How does the nose know? Obtaining insight into olfaction.

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I. INTRODUCTION

Smell is important; it is one of the five fundamental ways people perceive the world. Yet, it is not well understood. Smell relies on a sequence of steps: an airborne molecule diffuses through the mucus layer of the nose and binds with a receptor that causes a signal to be transduced to the neural pathway to the brain, where it is then processed to give an odor. The standing explanation for the step in this process where the receptors interact with and detect an odorant is Shape Theory. Studies that claim that the olfactory system, in particular the olfactory receptors, can detect the difference between a molecule and its isotopologues (which differ by one or more isotopic substitutions to the molecule) are challenging Shape Theory in favor of the rival Vibration Theory. This work will focus on evaluating that challenge.

II. SHAPE THEORY

Shape Theory is known as the "lock and key" theory of olfaction. The idea that odorants fit within the nose as a key fits within a lock is not particularly new, the idea can be traced back to the Greek philosophers. Advances in the understanding and importance of molecular structure during the early era of proteins championed by Pauling [1], however, gave the first biologically relevant context for such an idea. The modern foundation of Shape Theory originates with Moncrieff who, in 1949, stated that for a molecule to be odorous it must be volatile and have a molecular configuration that will fit the complementary [what we would now call the active] site of the olfactory receptors [2]. Moncrieff reasoned that there need only be a few olfactory receptors that give primary odors, and it is their combination that gives more complex odors.

Amoore further developed Moncrieff's idea by sorting through the literature for primary odor categories, characterizing the three-dimensional molecular geometries for odorants within a given category, and proposing receptor geometries that would accommodate the set [3]. These initial primary odors were: camphoraceous, musky, floral, peppermint, ether-like, pungent and putrid. The pungent and putrid receptors are sensitive to the electro/nucleophilicity of the odorant, respectively. Figure 1 gives a representative schematic of the shape-based receptor.

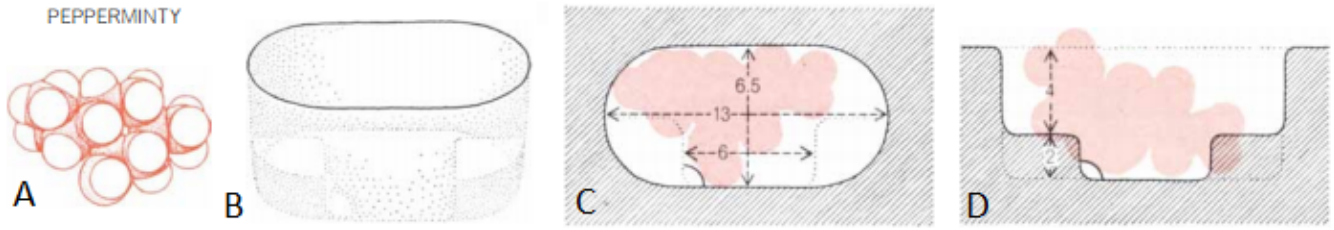

FIG. 1: Schematic of an agonist (A) and receptor(B-D) for one of the seven primary receptors (pepperminty) proposed by Amoore [3]

Axel and Buck discovered olfactory receptors (ORs) nearly 40 years after the development of this theory, an accomplishment for which they won the Nobel Prize [4]. ORs are transmembrane proteins in the cilia of the mucal layer of the nose (Figure 2) with an odorant docking site on the mucal side of the layer and a binding site on the cytoplasmic domain that couples to a heterotrimeric G-protein. When an odorant docks to the site, the G-protein sets off a signaling cascade that leads to the perception of smell [5]. The ORs are the largest class in the family of

G-protein coupled receptors (GPCRs). Estimates give approximately 350 different functional olfactory receptors in the human genome [6]. Individual odorants can activate multiple receptors and each receptor may respond to multiple odorants [7].

Experimental evidence has led to the refinement of the details of Shape Theory, of course, but the fundamental principles of structure-activity remain the essential component of this theory. There are a number of difficulties in establishing structure-activity relationships, because most of the literature supporting Shape Theory relies on assays that are obtained post neurological processing. Asking a person to detect the odd pair out in a vial or an animal to go this or that way in a maze tells more about structure-odor relationships than structure-activity. Structure-odor relationships are much more difficult to analyze, but are the ultimate goal [8]. There are a small number of promising studies however, that restrict their focus to structure-activity. Probably the best type of evidence for OR activity are experimental studies that measure the direct output of the neuron, as each neuron expresses only one OR gene type [9]. For example, Araneda et al. found that the I7 mouse odor receptor was selective towards aldehydes with strict steric requirements at the carbonyl group but fairly indiscriminate at the tail region [10]. To elucidate conformational changes that occur upon binding, a crystal structure would suffice. The difficulties with carrying out these studies are that: a) it has been hard to express ORs on heterologous cells and b) the ORs are transmembrane proteins, which are difficult to crystallize. Because of the difficulty in crystallization, other assays need to be performed to establish the structural evidence necessary to support Shape Theory. Highly variable regions within the OR family are believed to be the characteristic regions that lead to the specificity of the receptors. Man et al. performed an analysis of sequences for human and mouse ORs and determined 22 sequence positions that correspond to known ligand-contact positions for other GPCRs [11]. To further elucidate potential binding sites, others have used computational methods to do docking studies and compare their results to ligand specificities. Because there are no crystallographic structures of these membrane-bound proteins, tertiary structures are predicted from rhodopsin, a model GPCR. For example, Floriano et al. constructed an atomic level structural model for mammalian OR S25 [12]. They tested this model structure (Figure 3) for a potential active site and found affinities for simple aliphatic alcohols and acids. The order of binding energies corresponded with the experimental neuron activation observed by Malnic et al. where heptanol and hexanol were found to be the agonists for this site [7]. Floriano predicts that the specificity results from three key residues that select for functional group and the length of carbon chain: Lys-302 forms a weak hydrogen bond with the hydroxyl group, while Phe-225 and Leu-131 limit the carbon chain length. In a separate study, Katada et al. modeled mouse OR EG to determine the binding site and identified amino acids likely to be important within the binding site [13]. They carried out a series of mutations designed to test the types of interactions (static or van der Waals) with the ligand, the results are shown in Table I. Furthermore, they used this information about the interactions to modify the receptor for a new target molecule. First they did docking studies with the new target molecule, then supported this with experiment.

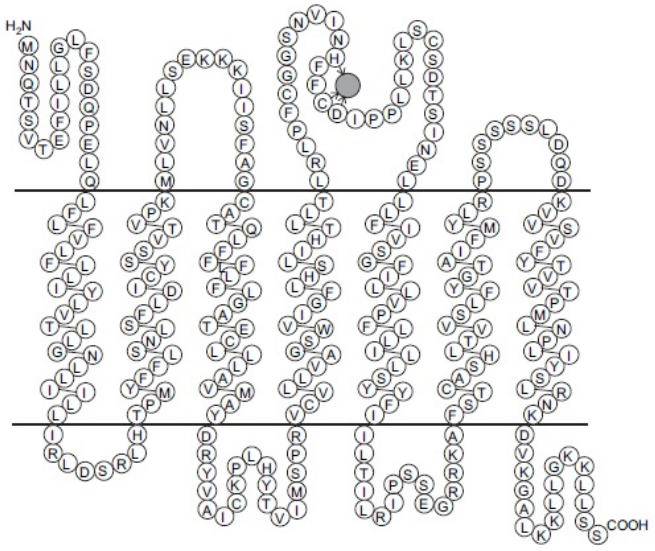

FIG. 2: Olfactory receptors are 7-transmembrane proteins within the cillia of the olfactory sensory neurons [14]

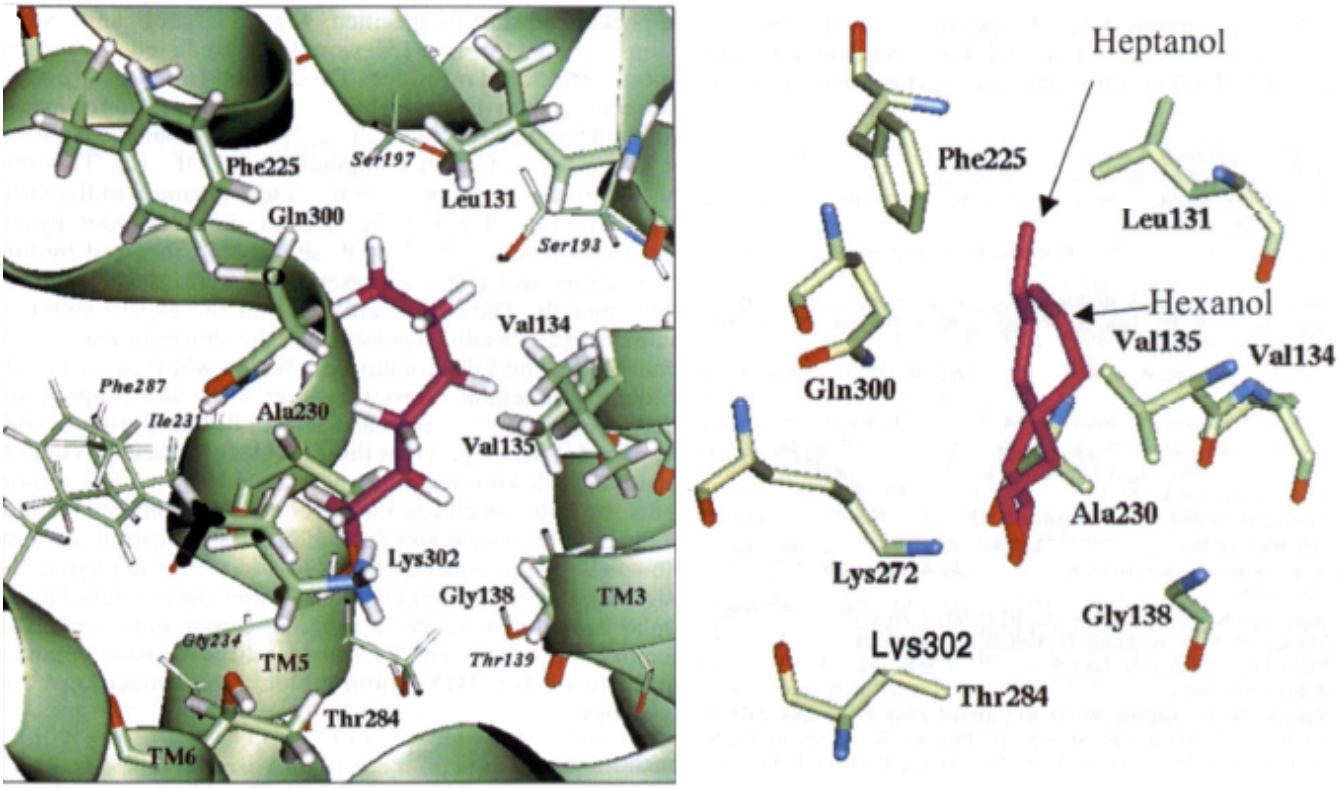

FIG. 3: Predicted binding site of OR S25 with agonists. Left) Longitudinal view with labeling to indicate relative position to the ligand. Residues within 3 A of the ligand (hexanol) have bold labels and have thicker display while residues within 3-5 A are labeled in italics. Right) Detail view with both agonists, the hydrogen atoms were suppressed. [12]

TABLE I: Point Mutations and their implication for ligand-receptor interactions[13].

Residue(s)	Implication
Ser113	Hydrogen donor for an oxygen atom at R4
Thr 211, 255, Leu 212, 259	van der Waals interaction
Arn 207	not conclusive, mutation may have caused folding error
Ile256	spatial configuration of binding pocket
Phe 206, Phe 252	ligand recognition
Thr 205, 280	No role

III. VIBRATION THEORY

A competing theory, Vibration Theory, incorporates certain aspects of Shape Theory, in that odorants have to be a certain shape to enter a geometrically constrained receptor. However, the underlying physical interaction that causes the odorant to activate the receptor is fundamentally different. According to Vibration Theory [15], the olfactory receptor has a pair of sites (electron donating, with energy E_D, coupled with electron accepting, E_A) separated by a junction. An electron can tunnel through the junction either elastically or inelastically. However, when a molecule is present in the receptor, if it has vibrational mode energy $E = \hbar * \omega_0$ where E is equal to the difference of E_D and E_A, then the electron can excite that vibrational mode as it tunnels to the acceptor site. This will increase the rate of inelastic tunneling relative to the elastic tunneling, which will initiate signal transduction. This is illustrated in Figure 4.

There are a few requirements, from a purely physical perspective, for this mechanism to be suitable explanation

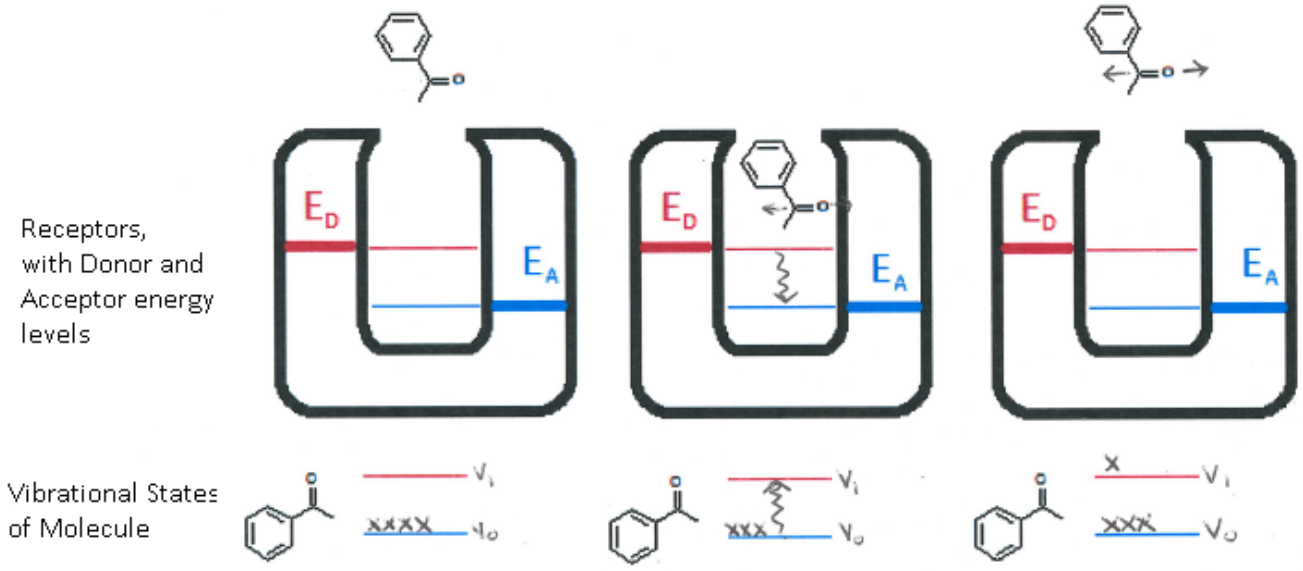

FIG. 4: Left: A molecule in its ground vibrational state approaches receptor. Center: The molecule has docked in the receptor cavity. The energy required to excite the molecule to its first vibrational mode matches the energy difference between E_D and E_A, so the molecule becomes vibrationally excited by the same quanta of energy as the electron loses when it tunnels to the acceptor site; there is an increase in the inelastic tunneling rate. Right: The molecule, now vibrationally excited, leaves the receptor.

for a biological system. The charge transfer rate has to be detectable, and it has to be detected over the background tunneling rate. Making assumptions about the receptor, odorant, and their interactions, Brookes et al. traced the electron's path : originating with a reducing species in the cell fluid, charge transfer to the donor of the molecule, tunneling, and to the release of the G-protein [16]. They estimated the time for the inelastic tunneling process τ_r to occur on the biologically relavant scale of μs to ms. By estimating the interactions, they observed that if the reorganization energy to return to the original state was sufficiently small, then the inelastic pathway could dominate. Solov'yov et al. have also used a similar theoretical approach, estimating physical parameters to justify Vibration Theory[17]. These studies provide some speculation as to how the physics of vibrationally assisted olfaction could be realized with the real protein. Turin, in the original paper [15], proposes that there is a conserved NADPH binding site in the ORs which could be the donor and Zn could be the acceptor, that leads to the severing the the G-protein. Brookes suggests that some (presently unknown) species in the cell serves as the source of electron, but the donor/acceptor pairs should be the HOMO/LUMO of residues with delocalized electrons, like tryptophan or phenylalanine. Solov'yov puts forth that metalic ions could be the source of electrons, and the donor/acceptor pairs could be phenylalanine, tyrosine, or histidine.

Similar to Shape Theory, most support for Vibration Theory relies on vibration-odor relationships (e.g. bitter almond odor: where structurally diverse compounds such as benzaldehyde and HCN share common vibrational frequencies and the "bitter almond" odor quality[15]), but there is some experimental evidence to support vibration-activity relationships. Guo and Kim analyzed the electrophysicological studies of odorant activities in *Drosophila*, and found a small, but non-random relationship between the vibrational spectrum and activity [18]. *Drosophila* has significantly less post-processing complications than other model systems. Franco et al. conducted a study with flies that compared four compounds : citronellyl nitrile, citronella, octanol, and deuterated octanol [19]. The first two form a pair of structurally similar compounds that is different from the last pair of structurally similar compounds. Flies trained to avoid citronellyl nitrile avoided deuterated octanol, and vice versa. However, flies trained to avoid citronella did not have a preference between octanol and deuterated octanol. Flies trained to avoid octanol did not have a preference for citronella or citronellyl nitrile. Since what citronellyl nitrile and deuterated octanol have in common is not shape, but vibrations in the 2200cm^{-1} range (CN and C-D bonds), this supports vibrational theory.

IV. ISOTOPOLOGUE DISCRIMINATION EXPERIMENTS : VIBRATION THEORY'S CHALLENGE TO SHAPE THEORY

Supporters of Vibration Theory claim that it can explain the phenomenon of isotopologue discrimination, while Shape Theory cannot. The rationale is that replacing atoms with a heavier isotope does not change the shape, so therefore if Shape Theory were true, then the activity (odor) of the molecule would be the same. If however, these isotopologues are perceived differently, there must be some physical mechanism underlying the differentiation–which is asserted to be vibrational in origin. Replacing an atom with a heavier isotope changes the force constant of the bond and leads to a different vibrational frequency, in the case of hydrogen with the heavier deuterium it is extremely pronounced (C-D stretch is 2200 cm^{-1} range while C-H stretch is in the 3000 cm^{-1} range). The following studies assess isotopologue discrimination in both flies and humans.

Franco et al. designed a study to test whether flies could differentiate between compounds and deuterated isotopologues [19]. The experimental set up was a standard T-maze where an uneven distribution of flies between the arms in the tube indicates a preference for one of the odorants. They tested acetophenone $C_6H_5OCH_3$ (AP) against three deuterated isotopologues: $C_6H_5OCD_3$ (AP-3), $C_6D_5OCH_3$ (AP-5), and $C_6D_5OCD_3$ (AP-8). Compared to air, the flies found AP to be attractive, with the deuterated compounds being relatively more adversive, in qualitative proportion to their degree of deuteration. The flies also preferred AP in direct comparison to each of the deuterated compounds. The authors also tested different pairs of normal and deuterated odorants (octanol (OCT) and $C_8D_{17}OH$ (OCT-17); benzaldehyde C_6H_5COH (BA) and C_6D_5COH (BA-D5)). The flies showed a spontaneous differential avoidance of OCT-17, but did not differentiate between BA and BA-D5. There were also a series of experiments where the flies were trained with foot shock to avoid either the deuterated or non-deuterated molecule of a test pair and were then confronted with a new pair of odorants, one deuterated and one not. Franco found if the flies were trained to avoid a deuterated molecule, they avoided the deuterated molecule of the novel pair, and if they were trained to avoid the non-deuterated molecule, then they did so in the novel pair (summarized in Table II).

TABLE II: Flies trained to avoid either deuterated or non-deuterated molecule in test pair exposed to a novel pair[19].

Trained against	Selected against
OCT	BA
OCT-17	BA-D5
AP	BA
AP-8	BA-D5
BA	AP
BA-D5	AP-8

Haffenden et al. performed a study to determine whether humans can detect the difference between BA (which has a bitter almond smell) and its isotopologues (BA and $C_6H_5^{13}COH$ (BA-C1) and $^{13}C_6H_5COH$ (BA-C6) and C_6D_5COD (BA-D6)) and then interpreting this result in light of the vibrational character of the molecules [20]. The discriminative test utilized in the study is the duo-trio test, with thirty trained panelists, not performed double blind. Each panelist received three sets of three samples, where two of the samples were the same. The panelists were asked to sniff the samples once (with one minute of recovery between sniffs) and then identify the odd sample. Subjects were able to identify the difference between BA-D6 and BA (but only BA-D6), even when the authors corrected for the probability of guessing right and an additional confounding variable, the order of presentation. Haffenden conducted an analysis of the infrared spectra of the isotopologues compared to BA and concluded the spectra with the greatest difference was that of BA-D6 where the aldehydic C-H stretches are shifted 700 cm^{-1}, as compared to the 200cm^{-1} of BA-C6, and no shift in the BA-C1. In Turin's theory of vibrational olfaction, each of the olfactory sensors span a 400 cm^{-1} segment of the vibrational spectrum. The authors thus conclude that the shift of BA-D6 moves it out of the sensor's range, the sensor necessary but perhaps not sufficient for the smell of bitter almond.

A second study, performed double blind, on whether humans can discriminate between isotopologues (using the pair AP/AP-8) was conducted by Keller and Vosshall [21]. Subjects were asked to rate the similarity of a pair of vials on a scale of one to ten. The scores given for the pair AP and AP-8 had no significant difference in rating compared to the pairs which contained the same odorant. In addition, a triangle test was performed, where the subjects had to identify the odd sample out of a group of three as well as the duo-trio test where the subjects were given two odors and asked to match one to the reference compound. In neither of these tests were subjects able to distinguish between AP and AP-8 with statistical significance.

V. DISCUSSION

The supporters of Vibration Theory cite the ability to differentiate between isotopologues as evidence against Shape Theory because there is no appreciable difference in the shape, but there is a difference both in the vibrational frequency and odor character of the molecule. The flaw in this argument is that Shape Theory has been simplified to the concept that shape and shape alone determines the odor character. However, even from the early work of Amoore, Shape Theory has been much more nuanced, incorporating the chemical/ physical properties of electrostatics. Possible alternative explanations for the differentiation between isotopologues that are still consistent with Shape Theory, providing that it can be established, are 1) the difference in binding energies for the heavier molecules [22] and 2) the spatial patterning of the receptors [23] causing a difference in the temporal processing of signal because the heavier molecules are slower.

As to whether, isotopologue are actually able to be selectively discriminated in experiment, there remains some disagreement. In the two studies with humans, one reported the ability to differentiate between odorant pairs and the other did not. The work of Haffenden et al. raises a few questions regarding its statistical validity. Firstly, the tests were not double blind. Secondly, the authors claim they are performing a duo-trio test, when they are really performing a triangular test. While these tests should be able to answer the same question, they do have different difficulties. These are separate concerns than the statistical oddities (chance guessing,order of presentation) that the authors raised in their own paper. They report that only BA-D6 is differentiable from BA with the corrections.The claim of this paper is that the aldehyde C-H stretch is the one key vibrational frequency is required to give BA and its derivatives a common odor (bitter almond), which requires that only BA-D6 will be different, because it has the greatest difference. However, with their recalculated p-values, both BA-D6 and BA-C1 should be considered differentiable from BA. They need to have stricter criterion than the standard p=0.05% to make the claim that only BA-D6 is differentiable or they need to revise their conclusions. Furthermore, the aldehyde stretch proposed to be necessary for the almond odor (2500 to 3000 cm^{-1}). This region is different than Turin's original prediction (fingerprint region) [15]. It also does not contain the frequencies of HCN which has an almond odor. Thus, these results actually counter the claim that vibrationally assisted mechanism is responsible for the almond odor. The criticism of levied on the work of Keller[21] is based on the premise that untrained panelists will not recognize fine nuanced difference that trained panelists will. To resolve these two experiments at a structure-odor level, the seven compounds could be tested using the methods of Keller but testing trained and untrained panelists and comparing their results.

The work of Franco et al. gives the best support both for the isotopologue discrimination phenomenon and Vibration Theory. While considered as individual pairs, the isotopologue effect can be reconciled with Shape Theory. However, Franco et al. showed that flies trained against a deuterated molecule, avoided other deuterated compounds. What feature other than the deuteration could they share that allows to discriminate in this way? The non-deuterated versions are known from Hallem et al. to activate different receptors [24]. Even stronger evidence for Vibration Theory in Franco et al. shows that flies can learn to recognize molecules that are structurally different but share the same vibrational frequency.

From the standpoint of vibration-odor relationships, while no clear picture can really be determined for humans, there seems to be much stronger evidence for *Drosophila*. That the results may differ for *Drosophila* and humans is not unexpected. While both receptors have portions that are 7-transmembrane proteins, the *Drosophila* proteins are topologically inverted from the human GPCRs [25]. Also, *Drosophila* expresses a co-receptor not present in vertebrates. Recent studies suggest that *Drosophila*'s receptors are heteromeric ligand-gated ion channels, which are fundamentally different than vertebrate GPCRs([26],[27]). One of the studies [27] suggested there may also be a G-protein mediated pathway, but this is highly controversial. The differences in the vertebrate and insect receptors has not been given due weight the in the discussion of the biological viability of vibrational theory.

These three isotopologue studies have attempted to shed light on how the interaction between odorants and receptors work by inference from molecule-odor data. Rather than any of the experimental modifications to the isotopologue studies presented earlier in the discussion, efforts would be better focused on attempting to directly observe the molecule-activity data instead. Because of the differing nature of their receptors, I propose two experiments, one for flies and one for vertebrates.

For the fly study, recall that Hallem et al. [24] tested a 24 odor receptors by observing the neuronal response to a variety of odorants including: OCT, AP, and BA. First, for control, the experiment should be repeated with these three odorants (chosen for consistency with previous work and commercial availability of their isotopologues). For preliminary experiments, it would be reasonable to focus only on the receptors that give different differential responses for each odorant. For example, when presented with BA, ON7a gives 200+ spikes. For AP, it gives less than 50 spikes, and OCT inhibits spiking to 50% of spontaneous firing rate. Then test the battery of isotopically substituted derivatives (BA derivatives: BA-C6, BA-C1, BA-D5, BA-D6; AP derivatives:AP-3, AP-5,AP-8; and OCT derivative: OC-17. If the responses of the neurons change at all, that is evidence for the differentiability of the

isotopologues. If the same receptors are activated, but to differing extents, then that is not support for either shape or Vibration Theory. If a new receptor is activated by all three, that was previously quiescent in each of the non-deuterated compounds, that would give strong support for Vibration Theory. It would also be interesting to look if spike rate could have some correlation with the relative number of heavier isotopes within a molecule, as Franco. et al. saw the adversiveness to AP-3 was less than that of AP-5, which was less than that of AP-8.

For vertebrates, there is the capacity to do a much more comprehensive study. There are fewer receptors that have been isolated and grown to determine the specificity in experiment in vertebrates than in insects. However, approximations can be made as to the three dimensional geometry for vertebrates receptors because they are part of the larger GPCR family, and a crystal structure is known for a model of that family. In insects, the receptors are fundamentally different and the structure has not yet been determined. A handful of vertebrate ORs have been thoroughly studied and have a prediction for a three dimensional structure and active site that is consistent with the activity. This allows for a complementary computational component (docking studies) in addition to experiments that directly measure the difference in neuron output for the agonists and their isotopologues. A suggested receptor for initial studies would be OR S25 because it already has a computational model [12] and it is highly specific to relatively simple compounds: aliphatic alcohols. Hexanol and heptanol are the agonists, and deuterated isotopologues are commercially available. Docking studies that compare the binding energies of deuterated and non-deuterated molcules could also be carried out. There is also room to continue the work of Floriano [12]; researchers could do a point mutation of Phe-225 and Leu-131 to see if a greater variety compounds with tails of different lengths and branching would activate the receptor, similar to what has been done by Katada et al. [13].

VI. CONCLUSIONS

The debate between Shape Theory and Vibration Theory has been quite heated in the literature [28], [29], [30], [31], [32]. This has been complicated because of the historical difficulty in obtaining direct observation of odorant-receptor interactions. Shape Theory has much greater evidence, especially in vertebrates. Isotopologue discrimination experiments provide a test that challenges Shape Theory and could provide support for Vibration Theory, however, the evidence is limited. In the current state, it seems like there is no evidence for isotopologue discrimination in humans or other vertebrate systems, but there is for flies. Thus, it stands that Shape Theory best explains vertebrate olfaction. For flies, there is evidence for isotopologue discrimination in support of Vibration Theory. Insects have a fundamentally different type of receptor, so this difference is not surprising and means that the mechanism, and therefore the theory of olfaction, for flies may well be different that of humans. However, the evidence suggests that vibrations play a minor role[18] and it may be possible that some small number of receptors are activated via vibrationally assisted means, rather than all of them. Thus, Vibration Theory should be incorporated into the theory of olfaction in insects, without suppressing the major contributions from Shape Theory. In this work, experiments are suggested that could further shed light on this area of research and lead to a resolution.

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