

The implausibility of the vibrational theory of olfaction

Eric Block^{a,1}, Seogjoo Jang^{b,1}, Hiroaki Matsunami^{c,1}, Sivakumar Sekharan^d, Bérénice Dethier^a, Mehmed Z. Ertem^{d,e}, Sivaji Gundala^a, Yi Pan^f, Shengju Li^f, Zhen Li^f, Stephen N. Lodge^a, Mehmet Ozbil^d, Huihong Jiang^f, Sonia Flores Penalba^a, Victor S. Batista^d and Hanyi Zhuang^{f,g,1}

^aDepartment of Chemistry, University at Albany, State University of New York, Albany, NY 12222; ^bDepartment of Chemistry and Biochemistry, Queens College, and the Graduate Center, City University of New York, Flushing, NY 11367; ^cDepartment of Molecular Genetics and Microbiology, Department of Neurobiology, Duke Institute for Brain Sciences, Duke University Medical Center, Durham, NC 27710; ^dDepartment of Chemistry, Yale University, New Haven, CT 06520; ^eChemistry Department, Brookhaven National Laboratory, Upton, NY 11973; ^fDepartment of Pathophysiology, Shanghai Jiaotong University School of Medicine, Shanghai 200025, China; ^gInstitute of Health Sciences, Shanghai Jiaotong University School of Medicine/Shanghai Institutes for Biological Sciences of Chinese Academy of Sciences, Shanghai 200031, China

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The vibrational theory of olfaction assumes that electron transfer occurs across odorants at the active sites of odorant receptors (ORs), serving as a sensitive measure of odorant vibrational frequencies, ultimately leading to olfactory perception. A previous study reported that human subjects differentiated hydrogen/deuterium isotopomers (isomers with isotopic atoms) of the musk compound cyclopentadecanone as evidence supporting the theory. Here, we find no evidence for such differentiation at the molecular level. In fact, we find that the human musk-recognizing receptor OR5AN1, identified using a heterologous OR expression system and robustly responding to cyclopentadecanone and muscone, fails to distinguish isotopomers of these compounds *in vitro*. Furthermore, the mouse (methylthio)methanethiol (MTMT)-recognizing receptor, MOR244-3, and other selected human and mouse ORs, responded similarly to normal, deuterated and ¹³C isotopomers of their respective ligands, paralleling our results with the musk receptor OR5AN1. These findings suggest that the proposed vibration theory does not apply to the human musk receptor OR5AN1, mouse thiol receptor MOR244-3, nor other ORs examined. Also, contrary to the vibration theory predictions, muscone-d₃₀ lacks the 1380-1550 cm⁻¹ IR bands claimed to be essential for musk odor. Furthermore, our theoretical analysis shows that the proposed electron transfer mechanism of the vibrational frequencies of odorants could be easily suppressed by quantum effects of non-odorant molecular vibrational modes. These and other concerns about electron transfer at ORs, together with our extensive experimental data, argue against the plausibility of the vibration theory.

olfaction | isotopomers | cyclopentadecanone | muscone | electron transfer

In 1870, the British physician William Ogle wrote: “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” (1, 2). Of the 29 different “theories of odour” listed in the 1967 edition of *The Chemical Senses* (3), nine associate odor with vibrations, particularly those theories championed by Dyson (4, 5) and Wright (6-8). However, the premise that olfaction involves detection of vibrational frequencies of odorants remains highly speculative since neither the structures of the ORs nor the binding sites or the activation mechanisms triggered upon odorant binding to ORs have been established. In 1996–1997, Turin (9-12) elaborated on the undulatory theory of smell, as considered in more detail below, and suggested that a mechanism analogous to inelastic electron tunneling spectroscopy (IETS) (13) may be involved, where tunneling electrons in the receptor probe the vibrational frequencies of odorants. In 2013, Gane *et al.* commented that “whether olfaction recognizes odorants by their shape, their molecular vibrations, or both remains an open and

controversial question” and that “a convenient way to address [this question] is to test for odor character differences between deuterated and non-deuterated odorant isotopomers since these have identical ground-state conformations but different vibrational modes.” Gane *et al.* also states that a particularly appropriate test case would involve odorants containing “more CH group ... [such as] musks [which] are among the largest odorants and typically contain 15–18 carbons and 28 or more hydrogens” (14).

In judging the plausibility of the vibration theory, we employ a multi-pronged approach. 1) We consider the concepts *shape* vs. *vibration theory*, and *odorant perception* vs. *reception*. 2) As a test of the vibration theory, we have prepared a series of isotopomers of musks and other compounds, containing up to 30 C–H or C–D bonds as test odorants, which are evaluated using *in vitro* activation of receptors identified by us and other groups as being highly responsive to these isotopomers. 3) We consider the confounding effects of impurities and isotope effects in interpreting odorant perception as well as the validity of requirements for specific infrared (IR) bands for recognition of musks by their receptors. 4) We examine the physical validity of the models developed to support the vibration theory. 5) We consider the specific limitations of our *in vitro* approach using isotopomers to evaluate the

Significance

The vibrational theory of olfaction posits detection of odorants through their vibrational frequencies, rather than solely through “hand-in-glove” substrate/enzyme-like odorants/odorant receptor interactions. To test the theory, we compare responses of different human and mouse odorant receptors toward deuterated and undeuterated isotopomers (isotopic atom isomers) of receptor-responsive odorants, since isotopomers should differ in their molecular vibrational frequencies. However, no differences in receptor response are seen with any tested labeled/unlabeled odorant-receptor pairs. Since published behavioral studies showed that humans could distinguish isotopomers, peri-receptor events or impurities, rather than receptor-level vibrational effects, are suggested. Because theoretical aspects of the vibration theory are also found wanting, the vibration theory is deemed implausible in the absence of compelling receptor-level experimental evidence to the contrary.

Reserved for Publication Footnotes

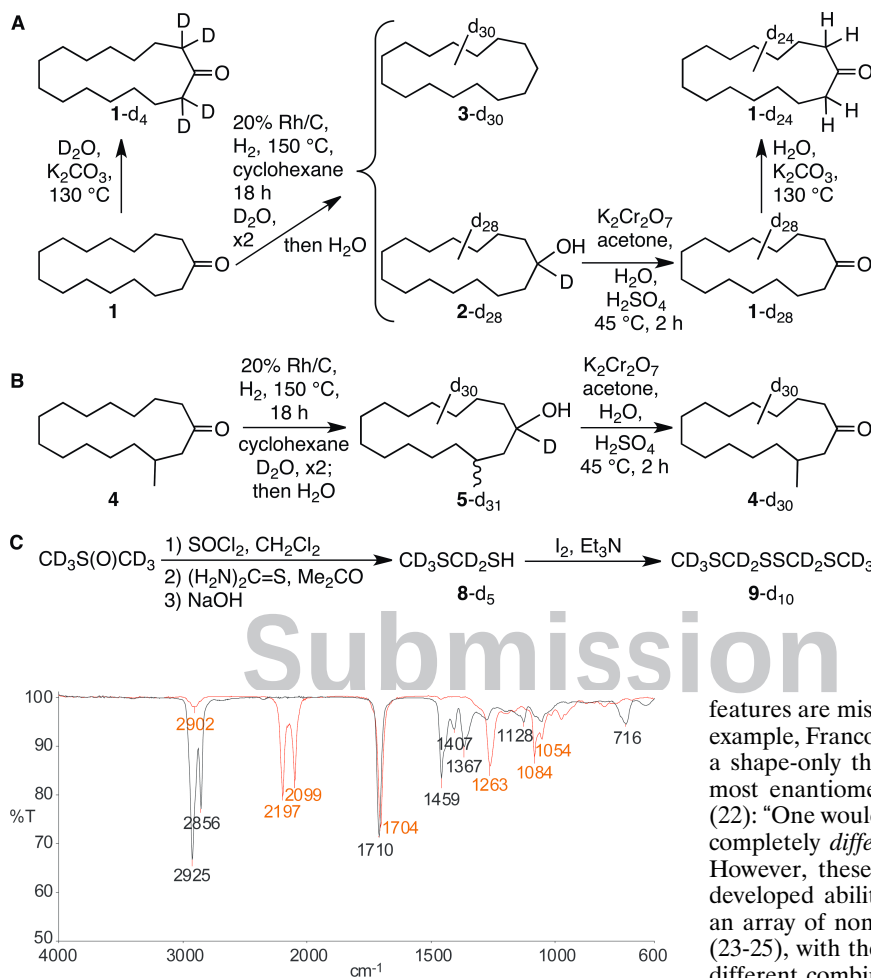


Fig. 2. Superimposed infrared spectra of muscone- d_{30} (4- d_{30} ; red trace) and undeuterated muscone (4; black trace) showing that 4- d_{30} is devoid of IR absorption in the 1380–1550 cm^{-1} region.

vibration theory based primarily on results obtained with a single identified human musk OR, in addition to other OR-ligand pairs. 6) We consider plausible non-vibration theory models for docking of musks to the human musk receptor OR5AN1 where the musk carbonyl groups function as hydrogen bond acceptors.

Gane *et al.* (14) have framed the argument for olfactory discrimination of hydrogen isotopomers as one of “shape” versus “vibration.” However, neither the binding modes of isotopomers nor their activation mechanisms are known. ORs belong to the superfamily of class A G protein-coupled receptors (GPCRs), which are known to be activated through allosteric conformational changes induced upon ligand binding even without triggering any kind of electron transfer processes. Ligand-receptor interactions can be both attractive and repulsive, involving hydrogen bonding, van der Waals, cation- π , π - π , ion-ion, dipole-dipole, steric, and hydrophobic interactions with the receptor, with water channels and bridging water molecules mediating hydrogen bonds, as well as metal-ion coordination, as we have recently demonstrated in the latter case (15, 16). Therefore, molecular “shape” can be considered a “straw-man” alternative to the vibration theory when describing the differing affinities of ligands bound to GPCRs (17, 18) including isotopomers (19, 20). Some of these attractive and repulsive interactions were identified in 1940 by Pauling and Delbrück (21), who note that interacting biomolecules “must have complementary surfaces, like die and coin, and also a complementary distribution of active groups.” In addition, shape-related

features are misrepresented by vibration theory proponents. For example, Franco *et al.* stated (17): “Given that proteins are chiral, a shape-only theory cannot account for the identical odors of most enantiomeric pairs,” echoing similar comments by Turin (22): “One would therefore generally expect enantiomers to have completely different smells. This is emphatically not the case”. However, these assertions are clearly at odds with the highly developed ability of mice and other mammals to discriminate an array of non-pheromonal chiral odorant enantiomeric pairs (23–25), with the divergent *in vitro* responses to enantiomers by different combinations of ORs (26) and, in particular, with the highly selective response of musk-sensitive mouse OR MOR215-1 to (*R*)-muscone (“*l*-muscone”) compared to (*S*)-muscone (“*d*-muscone”) (27).

In addition to our concerns regarding *shape*, a second issue relates to describing how different smells are perceived – that is, the *perception* of an odorant. It is known that *in vivo* perception of odorants reflects the totality of peri-receptor events as well as odorant-OR interactions (*reception*). Volatile odorants enter the nasal passage where they dissolve in the nasal mucus overlying the olfactory epithelium and are then rapidly detected by ORs on the cilia of the olfactory sensory neurons, ultimately leading to signaling (28, 29). It is the mechanism of odorant-OR interactions – the *reception* of the odorant – that we seek to examine with isotopomers to determine whether the vibration theory is plausible, displaying isotope effects, since *perception* could be influenced by isotope effects due to the peri-receptor events involving mucosal components such as enzymes, mucopolysaccharides, salts and antibodies.

Whether deuterated and non-deuterated odorant isotopomers can be distinguished by smell and, even if they can, whether this validates the vibration theory is a matter of contention. A 2001 paper (30) reported that benzaldehyde- d_6 gave a statistically significant difference in odor perception relative to normal benzaldehyde, in support of the vibration theory. However, this study has been criticized for lacking double-blind controls to eliminate bias, and because it used an anomalous version of the duo-trio test (31). Furthermore, the study failed to account for peri-receptor events, namely enzyme-mediated conversion of odorants that has been shown to occur in nasal mucus. For example, benzaldehyde is converted to benzoic acid (32), a reaction potentially subject to significant

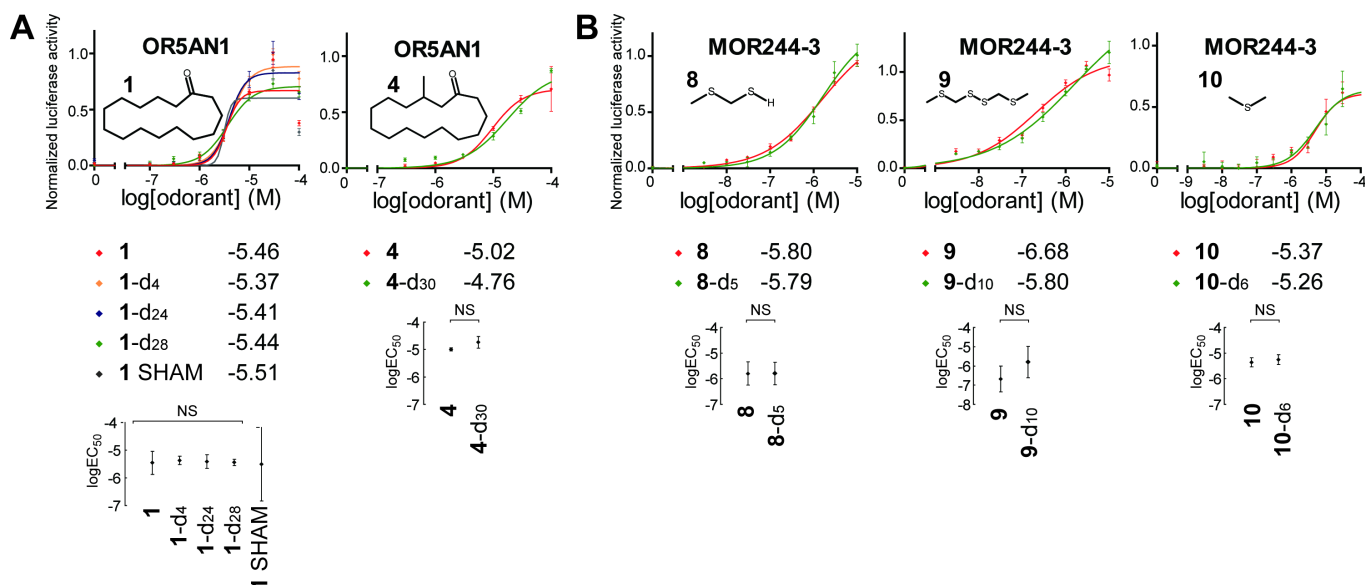


Fig. 3. Dose-response curves of (A) OR5AN1 to isotopomers of cyclopentadecanone (1) and muscone (4), and (B) MOR244-3 to isotopomers of MTMT (8), bis(methylthiomethyl) disulfide (9), and dimethyl sulfide (10). Best-fit logEC₅₀ values of the curves are shown alongside the graph legends (placed below the graphs). Scatter plots with 95% confidence interval logEC₅₀ values and indicating statistical significances between the logEC₅₀ values among isotopomers are also shown below the corresponding graphs. In B, 30 μ M of copper was added upon odorant stimulation. "SHAM" indicates non-deuterated cyclopentadecanone subjected to the same chemical synthetic procedures as the deuterated samples without D₂O addition. "NS", not significant. For all dose-response curve graphs, the chemical structures of the respective compounds are shown within the graphs and normalized responses are shown as mean $\pm$ SEM ($N = 3$).

primary isotope effects (2, 33, 34), which could explain the difference in odor perception for the benzaldehyde isotopomers. Earlier claims that human subjects can distinguish odors of acetophenone isotopomers (9, 35) have been shown to be untrue (14, 31). Recent studies indicate that *Drosophila melanogaster* can distinguish acetophenone isotopomers (36, 37) and that *Apis mellifera* L., the honey bee, can be trained to discriminate pairs of isotopomers (38). These studies differ from earlier insect studies in which isotopomer discrimination was not found. For example: systematic deuteration of 4-(*p*-hydroxyphenyl)-2-butanone acetate, a *Dacus cucurbitae* Coquillett (the male melon fly) attractant, did not affect the attractiveness of the compound to the fly (39); deuteration of alarm pheromones failed to alter the response toward these compounds by *Pogonomyrmex badius* worker ants (40); honey bees could not distinguish deuterated and non-deuterated nitrobenzene (41).

Concerns have been raised (42) about aspects of the *Drosophila* study (36), which is "behavioural and not at the receptor level" (2) (also a concern with the *Apis* study). Also, given that the ORs and their downstream signaling in *Drosophila* and human are completely unrelated, the *Drosophila* study should not be considered predictive of the ability of humans to distinguish isotopomers (2, 17). In view of the above discussion, it is interesting that in a blinded, behavioral study, smell panelists distinguished deuterated and non-deuterated isotopomers of cyclopentadecanone (1; Fig. 1A) and other musk odorants (14).

Here, we study the response of a human musk-sensitive OR, OR5AN1, identified through screening of heterologously expressed human ORs, to cyclopentadecanone (1) and muscone (4) isotopomers. We also present pharmacological data on the response of mouse ORs to deuterated and non-deuterated acetophenone and benzaldehyde, as well as selected ¹³C isotopomers. In addition, we present related studies on the response of various human and mouse ORs to other deuterated and non-deuterated odorants, including (methylthio)methanethiol (8; "MTMT"; Fig. 1C) and bis(methylthiomethyl) disulfide (9) studied in connection with our investigation of the role of copper

coordination in the recognition of both sulfur-containing odorants by the mouse OR MOR244-3 (15, 16). Insofar as the ability to distinguish odors of isotopomers directly tests the predictions of the vibration theory, the comparative response of human and mouse ORs to isotopomers of these selected ligands in the heterologous OR expression system constitutes a robust test of the vibration theory. Finally, we discuss the basis for recent vibration theories of olfaction and supporting computational evidence (37, 43-47) in light of well-established electron transfer theories (48). We point out that key assumptions underlying the vibration theory lack experimental support and are missing important physical features expected for biological systems.

Experimental Results

Response of a human musk OR to deuterated and non-deuterated muscone and other musk compounds

Since human subjects are reported to discriminate fully deuterated and non-deuterated cyclopentadecanone (1; Exaltone®) (14), we sought to perform a corresponding receptor activation assay *in vitro*.

First, from a commercial sample of cyclopentadecanone (1, Fig. 1A), we synthesized 1-d₂₈ (fully deuterated), 1-d₂₄ and 1-d₄. Treatment of 1 (twice) with Rh/C in D₂O at 150 °C (49) gave cyclopentadecanol-d₂₉ (2-d₂₉), which was oxidized with K₂Cr₂O₇/acetone (50) and repeatedly recrystallized to give 1-d₂₈. Deuterium was selectively introduced into 1, or selectively removed from 1-d₂₈, adjacent to the carbonyl group using D₂O/K₂CO₃ or H₂O/K₂CO₃ (51), giving 1-d₄ and 1-d₂₄, respectively, at 130 °C. A sham sample of 1, which underwent all of the procedures as 1-d₂₈, but with H₂O instead of D₂O, was also included as a negative control. Both 2/2-d₂₉ and cyclopentadecane-d₃₀ (3-d₃₀), a byproduct in the catalytic reduction of 1, and non-deuterated 3 were also tested for receptor activation. Similarly, (*R,S*)-muscone (4; (*R,S*)-3-methylcyclopentadecanone) was converted to 4-d₃₀ by way of *cis/trans*-3-methylcyclopentadecanol-d₃₁ (5-d₃₁) (Fig. 1B). Compounds 4 and 4-d₃₀ are baseline-resolved by gas chromatography and show very different IR spectra (see Fig. 2; see SI for exper-

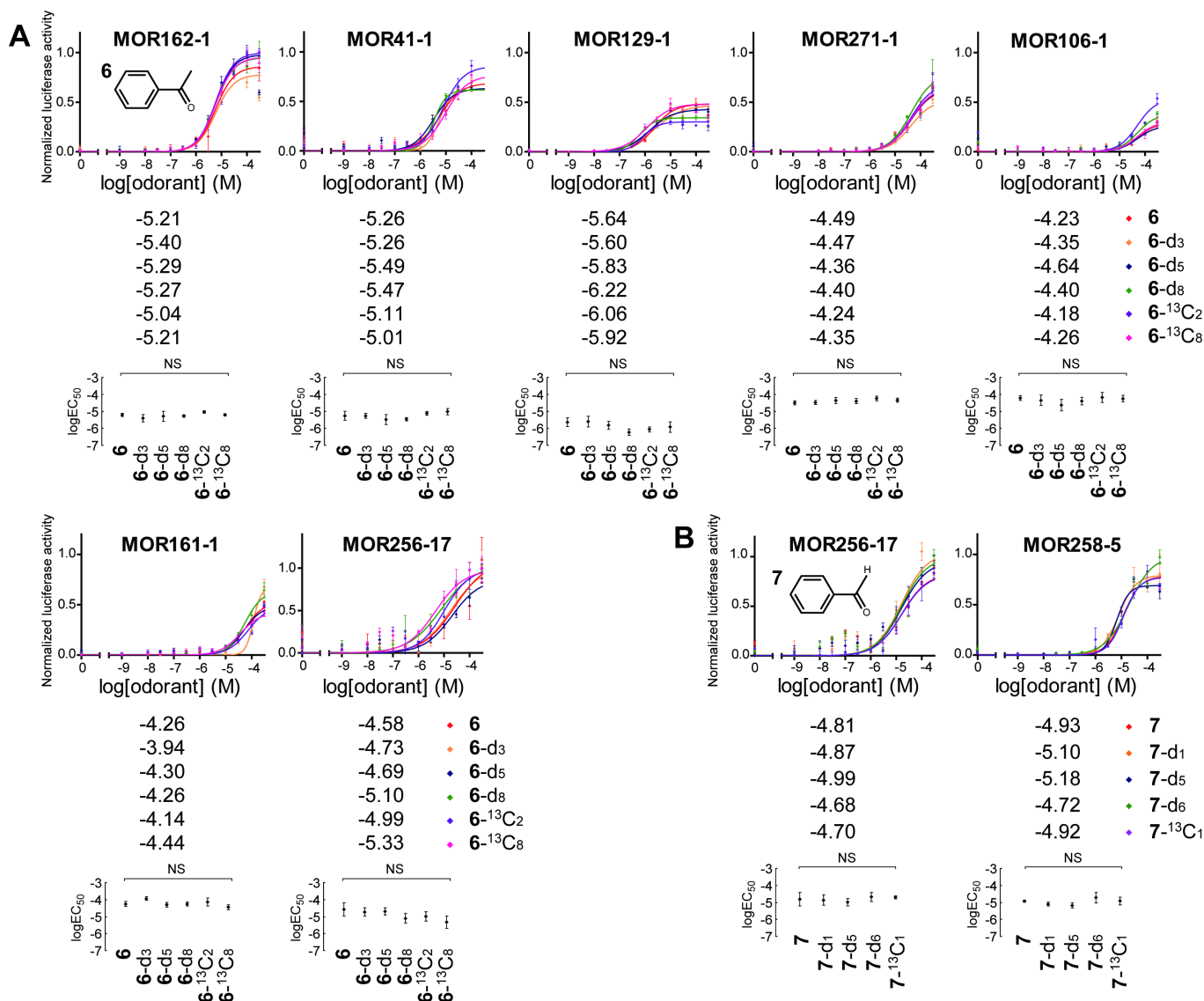


Fig. 4. Dose-response curves of various human and mouse receptors to isotopomers of (A) acetophenone (6) and (B) benzaldehyde (7). Best-fit logEC₅₀ values of the curves are shown alongside the graph legends (placed below the graphs). Scatter plots with 95% confidence interval logEC₅₀ values and indicating statistical significances between the logEC₅₀ values among isotopomers are also shown below the corresponding graphs. "NS", not significant.

imental details of synthesis and characterization of deuterated compounds 1-5). Contrary to statements by Gane *et al.* (14) that a musk receptor "detects vibrations in the 1380–1550 cm⁻¹ range," and that musk odor requires that "the molecule has intense bands in that region," the infrared spectrum of muscone-d₃₀ is devoid of 1380–1550 cm⁻¹ absorption (Fig. 2).

Second, using a heterologous OR expression system (52, 53), we performed parallel screenings of all deuterated and non-deuterated versions of 1 on the human OR repertoire. Among all 330 human ORs screened, we identified one OR, OR5AN1, that is a *bona fide* receptor for 1 and its isotopomers (Fig. S3.1). OR5AN1 also responds strongly to other related musk analogs, including muscone, ω-pentadecalactone (Exatolide®), and less strongly to cyclopentadecanol (Fig. S3.3). This is consistent with a recent report (27), in which OR5AN1 was identified as a human muscone OR based on homology to the mouse OR MOR215-1, functionally cloned from muscone-responsive glomeruli, a second report on OR5AN1 as the only functional human homolog of mouse muscone ORs *in vivo* (54), and a third report that only a small number of receptors are thought to be involved in sensing

musk odor (55). Our screening and the following confirmation experiments did not reveal any human OR that responded to only one, two, or three of the four isotopomers of 1.

Third, we tested whether or not OR5AN1 responded similarly to isotopomers of the different musk analogs. We found that all four different isotopomers of 1 gave highly similar responses and the EC₅₀ values of the respective dose-response curves were not significantly different (Fig. 3A, left, and Table S3.2A). In addition, we also tested 2 alongside fully C–D deuterated isotopomer 2-d₂₉, and found that even though this compound evoked a much smaller response, similar response levels were seen between the deuterated and non-deuterated versions of this compound (Fig. S3.2B and Table S3.2B). We also found the hydrocarbon analogs cyclopentadecane (3) and cyclopentadecane-d₃₀ (3-d₃₀) to be inactive (Fig. S3.2B). We also tested whether or not OR5AN1 responded similarly to isotopomers of (R,S)-muscone (4). Again, we found similar responses between the undeuterated (R,S)-muscone and its fully deuterated d₃₀ isotopomer (4-d₃₀) (Fig. 3A, right, and Table S3.2A).

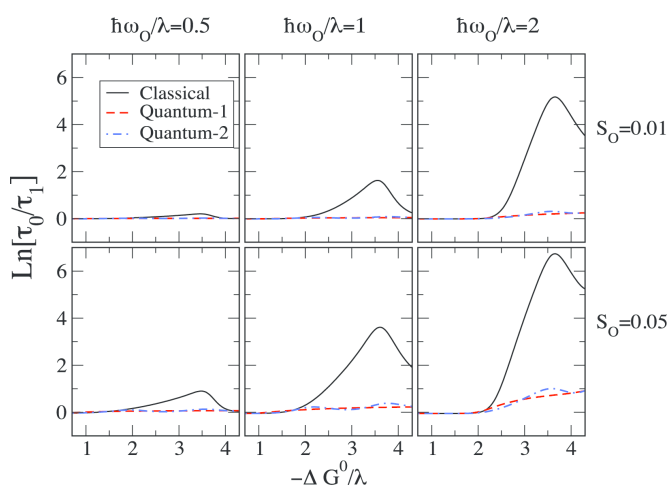


Fig. 5. The natural logarithms of the ratios of τ_0/τ_1 versus $-\Delta G^0/\lambda$ (negative Gibbs free energy of reaction in the unit of reorganization energy) corresponding to the results in Figs. S1-3. The "Classical" refers to the classical limit of Ohmic bath, Eq. (18) of SI, with parameters for Fig. S1.1. The "Quantum-1" refers to the quantum regime of the Ohmic bath with parameters for Fig. S1.2. The "Quantum-2" refers to the case of classical Ohmic bath plus one quantum mode in the bath with parameters for Fig. S1.3. Each column represents different value of $\hbar\omega_O/\lambda$, where ω_O is the angular frequency of the odorant oscillator and λ is the reorganization energy of the protein environments. The upper panels are for $S_O = 0.01$ and the lower panels are for $S_O = 0.05$, where S_O is the Huang-Rhys factor for the odorant oscillator.

Mouse ORs for acetophenone and benzaldehyde show similar responses to all isotopomers tested

We assayed isotopomers of acetophenone (**6**) and benzaldehyde (**7**) in our system using cognate mouse ORs. Similar to the case of cyclopentadecanone, no significant difference was seen between non-deuterated and deuterated versions for all of the ORs tested (Fig. 4A-B and Table S3.2A). In addition, ^{13}C -labeled isotopomers may present a good test for validating/invalidating the vibration theory as they do not significantly alter vibrational frequencies (42). We also included **6- α,β - $^{13}\text{C}_2$** and **6- $^{13}\text{C}_8$** as well as **^{13}CHO -**7- $^{13}\text{C}_1$** to test against their ^{12}C counterparts in our odorant panel. We again found no significant differences among all isotopomers tested (Fig. 4A-B and Table S3.2A), indicating that neither vibrational frequencies nor other factors, such as association/dissociation rates, are contributing to the level of OR activation.**

Additional ORs respond similarly to isotopomeric ligands in heterologous cells

We investigated whether other ORs respond differently to isotopomer pairs by assaying 14 other known receptor-ligand pairs using 10 human and mouse ORs and 10 odorous ligands with purchased or synthesized H/D isotopomers, including octanol and octanol- d_{17} , discriminated by *Drosophila* in the aforementioned study (36). Our assay included (methylthio)methanethiol (**8**) and (methylthio)methanethiol- d_5 (**8- d_5**) (Fig. 1C), as well as bis(methylthiomethyl) disulfide (**9**) and bis(methylthiomethyl) disulfide- d_{10} (**9- d_{10}**), ligands for mouse receptor MOR244-3, notable for requiring copper for ligand binding, and whose active site we have modeled (15, 16). Deuterated compound (**8- d_5**) was prepared in several steps from dimethyl sulfoxide- d_6 and then oxidized to **9- d_{10}** ; these compounds had $\sim 100\%$ d_5 and d_{10} deuterium incorporation according to GC/MS. When **8** and **9** were tested in the presence of $30\ \mu\text{M}\ \text{Cu}^{+2}$ no differential receptor activity was seen, which was also the case for the other isotopomeric pairs tested (Figs. 3B and S3.2; Table S3.2A-B).

Concerns involving impurities

When comparing odors of isotopomers it is essential to ensure that what is being measured is the odor of pure isotopomers devoid of impurities, since trace impurities could lead to differential response at the organism or receptor level. For example, despite Turin's claims of different odors for deuterated and non-deuterated dimethyl sulfide (35), no differences were seen in the OR response to dimethyl sulfide (**10**) and dimethyl sulfide- d_6 (**10- d_6**) (Fig. 3B and Table S3.2A). These results are consistent with reports that the odor of commercial samples of dimethyl sulfide is due to impurities, which can be removed by washing with aqueous HgCl_2 (56). We suggest that commercial samples of dimethyl sulfide- d_6 are of much higher purity than samples of dimethyl sulfide; the former may have lower levels of these impurities. In general, this difference in purity is anticipated between undeuterated and deuterated isotopomers, based on the multistep procedures involved in isotopic labeling, and expectations based on the much higher cost of the deuterated compounds.

In a more pertinent example, Gane *et al.* (14) report the ^1H NMR data for deuterated cyclopentadecanone (**1**), purified by silica gel chromatography using 9:1 hexane-ether, as " δ 2.37 (m, 0.2H), 1.59 (m, 0.22H), 1.30–1.20 (m, 1.72H), 0.84–0.87 (m, 0.25H)." Notably, the ^1H NMR spectrum of pure, commercial **1** (Figure 1 in Gane *et al.* and Fig. S2.7) shows the highest field peak at δ 1.30–1.20, with no evidence of absorption at δ 0.84–0.87, which leads us to question the assertion of Gane *et al.* for their deuterated **1** that "No impurities are seen in the spectra." In our hands, the ^1H NMR spectra (Fig. S2.6) for chromatographed and repeatedly recrystallized samples of **1- d_{28}** lack the unidentified impurity peak at δ 0.84–0.87 in the Gane *et al.* deuterated **1**, not seen in the ^1H NMR spectrum of their (or our – see SI) commercial **1**. This impurity peak, seen in our deuterated samples when commercial, unpurified chromatography grade hexanes were used for chromatography, but not with redistilled pentane, could possibly have compromised the smell testing performed by Gane *et al.*, given that for odor evaluation by Gane *et al.*, "after silica gel purification, aliquots of the deuterated musks were diluted in ethanol and their odor character assessed on smelling strips," and that the δ 0.84–0.87 impurity peak constituted 10% by integration of all residual proton signals. With regard to GC purification prior to additional smell testing, it is not known whether or not the compound(s) responsible for the additional δ 0.84–0.87 impurity peak, or decomposition products of the compound(s) in the hot injection port, coelute with deuterated musks.

Concerns involving isotope effects

While differences in perception of hydrogen/deuterium isotopomers has been invoked as evidence supporting the vibration theory, it is important to recognize that changing H to D not only changes vibration, but also intermolecular interactions, due to the lowering of zero point energy of bonds to D compared to H. Thus, the acidity of D_2O and H_2O are different, hydrogen-bonding of O–H and O–D bonds are different, boiling points and freezing points are different, etc. In particular, the gas chromatographic retention times of isotopomeric pairs in the present study are significantly different in all cases examined.

The lack of isotope effects of isotopomers **1–8** when interacting with the corresponding receptors is not unexpected given that C–H/C–D bonds are not likely to be broken during docking with the receptor. Comparative isotopomer-receptor interactions can be probed computationally. In fact, we have reported (16) a QM/MM model for the mouse OR MOR244-3 in complex with the organosulfur odorant MTMT (**8**). The proposed binding site consists of a copper ion coordinated to the thioether sulfur atom of MTMT as well as to the N, S and O atoms of H105, C109 and N202 residues. The QM/MM calculations indicate that both the deuterated (**8- d_5**) and non-deuterated odorants (**8**) have similar binding affinities, and that no difference in response is predicted

upon deuteration, consistent with the experimental observations (see SI).

In addition, we point out that isotope effects in odorant response at the behavioral/organismal level are not necessarily evidence in favor of the vibration theory. Peri-receptor events and/or psychophysical processes are known to be important in olfaction (2, 57) and may result in different olfactory percept of isotopomers. For example, it has been proposed that the nasopharyngeal mucus "behaves like a polar chromatographic column" (58), with differential diffusion rates, air/mucus partition coefficients (59) and solubility towards dissolved odorants (60), potentially leading to separation of isotopomers. Since **1** and **1-d₂₈** are separated by several minutes on a gas chromatographic column (14), and HPLC separation of H/D isotopomers is well known (61), isotopomer fractionation could contribute to perceived differences.

Furthermore, as noted by Brookes *et al.* (46), biotransformation enzymes reside within the mucus layer and "comparisons of odors could well be affected even by small differences in metabolism, for instance from reaction rates depending on isotope[s]." Since the Baeyer-Villager (B-V) reaction is known to be mediated (62) by oxidative enzymes (such as cytochrome P450), which are present in the nasopharyngeal mucus layer (2, 32, 63, 64), and the B-V reaction of deuterated cyclic ketones forming deuterated lactones is known to show an isotope effect (62), such a reaction might affect odor perception of pairs such as **1** and **1-d₂₈**. Indeed, we have confirmed that **1-d₂₈** undergoes peracid-mediated B-V oxidation *faster* than non-deuterated **1**, in accord with literature results (62), although a full kinetic analysis in the case of **1/1-d₂₈** was not possible due to partial overlap of ketone and lactone peaks under GC-MS conditions. Deuterium substitution is well known to effect drug pharmacokinetics, e.g., for drugs metabolized by aldehyde oxidase (65), and can change many intermolecular interactions.

Critique of current theoretical proposals

Turin's idea that electron transfer occurs at ORs and that these ORs can detect odorant vibrational frequencies gained traction in recent years (37, 43-47). These theoretical works (37, 43, 47) are in support of the vibration theory but remain largely tentative because they admittedly rely on unconfirmed assumptions, lacking experimental evidence, in order to make the proposal appear to be feasible. Electron transfer in biological environments is not uncommon. There is a substantial amount of literature reporting various experimental and theoretical studies. However no evidence exists that GPCRs require electron transfer for their activation. The proposed mechanism (43) of delivering electrons to ORs is also too unreliable to set the stage for a fail-safe mechanism of detecting odorants' vibrational frequencies. In addition, biological electron transfer processes are sensitive to chemical bonding characteristics, local molecular environments, and dynamic fluctuations, which can affect the transfer rate by orders of magnitudes. OR sites are floppy and open to numerous and diverse-sized odorants, and are susceptible to these effects. Current theoretical proposals supporting the vibration theory, as summarized below, are oblivious of these complex issues.

Brookes *et al.* (43) proposed a model based on the standard spin-boson type Hamiltonian for electron transfer (66), utilizing Jortner's expression (67) of electron transfer rate for quantum vibrational modes. Two electron transfer times, τ_0 for electron transfer without odorant and τ_1 for electron transfer exciting one vibrational quantum of the odorant, were introduced. Assuming that all vibrational modes coupled to electron transfer are *classical*, except for the odorant oscillator, they obtain Marcus' expression (68) for $1/\tau_0$ and Jortner's expression (67) for $1/\tau_1$ (see SI). Approximating the odorant oscillator as a classical point dipole, they estimated the Huang-Rhys factor of the odorant oscillator to be $S \approx 0.01$. This is a very small value, which would be difficult

to detect unless high quality samples and sensitive spectroscopic techniques are used. Brookes *et al.* (37) recognize this issue and propose that: (i) An OR site is finely tuned so that the energy difference between the electron donor and acceptor matches the vibrational quantum of the odorant oscillator, $E_D - E_A = \hbar\Omega$. (ii) The reorganization energy of the protein environment is assumed to be very small, ca. $\lambda = 30$ meV. Under these conditions, they conclude that electron transfer could detect odorant vibrational frequencies.

While Brookes *et al.* (43) bring the vibration theory to a more concrete theoretical level, none of the key assumptions has supporting experimental evidence. Furthermore, their estimate for the reorganization energy of the electron transfer-coupled protein environment is unusually small even when compared to other confirmed biological electron transfer processes (69). The reorganization energy for electron transfer in well secured hydrophobic pockets of proteins can be small (70), but the assumed value (43) relies on old literature data (71), which is smaller by an order of magnitude than more recent estimates (72). While the restriction on the reorganization energy can be relieved somewhat by modification of the resonance condition (see SI), Solov'yov *et al.* (47) estimates that the reorganization energy needs to be smaller than 0.1 eV for the vibration theory to be feasible. This is still substantially smaller than commonly known values (69, 70). Clear experimental or computational evidence supporting such estimate is lacking.

Another fundamental issue with the proposed theoretical models (43, 47) is the neglect of quantum contributions from molecular vibrational modes other than those of the odorant oscillator, leaving the window of vibrational frequencies open only for odorant molecules. This is tantamount to neglect of molecular level structural information on OR sites available from homology models (47). The metal-ligand bonds and peptide bonds in the postulated electron-donor or acceptor sites could have similarly high frequency modes with inelastic effects at least comparable to those of odorants. Full consideration of such high-frequency modes can easily alter the qualitative nature of electron transfer (73, 74), and could mask the vibrational frequencies of odorants, as illustrated in Fig. 5, making a cursory analysis of the vibration theory highly unreliable (see SI for more detailed description).

The resulting values of detection efficiency τ_0/τ_1 , plotted in Fig. 5, show that a modest amount of coupling to quantum vibrational modes of the environment could be sufficient to suppress the proposed odor detection mechanism of the vibration theory (43). In addition, considering the prevalence of C-H bonds in protein environments, it is unclear how the effects of deuterating the odorants, as proposed by Gane *et al.* (14), can stand out even if the proposed electron transfer mechanism were true. Although Solov'yov *et al.* (47) made significant improvement to the work by Brookes *et al.* (43) by including the effects of more than one vibrational modes of odorants, calculating the Huang-Rhys factor in the presence of the field, and recognizing the importance of structural fitting of odorants, they also omitted consideration of non-odorant quantum vibrational modes. Thus, the issues raised above remain unresolved.

Bittner *et al.* (37) proposed a model where the electron transfer can occur only through the odorant as a bridge. The sensitivity to molecular vibration in this model also originates from resonance effects, assuming that internal modes of the odorant are excited impulsively during hole transfer from a donor to acceptor site on the receptor, along the direction of the gradient of the Born-Oppenheimer potential for its oxidized form. These assumptions lead to an interesting expression for the detection efficiency with direct correlation to IR signals and with some predictive capability. However, the final rate expression does not depend on the electronic energy of the odorant, which is at odds with most known multistate electron transfer processes (75). Most

importantly, the model does not include the reorganization energy in the resonance condition, let alone the effects of quantum vibrational modes of donor and acceptor sites.

Other important issues that are not considered by all current theoretical proposals are the effects of disorder, dynamical fluctuations, and the sensitivity of electron couplings to bonding characteristics. For example, it is well established (75) that effective donor-acceptor electronic coupling is very sensitive to chemical characteristics and conformational details of the bridge (odorant) molecules, e.g., as shown by recent single molecule conductance measurements where substantial fluctuations of conductance were seen even when metallic electrodes are used under well controlled bias potentials (76). Dynamical modulation of these electronic couplings is also very likely, resulting in fundamentally different kinetics (77, 78). Therefore, all of these factors can easily alter the electron transfer rate by orders of magnitude, becoming as important or more so, than the proposed Jortner-type vibronic effect (43). A theory that can genuinely support Turin's idea needs to demonstrate that electron transfer can indeed amplify small vibrational contributions of odorants despite all of these complicating effects typical of electron transfer in biological environments.

Discussion

In the absence of OR structural models, theoretical work is limited to the construction of phenomenological models consistent with available experimental observations. The principal experimental evidence supporting the vibrational theory has been the deuterium isotope effect at the perceptual (behavioral) level (14, 36, 38). However, we find no experimental evidence supporting the theory at the molecular level. We focused on the functional analysis of a human OR tuned to the same musk compounds that were recently promoted as an important experimental evidence, with the aim of specifically testing the electron tunneling mechanism at the receptor level. However, the experimental data reported in our study shows a dramatic lack of correlation between OR level signals and isotope effects over an extended set of 26 receptor-ligand pairs, with at least one deuterated counterpart to each of these ligands. In addition, we find that the assumptions of current theoretical models lack experimental support and do not necessarily fit into the general picture of typical electron transfer processes in biological environments. Thus, our combined experimental results and theoretical analysis present a comprehensive set of observations questioning the validity of the vibration theory as a plausible description of odor detection.

Gane *et al.* (14), finding that cyclopentadecanone (**1**) and **1-d₂₈** can be distinguished by human smell, speculated that "a small number of receptors, possibly just one, are involved in sensing musk odor," in accord with an earlier, similar conclusion (55). A recent study (27) using heterologous cell assays and a c-fos induction assay in the olfactory bulb identified MOR215-1 as a strong musk-responding mouse OR. This study observes that "6% of humans are muscone anosmic...; therefore, muscone may be recognized by only a small set of ORs, including OR5AN1 in humans, and genetic variation in these receptors may cause muscone anosmia." McClintock *et al.* identified five highly-related ORs including MOR215-1 that are likely to be activated by muscone in freely-behaving mice (54), supporting multiple muscone receptors. Importantly, however, the only functional human counterpart of these ORs is OR5AN1. Here, we identify OR5AN1 through a thorough screening of the human OR repertoire using all four isotopomers of **1** and find that this OR responds similarly to these isotopomers. In summary, despite extensive screening, multiple research groups have identified only OR5AN1 as a human musk receptor. Nevertheless, failure to identify other human musk receptors in addition to OR5AN1 still leaves open the possibility that there are other human musk ORs.

Future studies with genetic association with the OR5AN1 locus and/or development of OR5AN1-specific antagonist(s) could show whether this is the only OR that mediates behavioral responses to the musk compounds.

We supplement our study of the response of OR5AN1 to isotopomers with the analysis of the response of copper-requiring mouse receptor MOR244-3 to isotopomers of its most active ligands as well as with the study of several other human and mouse receptors responding to cognate ligands. The consistent lack of difference found in the responses of all of these human and mouse receptors to isotopomers lessens our concern about possibly missing key receptors that are differentially responsive to isotopomers. Furthermore, we note it would be unusual for some, but not all, ORs strongly responsive to a particular ligand to demonstrate isotopomer discrimination.

In addition to OR5AN1, we describe here OR-ligand pairs of isotopomers of compounds **6–9**. We found that none of the tested receptors exhibit different responses to isotopomers, although the infrared spectra of the non-deuterated parents are strikingly different from the fully deuterated analogs (see Fig. 2 and SI) and the inelastic electron-tunneling vibration-frequencies would also be expected to differ significantly. Our experiments sought to examine the validity of the vibration theory at the receptor level by comparing the differential response to isotopomers, using a cell-based OR expression system, as compared to differential response found from animal behavioral studies, or human odorant perception. So far, many studies have been able to correlate functional responses from intact neurons to that of *in vitro* OR pharmacology. For example, by comparing the response profiles of several mouse ORs to cognate ligands using functional imaging of the olfactory bulb against heterologous ORs, Oka *et al.* showed that ligand selectivity of the ORs is comparable, although the responses vary in efficacy (79). In addition, *in vitro* activity of human receptors in heterologous cells has been shown to predict human perception for several different ORs, also suggesting that the heterologous system at least partly mimics *in vivo* function (80, 81). Nonetheless, it should be noted that the current *in vitro* method is not without limitations. The experimental setup may lack the source of electrons assumed in the vibration theory. We cannot exclude the possibility that some ORs simply may not function in our system, thus reflecting only a fraction of OR responses that may be present at the perceptual level. One possibility is that the activation of certain ORs may lead to alternative signaling pathways, and that our cAMP-based assay may not be able to detect such activation. In addition, the absence of a nasal mucosal environment also prevents the evaluation of the significance of peri-receptor events.

With the limitations noted above, given the absence of an effect of deuteration on OR response, as demonstrated in the present work, and the lack of experimental evidence supporting the fundamental assumptions of current theoretical models of the vibration theory, we conclude that the perceived differences in smell and olfactory response are likely due to peri-receptor processes or impurity of the tested odorants and not to inelastic electron tunneling assisted by vibrational modes.

Conclusion

Since Ogle's original proposal for the vibration theory more than 140 years ago (1), the idea has been embraced by Dyson (4, 5), Wright (6-8), and Turin (9, 10). However, we find that it does not apply to the human musk receptor OR5AN1, or the mouse thiol receptor MOR244-3, as shown by the clear absence of isotope effects with deorphaned human and mouse ORs on exposure to the specific deuterated, ¹³C and non-labeled ligands for these ORs. Our testing included OR5AN1, which strongly responds identically to both muscone and muscone-d₃₀. We also find that muscone-d₃₀ lacks IR absorption in the 1380–1550 cm⁻¹ range (see Fig. 2), clearly at odds with the claims of Gane *et al.*

(14) that a musk receptor “detects vibrations in the 1380–1550 cm⁻¹ range,” and that musk odor requires that “the molecule has intense bands in that region.” Muscone-d₃₀ has even more C–D bonds than found in ligands previously tested by Gane *et al.*, who claim that it is the number of hydrogen vibrational modes that is “essential for detecting the difference between isotopomers” (14).

Our experimental results are consistent with the ability of mice (and other mammals) to discriminate between a large array of non-pheromonal chiral odorant enantiomeric pairs, as well as with the ability of mouse receptor MOR215-1 to discriminate (*R*)- and (*S*)-muscone (27). While it is known that the muscone enantiomers “differ from each other with regard to odor quality and the odor detection threshold in humans (27),” data is not yet available on the response of OR5AN1 to muscone enantiomers. We agree with the suggestion that the “muscone receptor is specific to C15 and C16 macrocyclic ketone compounds and that the ketone moiety may function as a hydrogen bond acceptor” (27). While QM/MM and mutagenesis studies should elucidate the nature of the interaction of musks with OR5AN1, such a suggestion would be in accord with specific hydrogen bonding interactions as observed for the mouse eugenol receptor mOR-EG (82), as well as our observation that hydrocarbon analog cyclopentadecane (3), which lacks the carbonyl group of 1, is inactive toward OR5AN1 (Fig. S3.2).

While some insect and human behavioral/psychophysical studies showed perceptual differences for isotopomers, perireceptor events or trace impurities may be sufficient to explain any isotope effect (2). Finally, with regard to the plausibility of the vibration theory, it has been argued that rather than being causal, any non-isotopic relationship between vibrational frequency and odor may come about indirectly as a consequence of “similar molecules having similar properties” (83) and because “the vibration spectrum of a molecule reflects its structure” (84). Our findings that the vibration theory is not supported by rigorous analysis of the response of OR5AN1 to diverse isotopomers reinforce Sell’s recommendation that those “wishing to study the nature of odorant-receptor recognition should use receptor activation rather than odor as input data” (85).

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Materials and Methods

Chemicals. All odorants were purchased from Sigma-Aldrich, J&K, Chemsy, or synthesized in-house. Deuterium incorporation into compounds 1–4, 8 and 9 was accomplished by literature methods from undeuterated or deuterated commercially available starting materials as described in the SI, with full characterization of all compounds, following purification by chromatography and recrystallization to constant melting point (when possible), by ¹H and ¹³C NMR, IR spectroscopy and GC-MS. Spectra and GC-MS traces are included in the SI. The chemicals were dissolved in DMSO or ethanol and diluted further into working concentrations before experiments.

Heterologous expression of odorant receptors. HEK293T-derived Hana3A cell line was grown in Minimum Essential Medium (Hyclone) containing 10% fetal bovine serum at 37 °C with 5% CO₂. Lipofectamine 2000 (Invitrogen) was used for transfection. Luciferase assays are performed as previously described. After 18 to 24 h, OR, the accessory OR protein, mRTP15, and constructs for firefly luciferase and *Renilla* luciferase expression were transfected into cells. Twenty-four hours after transfection, the cells were stimulated with odorants (plus 30 μM of Cu²⁺ ions when the ligands were (methylthio)methanethiol and bis(methylthiomethyl) disulfide dissolved in CD293 (Invitrogen). We used the Dual-Glo® Luciferase Assay System (Promega) and followed the manufacturer’s instructions for measuring chemiluminescence.

Statistical analyses. One-way ANOVA or unpaired Student’s *t* test was used to compare the 95% confidence interval logEC₅₀ values among isotopomers for each receptor-odorant pair in Figures 3 and 4. Level of significance was * *P* < 0.05. *F* test was used to compare the best-fit values of EC₅₀, Hill Slope, and top of the dose-response curves between the original hydrogenated odorant and its isotopomers in Figures 3, 4, and S3.2. Bonferroni correction was applied to the *F* tests to account for multiple comparisons. Level of significance was * *P* < 0.00076 before correction and * *P* < 0.05 after correction.

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