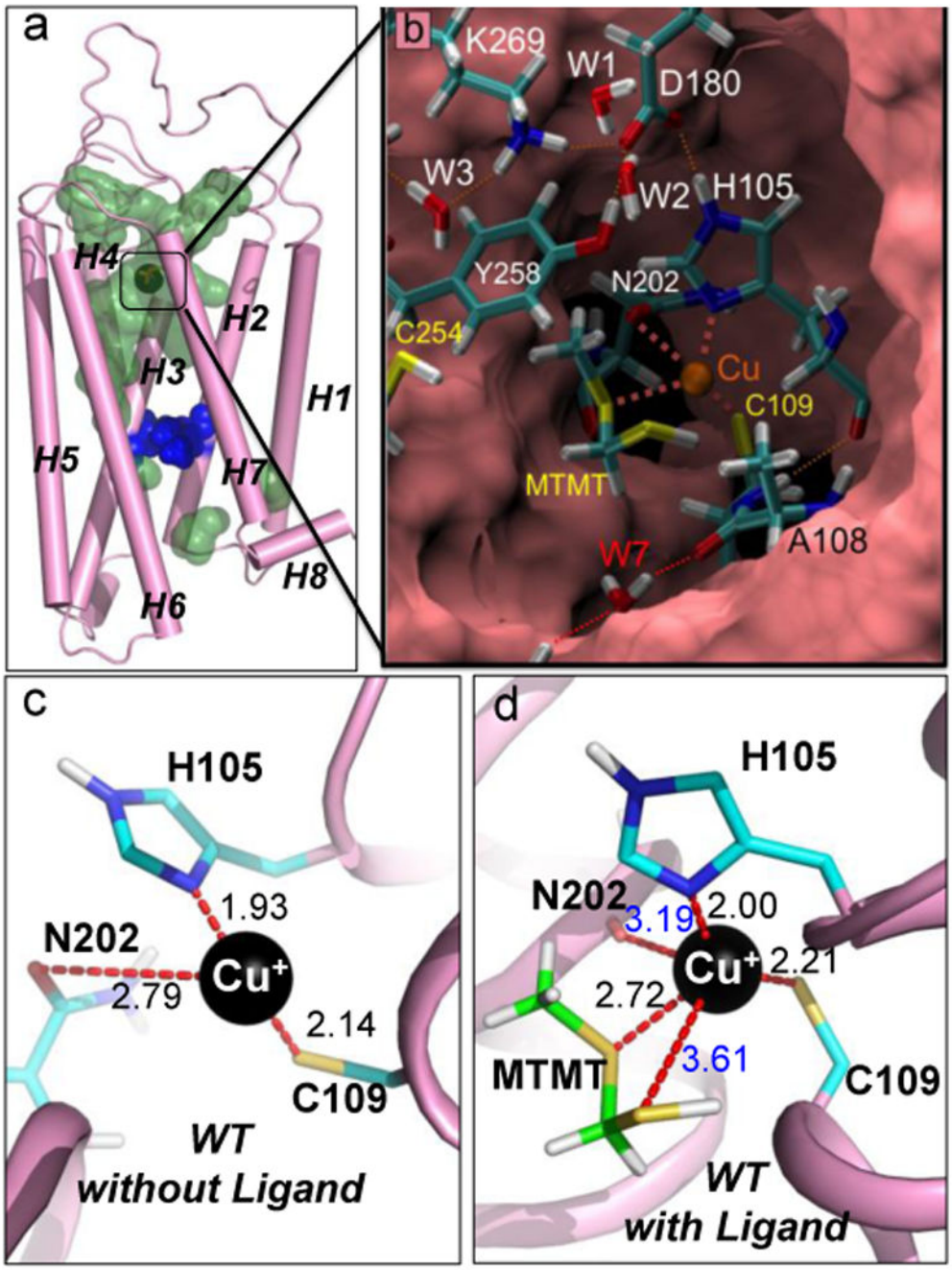


Fig. 15.

(Top) (a) QM/MM model of the MOR244-3, including an aqueous channel (green) inside the barrel of TM α-helices (pink). (b) MTMT bound to Cu^{+} coordinated to the heteroatoms of H105 and C109, and surrounded by a cage of H-bonds linking H105, D180, K269, Y258, and water molecules. (Bottom) The active site of MOR244-3 without (c) and with (d) the MTMT ligand.[40]

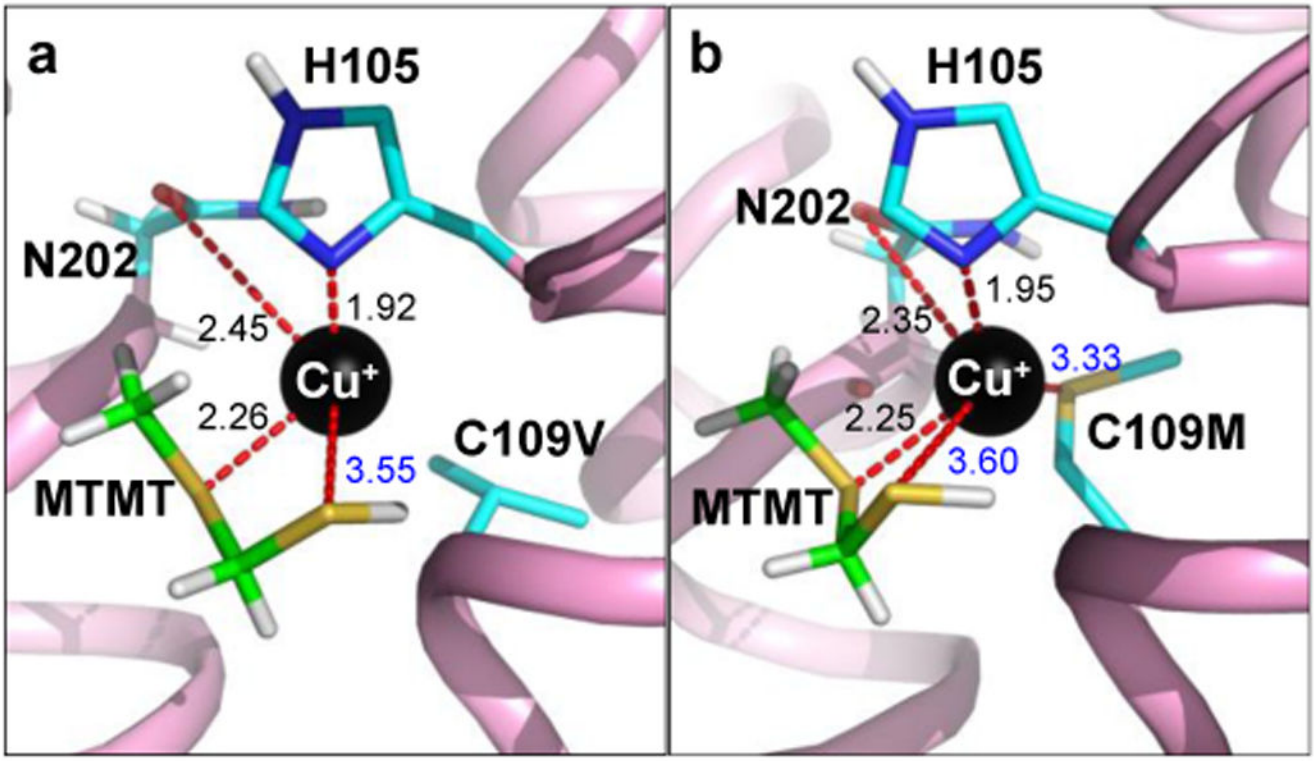


Fig. 16.

QM/MM optimized models of the MOR244-3 C109V (a) and C109M (b) mutants with MTMT bound to Cu^{+}. Coordination distances (dashed lines) are in Angstroms.[40]

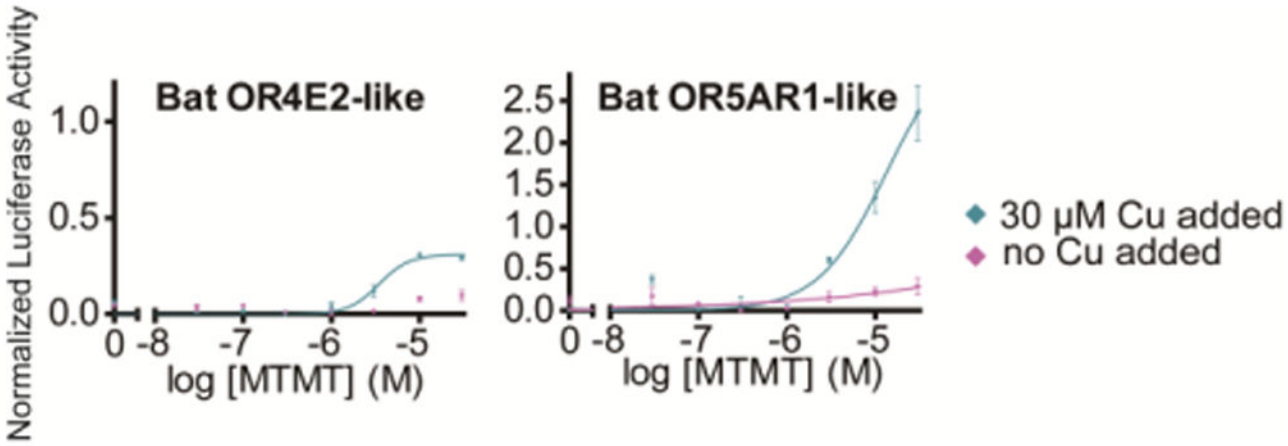


Fig. 17.

Copper enhances the activation of the ortholog of MOR244-3 in response to MTMT in *Myotis lucifugus* (the little brown bat).

Fig. 18.

On March 18, 1937 in New London, Texas, a school tragedy sparked the need for gas odorization (photo courtesy of Dr. T. J. Bruno).

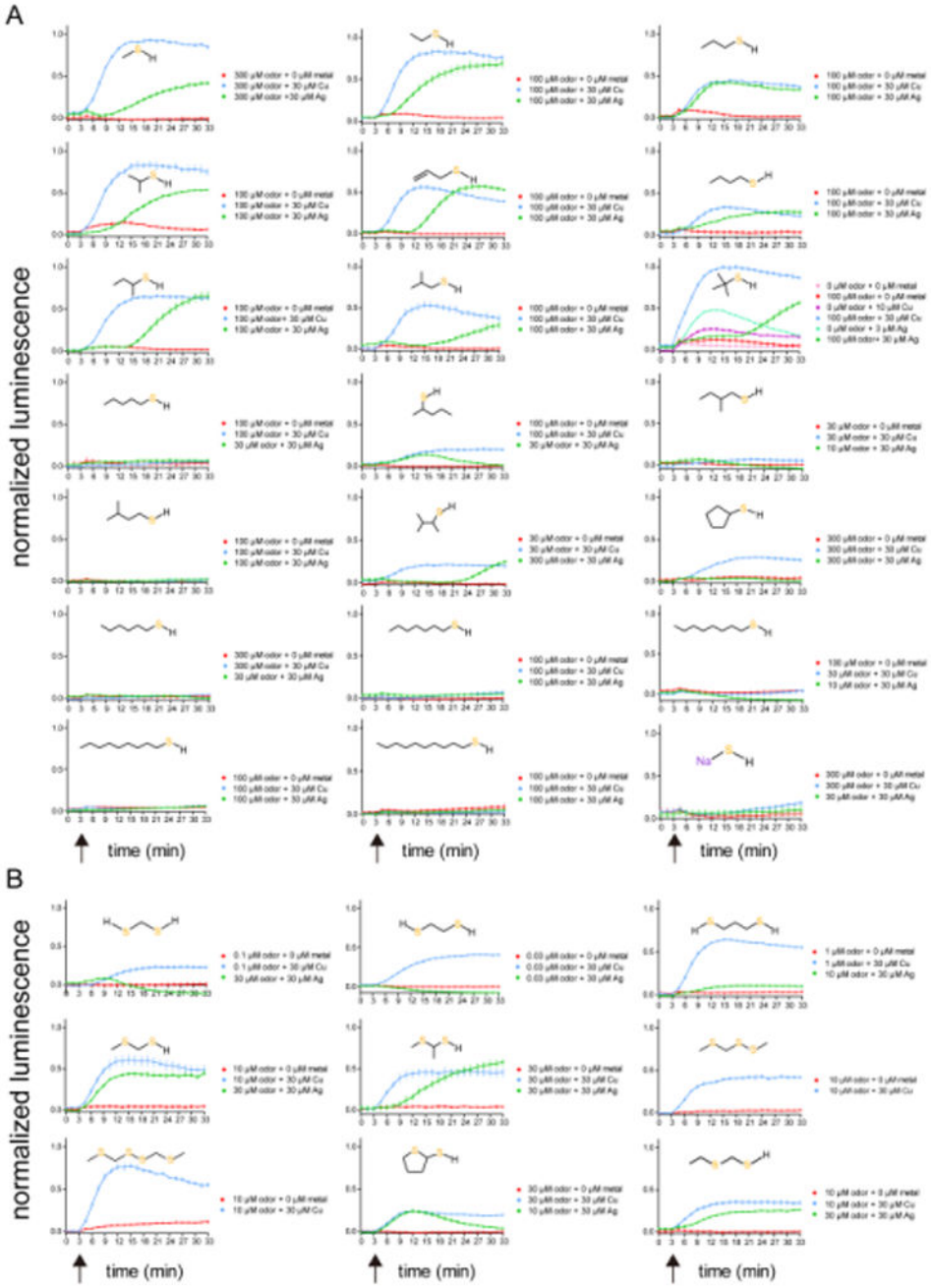


Fig. 19.

OR2T11 responds to selected thiol compounds in the GloSensor™ cAMP assay. Real-time measurement of OR2T11 activation in response to (A) monothiols and sodium hydrosulfide (at pH 6.14), and (B) dithiols and α-mercaptothioethers as detected within 30 min of odorant addition. Metals used in the assay were $CuCl_2$ and $AgNO_3$, except for methanethiol and sodium hydrosulfide, where colloidal silver was used. The arrow along the *x*-axis indicates the time point of odorant addition; *y*-axis indicates normalized luminescence±SEM ($N = 3$). All responses are normalized to the highest response of OR2T11 to TBM.[71] Reprinted with

permission from S. Li, L. Ahmed, R. Zhang, Y. Pan, H. Matsunami, J. L. Burger, E. Block, V. S. Batista and H. Zhuang. *J. Am. Chem. Soc.,* 2016, **138**, 13281–13288. Copyright 2016 American Chemical Society.

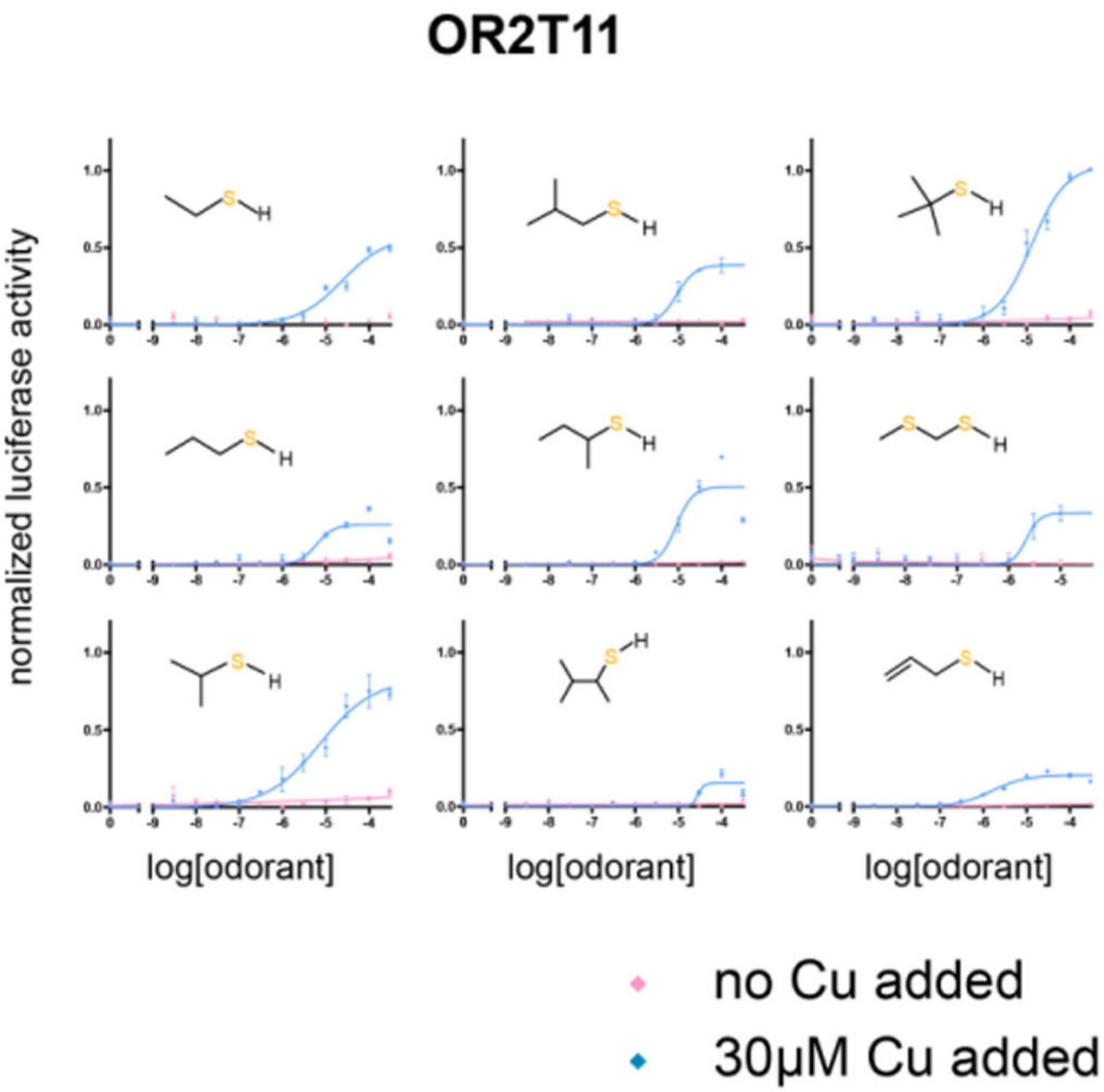


Fig. 20.

OR2T11 responds with a copper effect to unbranched and branched alkanethiols with 1-4 carbons, as well as MTMT and 2-propenethiol in the luciferase assay.[71] Reprinted with permission from S. Li, L. Ahmed, R. Zhang, Y. Pan, H. Matsunami, J. L. Burger, E. Block, V. S. Batista and H. Zhuang. *J. Am. Chem. Soc.,* 2016, **138**, 13281–13288. Copyright 2016 American Chemical Society.

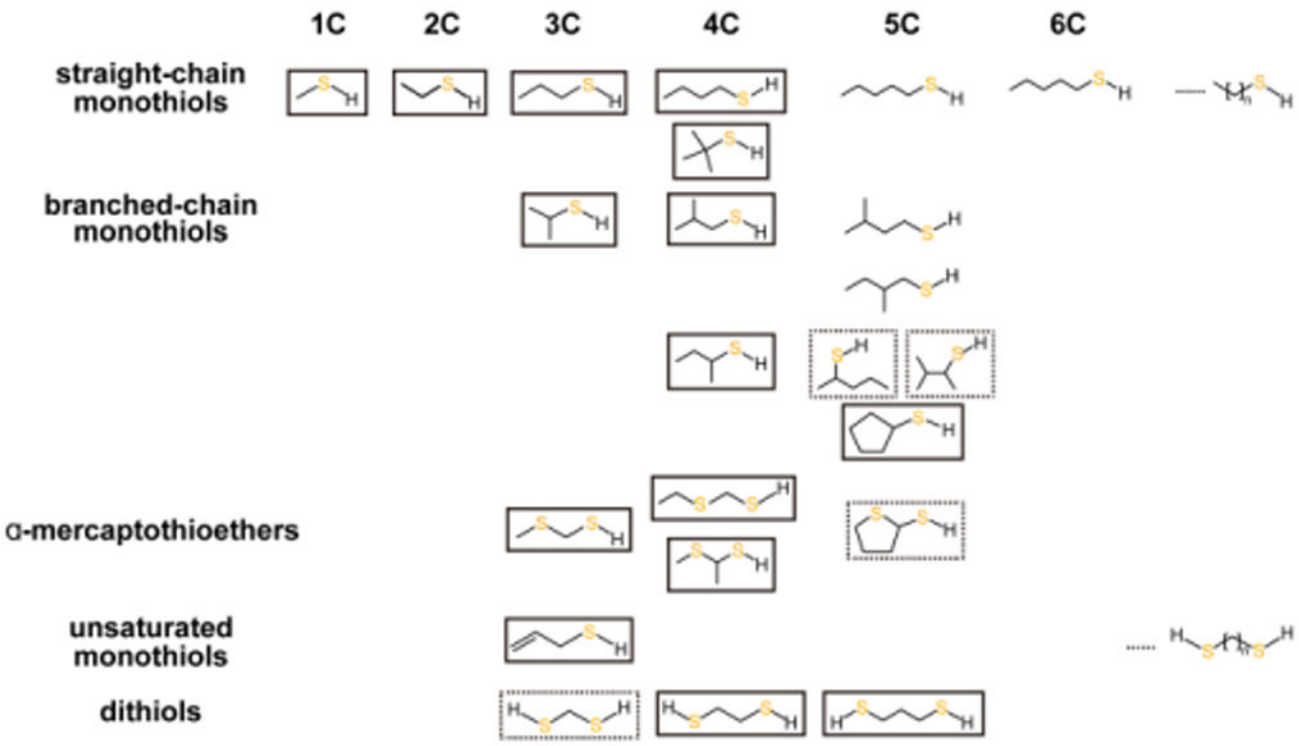


Fig. 21.

A schematic diagram of sulfur-containing compounds screened with OR2T11. Odors boxed with solid lines showed prominent responses in the presence of 30 μM Cu^{2+} and odors boxed with dashed lines showed less prominent responses, as defined by a more than 70% reduction in efficacy compared with TBM in the GloSensor™ cAMP assay. “1C” through “6C” refer to the number of the carbon atoms in the original straight-chain monothiol compounds. Straight-chain monothiols with 10 > C > 5 were tested and deemed inactive.[71] Reprinted with permission from S. Li, L. Ahmed, R. Zhang, Y. Pan, H. Matsunami, J. L. Burger, E. Block, V. S. Batista and H. Zhuang. *J. Am. Chem. Soc.,* 2016, **138**, 13281–13288. Copyright 2016 American Chemical Society.

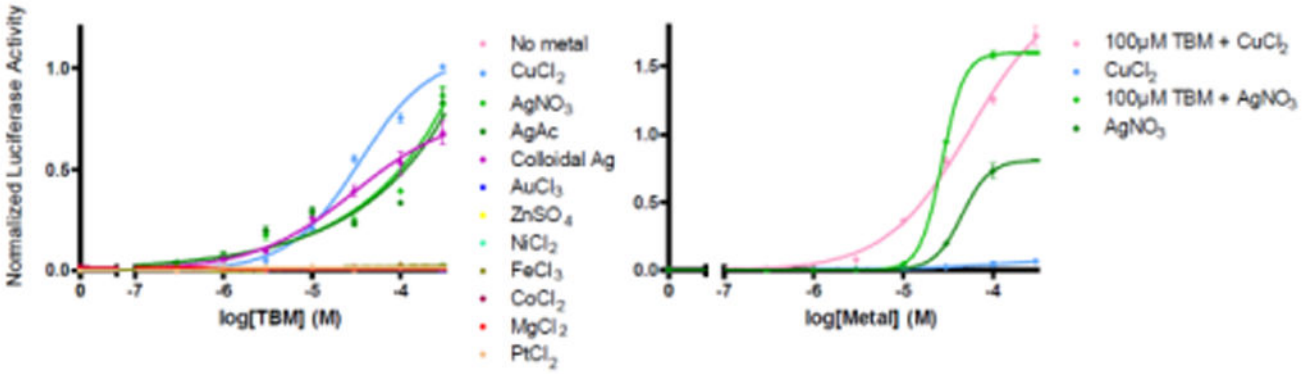


Fig. 22.

Human thiol receptor responds to *tert*-butyl mercaptan (TBM) with silver as well as copper effect; it also responds to silver but not copper without added thiol.[71] Reprinted with permission from S. Li, L. Ahmed, R. Zhang, Y. Pan, H. Matsunami, J. L. Burger, E. Block, V. S. Batista and H. Zhuang. *J. Am. Chem. Soc.,* 2016, **138**, 13281–13288. Copyright 2016 American Chemical Society.

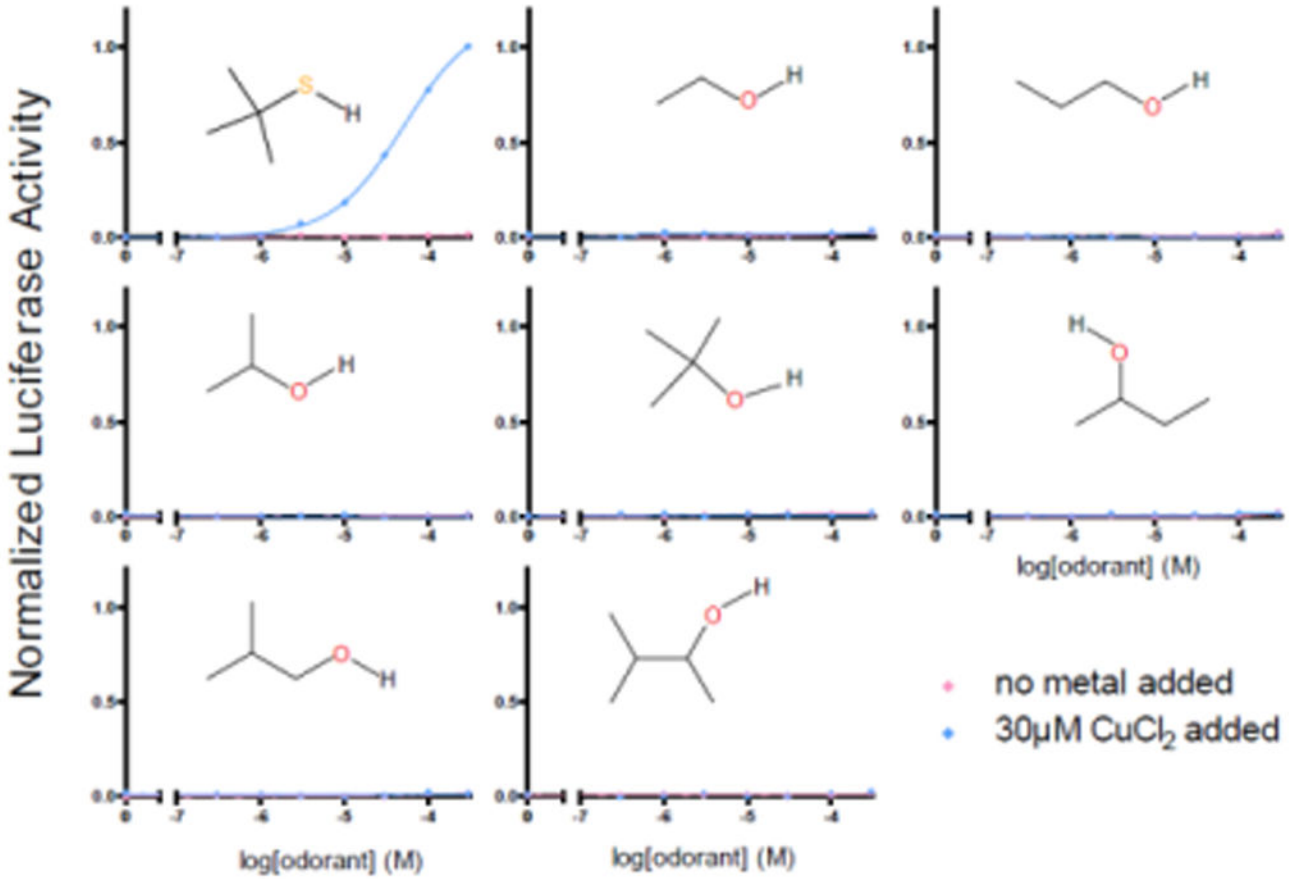


Fig. 23.

Dose-response curves for human OR2T11 and selected alcohols; y-axis indicates normalized response ± SEM (N = 3). Responses are normalized to the highest thiol response of each OR.[71] Reprinted with permission from S. Li, L. Ahmed, R. Zhang, Y. Pan, H. Matsunami, J. L. Burger, E. Block, V. S. Batista and H. Zhuang. *J. Am. Chem. Soc.,* 2016, **138**, 13281–13288. Copyright 2016 American Chemical Society.

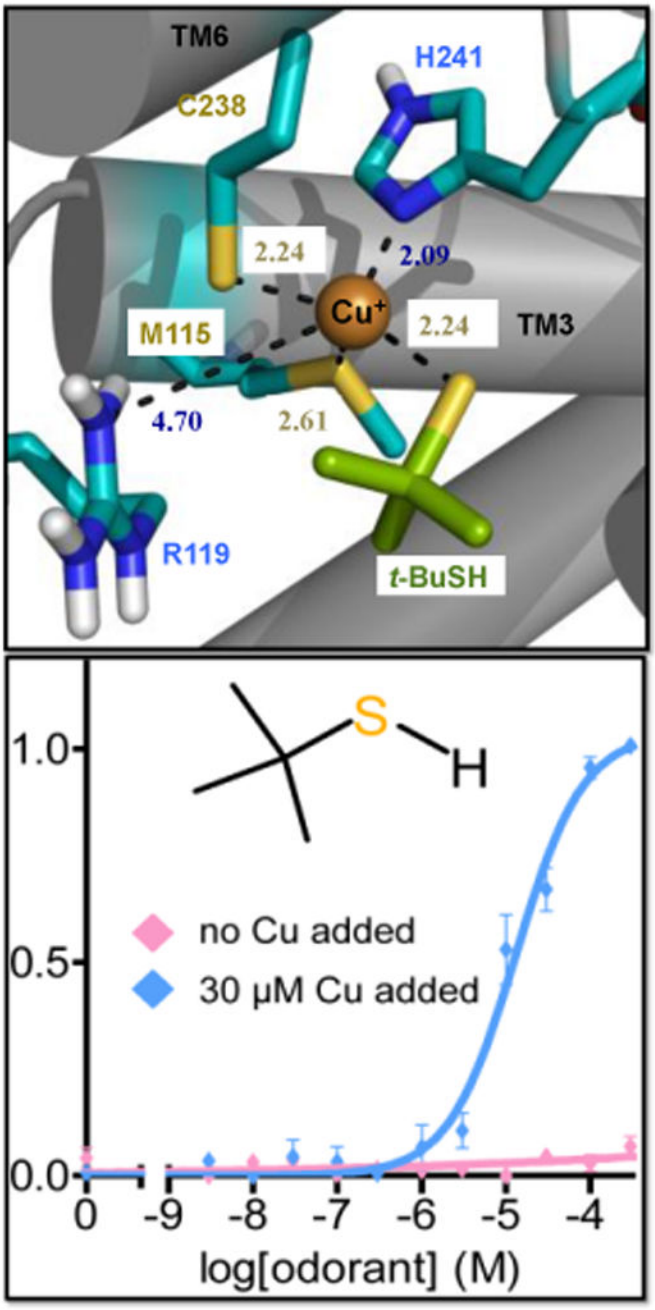


Fig. 24.

Human thiol receptor OR2T11: QM/MM modeling, showing binding of Cu^{+} to TBM and to C238, H241, R119 and M115.

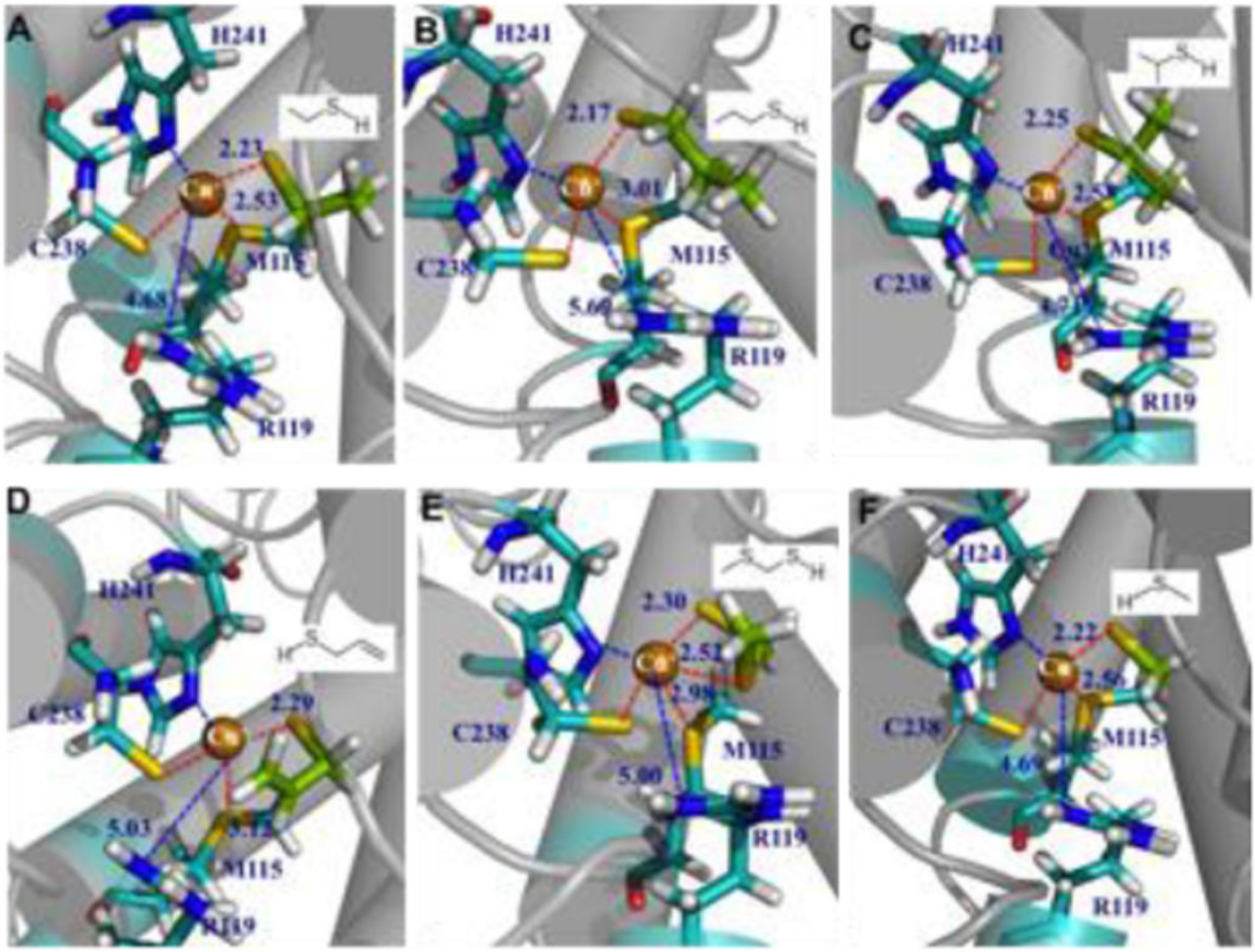


Fig. 25.

QM/MM optimized models of (A) EtSH, (B) *n*-PrSH, (C) *i*-PrSH, (D) 2-propenethiol, (E) (methylthio)methanethiol, and (F) methanethiol, all bound to the Cu^{+} ion in the OR2T11 site consisting of M115, C238 and H241. The cysteine and ligands are in the thiolate form.[71] Reprinted with permission from S. Li, L. Ahmed, R. Zhang, Y. Pan, H. Matsunami, J. L. Burger, E. Block, V. S. Batista and H. Zhuang. *J. Am. Chem. Soc.,* 2016, **138**, 13281–13288. Copyright 2016 American Chemical Society.

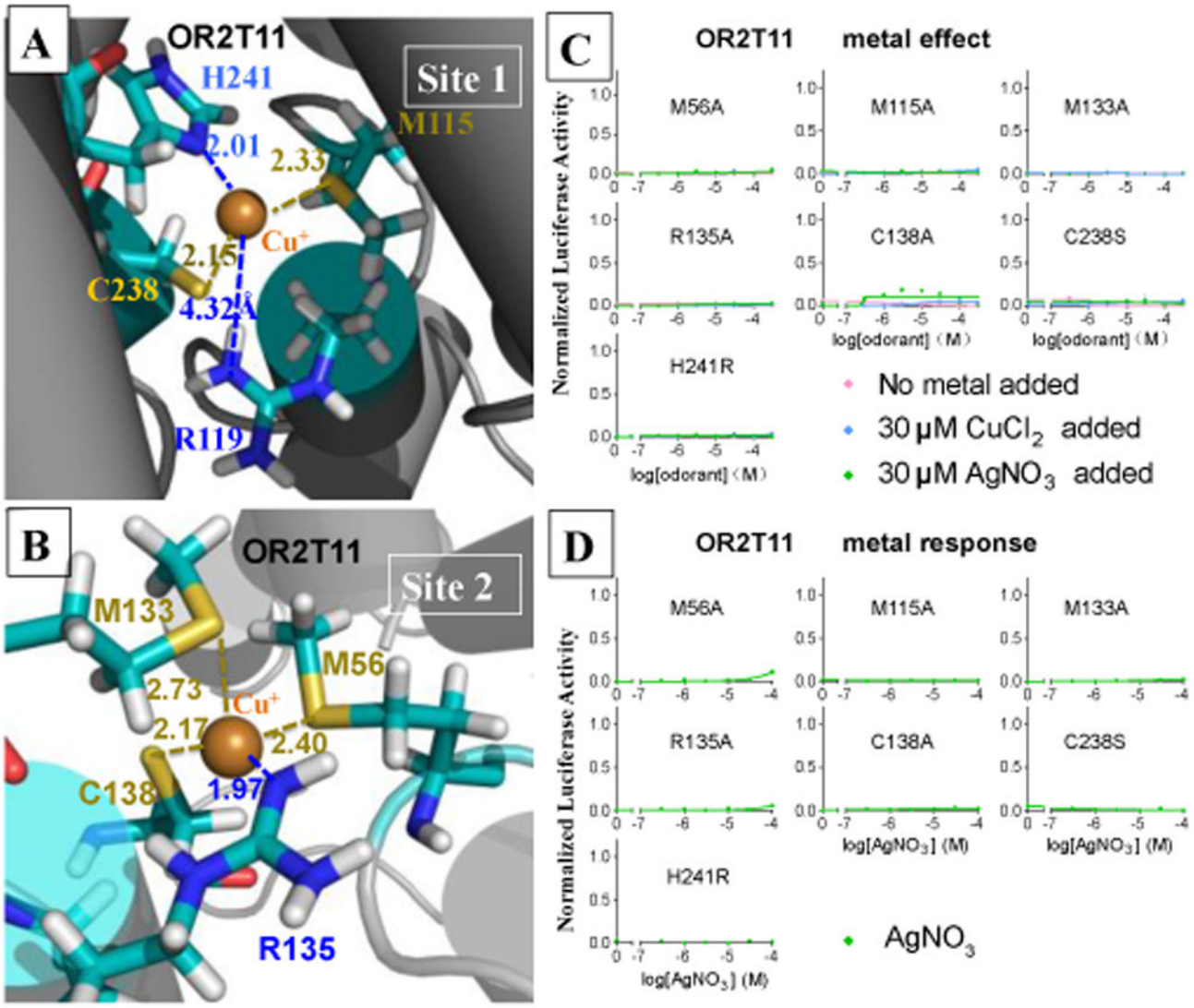


Fig. 26.

Binding sites of OR2T11. Panels (A) and (B) show the two binding sites of OR2T11 consisting of M115, C238 and H241, and M56, M133, R135 and C138, respectively; panels (C) and (D) show the mutagenesis studies on the corresponding amino acid residues in the binding site of OR2T11 (e.g., in (C), M56A indicates that methionine 56 has been mutated to alanine). The cysteine is in the thiolate form.[71] Reprinted with permission from S. Li, L. Ahmed, R. Zhang, Y. Pan, H. Matsunami, J. L. Burger, E. Block, V. S. Batista and H. Zhuang. *J. Am. Chem. Soc.,* 2016, **138**, 13281–13288. Copyright 2016 American Chemical Society.

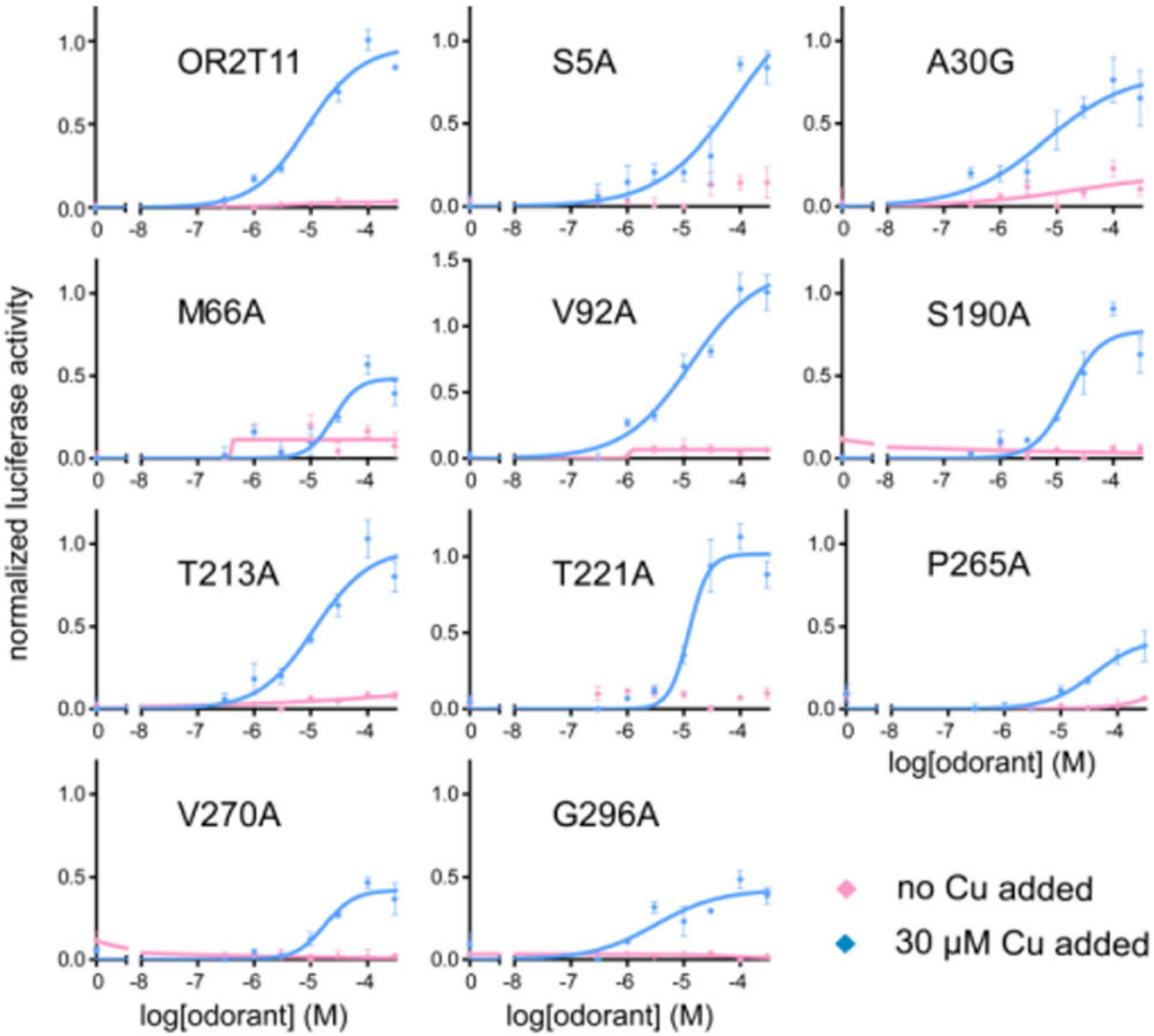


Fig. 27.

OR2T11 control mutants. Dose-response curves of OR2T11 control mutants to TBM. The *y*-axis indicates normalized response±SEM ($N = 3$). All responses are normalized to the highest response of wild type OR2T11 to TBM with 30 μM of Cu added.[71]

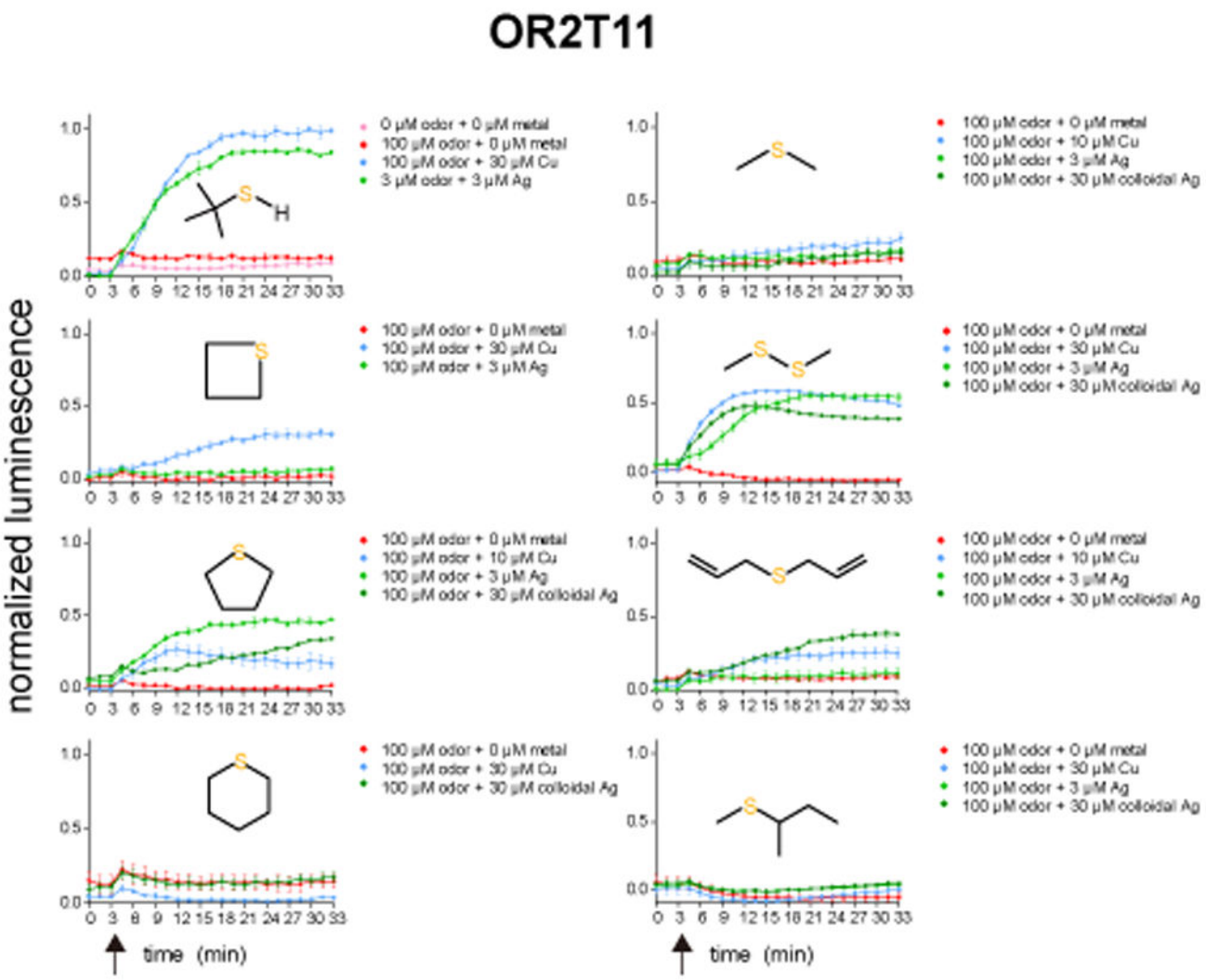


Fig. 28.

Response of sulfur compounds to OR2T11 in the presence or absence of copper and silver salts and colloidal silver in the GloSensor cAMP assay. Real-time measurement of OR2T11 activation is shown as detected within 30 min of odorant addition. The arrow indicates the time point of odorant addition. y-axis indicates normalized luminescence±SEM ($N = 3$). All responses are normalized to the highest response of OR2T11 to TBM.[71] Reprinted with permission from S. Li, L. Ahmed, R. Zhang, Y. Pan, H. Matsunami, J. L. Burger, E. Block, V. S. Batista and H. Zhuang. *J. Am. Chem. Soc.,* 2016, **138**, 13281–13288. Copyright 2016 American Chemical Society.

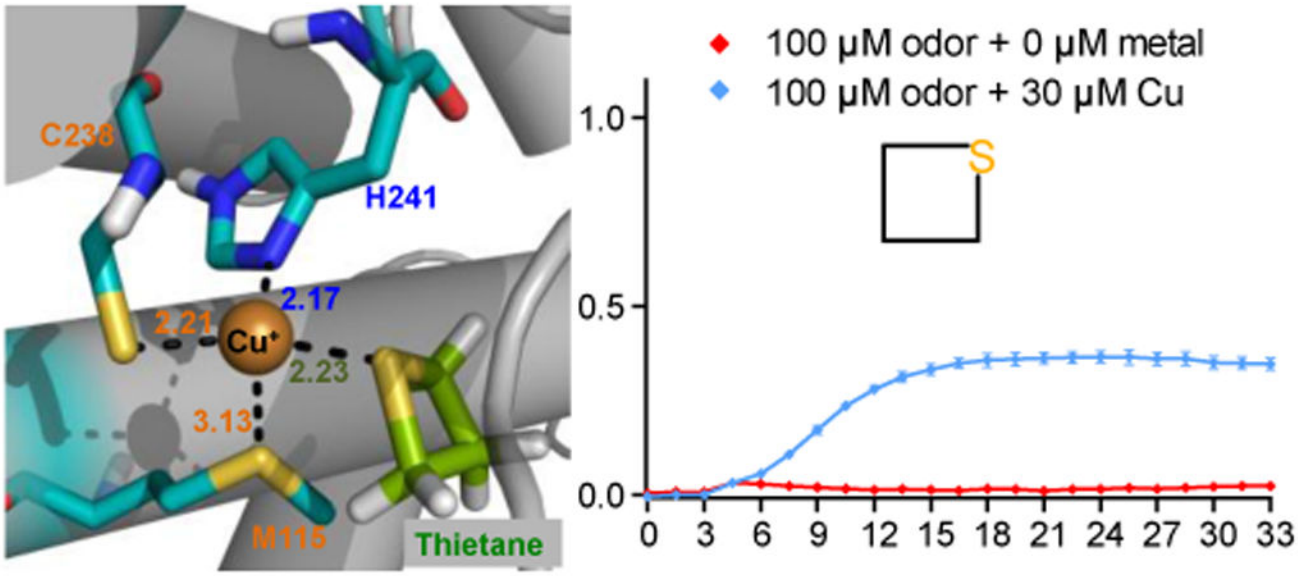


Fig. 29.

QM/MM optimized models, with indicated distances, of thietane bound to the Cu^{+} ion in the OR2T11 site consisting of M115, C238 and H241. The cysteine is in the thiolate form.

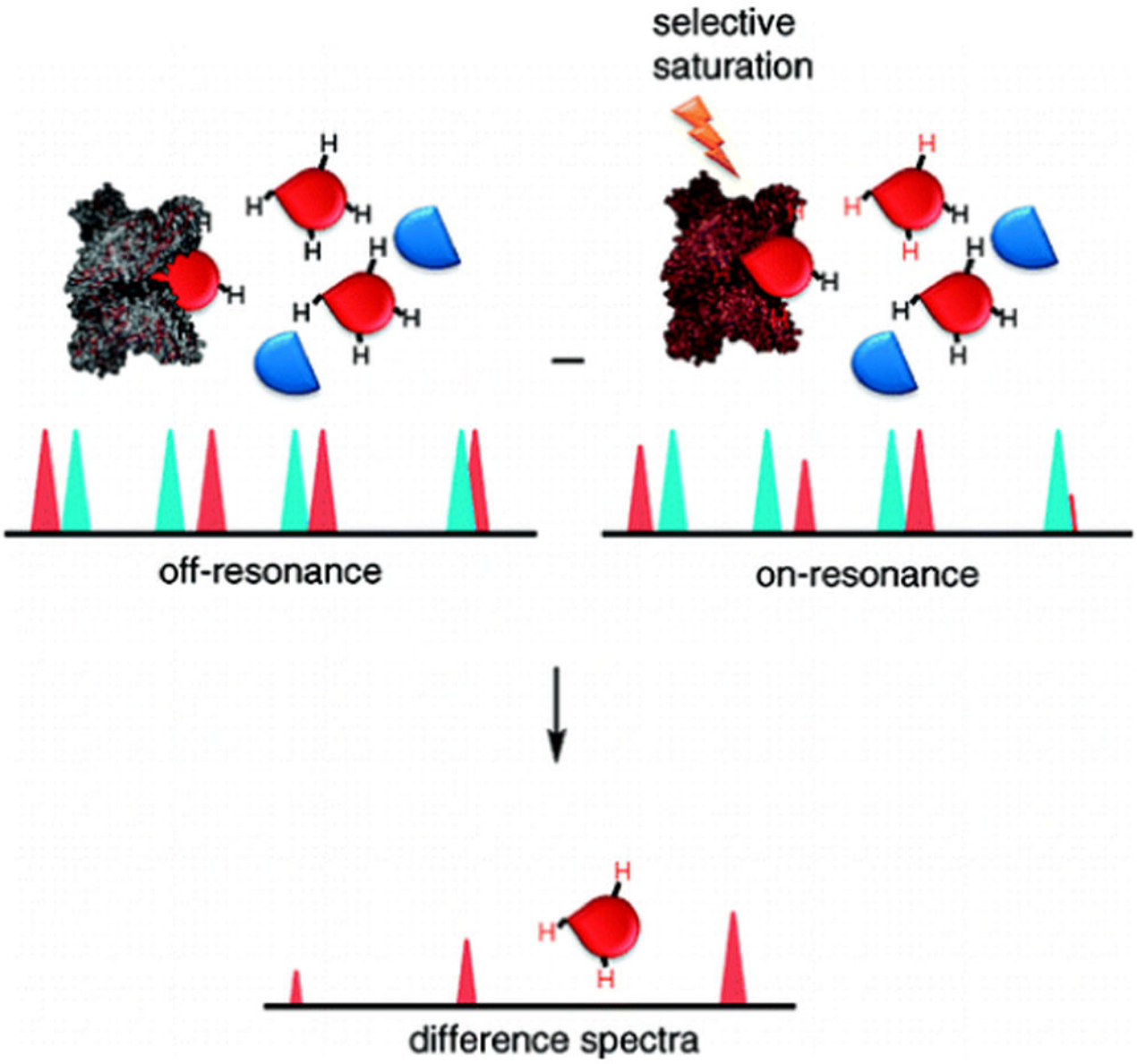


Fig. 30.

Saturation-transfer difference (STD) NMR spectroscopy. Reprinted with permission from Viegas, J. Manso, F. L. Nobrego and E. J. Cabrita, *J. Chem. Educ.*, 2011, **88** (7), 990–994. Copyright 2011 American Chemical Society.

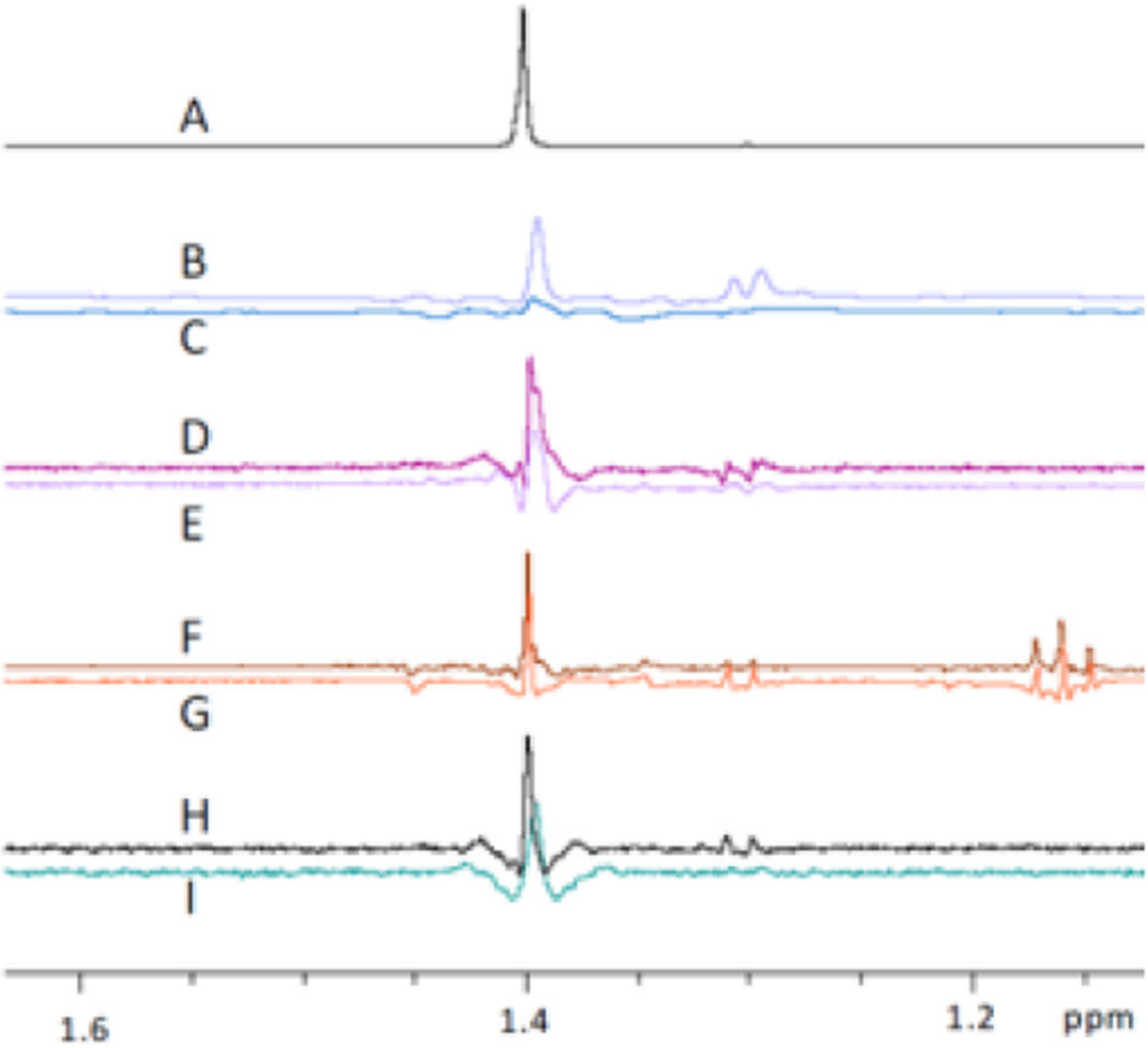


Fig. 31.

(A) NMR spectrum of TBM in acetone-d_6; STD spectrum of cells transfected to express OR2T11 in HBSS/D_2O with TBM and (B) with $CuCl_2$ or (C) prior to $CuCl_2$ addition. STD spectrum of cells transfected to express OR2T11 in HBSS/D_2O with TBM and (D) with $AgNO_3$ or (E) prior to $AgNO_3$ addition. STD spectrum of cells transfected to express MOR244-3 in HBSS/D_2O with TBM and (F) with $CuCl_2$ or (G) prior to $CuCl_2$ addition. STD spectrum of cells transfected to express MOR244-3 in HBSS/D_2O with TBM and (H) with $AgNO_3$ or (I) prior to $AgNO_3$ addition.[71] Reprinted with permission from S. Li, L. Ahmed, R. Zhang, Y. Pan, H. Matsunami, J. L. Burger, E. Block, V. S. Batista and H. Zhuang. *J. Am. Chem. Soc.,* 2016, **138**, 13281–13288. Copyright 2016 American Chemical Society.

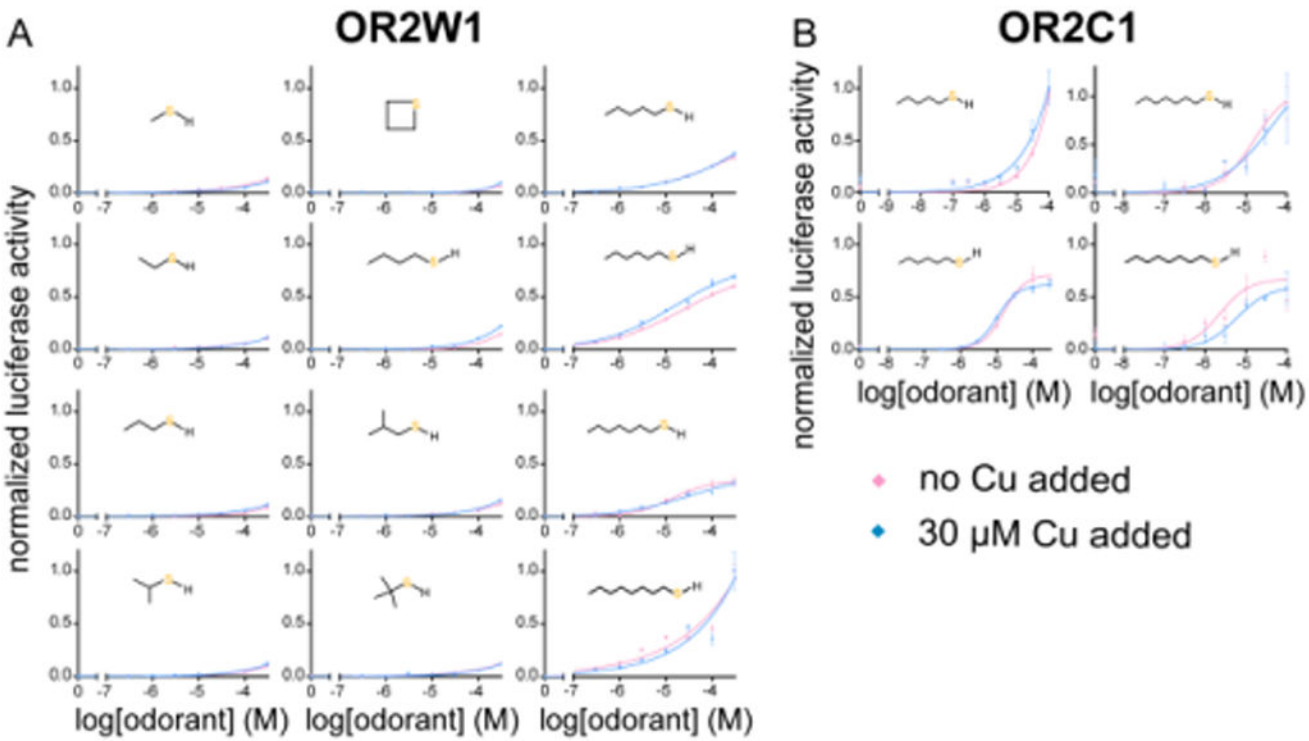


Fig. 32.

OR2W1 and OR2C1 respond to selected monothiols. Dose-response curves of (A) OR2W1 and (B) OR2C1 to various monothiols in the luciferase assay. The *y*-axis indicates normalized response±SEM ($N = 3$). All responses are normalized to the highest response of each receptor ($N = 3$).[71] Reprinted with permission from S. Li, L. Ahmed, R. Zhang, Y. Pan, H. Matsunami, J. L. Burger, E. Block, V. S. Batista and H. Zhuang. *J. Am. Chem. Soc.,* 2016, **138**, 13281–13288. Copyright 2016 American Chemical Society.

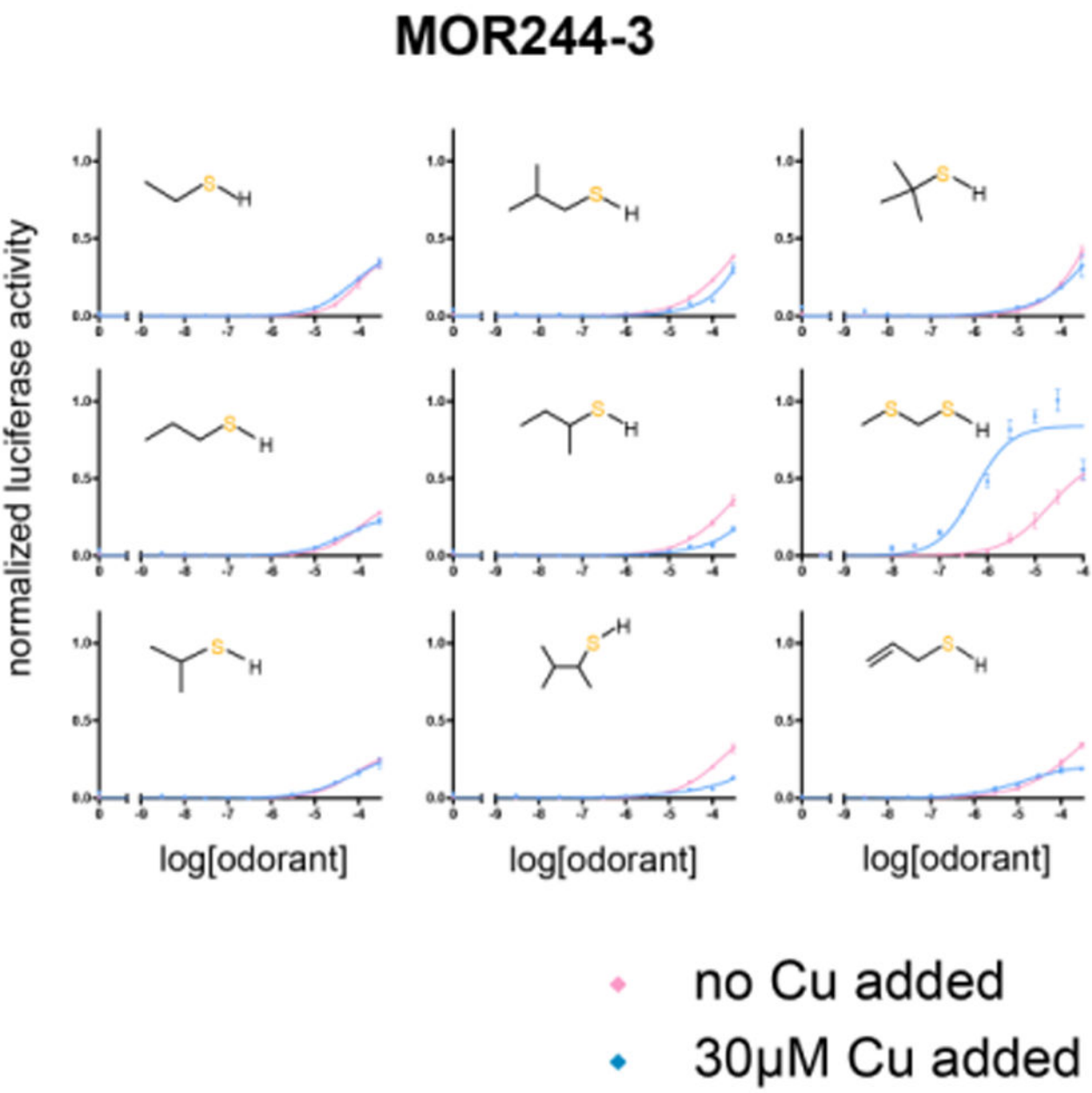
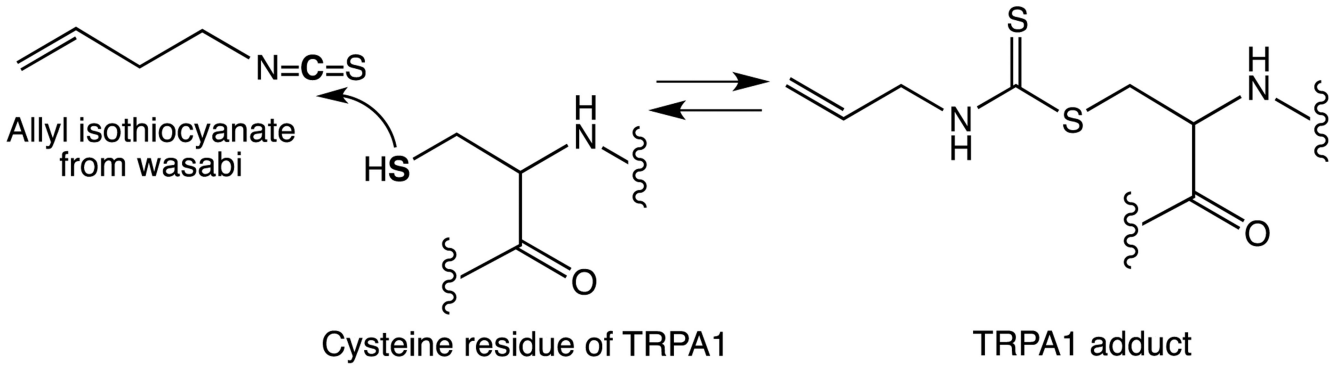


Fig. 33.

MOR244-3 selectively employs copper in binding to thiols, only showing a copper effect with MTMT; y-axis indicates normalized response ±SEM (N = 3). All responses are normalized to the highest thiol response of each OR.[71] Reprinted with permission from S. Li, L. Ahmed, R. Zhang, Y. Pan, H. Matsunami, J. L. Burger, E. Block, V. S. Batista and H. Zhuang. *J. Am. Chem. Soc.,* 2016, **138**, 13281–13288. Copyright 2016 American Chemical Society.

Fig. 34.

Multiple sequence alignment of the human M2 muscarinic receptor, human olfactory receptor OR2T11, mouse olfactory receptors MOR244-3 and MOR244-2.[71] Reprinted with permission from S. Li, L. Ahmed, R. Zhang, Y. Pan, H. Matsunami, J. L. Burger, E. Block, V. S. Batista and H. Zhuang. *J. Am. Chem. Soc.,* 2016, **138**, 13281–13288. Copyright 2016 American Chemical Society.



Scheme 1.

Proposed reaction pathway for the reversible activation of TRPA1 by allyl isothiocyante from wasabi.[21g]

Table 1

ONIOM extrapolated energies of QM/MM optimized structures, single point energy of odorants, and calculated binding energy for selected thiols and H_2S in the gas phase in OR2T11 binding site 1, involving M115, C238 and H241.[71]

Odorant	ONIOM extrapolated energy of the complexes (a.u.)	Single point energy of odorants (a.u.)	Binding energy (kcal/mol)
2-methylpropane-2-thiol (*t*-BuSH)	-2598.363506	-556.009804	-36.2
propane-2-thiol (*i*-PrSH)	-2559.048719	-516.6979299	-34.4
propane-1-thiol (*n*-PrSH)	-2559.045017	-516.694362	-34.3
ethanethiol (EtSH)	-2519.736399	-477.387503	-33.2
(methylthio)meth-anethiol (MTMT)	-2917.900232	-875.5557832	-30.4
methanethiol (MeSH)	-2480.422901	-438.078609	-30.3
prop-2-ene-1-thiol (AllSH)	-2557.807195	-515.4663709	-28.1
butane-2-thiol (*sec*-BuSH)	-2598.342528	-556.0059838	-25.4
hydrogen sulphide (H_2S)	-2441.118773	-398.7834945	-24.6
2-methylpropane-thiol (*i*-BuSH)	-2598.33847	-556.0070369	-22.2
3-methylbutane-2-thiol	-2637.630407	-595.3139794	-12.8