Molecular vibration-sensing component in *Drosophila melanogaster* olfaction

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A common explanation of molecular recognition by the olfactory system posits that receptors recognize the structure or shape of the odorant molecule. We performed a rigorous test of shape recognition by replacing hydrogen with deuterium in odorants and asking whether *Drosophila melanogaster* can distinguish these identically shaped isotopes. We report that flies not only differentiate between isotopic odorants, but can be conditioned to selectively avoid the common or the deuterated isotope. Furthermore, flies trained to discriminate against the normal or deuterated isotopes of a compound, selectively avoid the corresponding isotope of a different odorant. Finally, flies trained to avoid a deuterated compound exhibit selective aversion to an unrelated molecule with a vibrational mode in the energy range of the carbon–deuterium stretch. These findings are inconsistent with a shape-only model for smell, and instead support the existence of a molecular vibration-sensing component to olfactory reception.

Olfactory systems perform remarkable feats of molecular recognition, but although much is known about the neurophysiology of olfaction (1–5), how olfactory receptors “read” molecular structure remains unknown. Parts of odorant molecules (odotopes) have been proposed to engage particular receptors in a “lock-and-key” manner and this molecular shape recognition mechanism is thought sufficient for odor discrimination (2). An alternative hypothesis (6) posits that molecules vibrate all atoms, or of particular functional groups of odorant molecules, contribute to odor recognition, and odorants with similar vibrational spectra should elicit similar olfactory responses (7). Molecules in which deuterium replaces nonexchangeable hydrogens constitute appropriate probes to test these alternatives because deuteration does not alter atom size or bond length or stiffness (8). Thus, the conformation of a deuterated molecule should be identical to that of a hydrogen-only (i.e., normal) odorant and, according to the molecular recognition theory, the two isotopes should smell identical. However, atoms in a molecule vibrate in normal modes at particular energies that depend on the molecular structure. The doubling of nuclear mass upon deuteration will change all the vibrational modes of an odorant molecule to differing extents. The second hypothesis predicts that deuteration will alter its smell in comparison with the hydrogen-only isotope. A recent attempt to test the latter hypothesis in humans was rather inconclusive, potentially confounded by interference from previous experience, olfactory training or habituation of the subjects, or shortcomings of human olfaction as suggested by the authors (9). A major experimental difficulty is that, in general, isotopes are prepared and purified differently, and therefore perceived differences in smell could be attributed to different impurities. Purity can, in principle, be ensured by smelling single-molecule peaks exiting a gas chromatographic column, but this makes simultaneous two-compound comparisons difficult and animal experiments harder still.

Here we describe a different approach to test whether molecular vibrations contribute to recognition of odor character. We assumed that, if molecular vibrations are indeed detected, the deuterated isotopes of different odorants may share a common odor character, as deuteration shifts particular peaks—e.g., the carbon–deuterium (C–H) stretch—of the compound-specific vibrational spectra to lower frequencies. If so, the deuterium odor character could be distinct and identifiable, irrespective of the structure and chemical properties of the odorant molecules that carry it. Significantly, we used *Drosophila* as unbiased and objective subjects to address this issue. They possess a relatively well understood olfactory system (10–13), exhibit keen olfactory discrimination (14–16), and can be conditioned to selectively avoid or seek odors with the use of established methodology (17, 18). We ask whether *Drosophila* can detect deuterium as a distinguishing molecular feature in odorant isotopes and a salient cue for conditioning. The results of these experiments provide support for the notion that flies can smell molecular vibrations.

Results

Spontaneous Differential Responses to Deuterated Odorants. Although deuterium does not appreciably change molecular shape, atom size, or bond length or stiffness, it doubles hydrogen mass, thus affecting the overall vibrational modes of an odorant. Therefore, if recognition of molecular shape alone was the sole determinant for odor character (2, 3), then flies should not respond differentially to deuterated [d, where d\(x\) denotes replacement of \(x\) nonexchangeable hydrogens with deuterium atoms] and nondeuterated/normal (i.e., H-) odorants. To address this hypothesis, we took advantage of the commercial availability of acetophenone (ACP) carrying three, five, or eight deuterium atoms (d\(_3\), d\(_5\), and d\(_8\)) in place of the respective hydrogens in the normal molecule (h-ACP). Equal amounts (75 µL) of each odorant were diluted to 1 mL in isopropyl myristate and we quantified (Fig. 1A) the response of groups of flies to each odorant versus unscented air traversing the arms of a standard T-maze (Materials and Methods) (15, 20). When given a choice between normal ACP (i.e., h-ACP) and air, the flies present in the odor-delivering maze arm indicated that, at the dilution used, the odorant was attractive. However, replacing only three hydrogen atoms with deuterium eliminated this spontaneous preference as the flies distributed nearly equally in the arms of the maze. The d\(_3\)-ACP was mildly aversive and the fully substituted d\(_8\)-ACP was the most aversive of the four isotopes. Moreover, flies consistently avoided d\(_3\), d\(_5\), and d\(_8\) ACP against equal concentration of h-ACP (Fig. S1). Therefore, a spontaneous switch in osmotaxis (14) to ACP was precipitated by substituting hydrogen atoms with deuterium.

To verify that flies are generally capable of spontaneous discrimination between airborne deuterated odorants and their normal counterparts, we additionally presented them with equal concentrations of normal and deuterated 1-octanol, normal and d\(_2\)-benzaldehyde, or a normal–perdeuterated ACP pair. In confirmation of the previous results, h-ACP was preferred versus an equal concentration of the d\(_8\) odorant (Fig. 1B). Reducing the concentration of the deuterated compound by 50% resulted in...
Fig. 1. Differential spontaneous responses to odorants containing deuterium. The mean relative distribution of flies in the arms of the maze (% excess flies) carrying the indicated odorants ± SEM is shown in all graphs. This metric reflects the prevailing distribution within the arms of the maze of groups of 40 to 60 flies tested each time. The total number of flies tested in each group is shown and the number of groups tested is n ≥ 6 for all groups. (A) Spontaneous responses to 75 μl normal or d3-, d5-, or d8-ACP, each diluted with isopropyl myristate (IPM) to 1 mL. Flies spontaneously preferred h-ACP over the solvent. In contrast, incorporation of five or eight deuterium atoms results in significantly different distribution (P < 0.001) from that toward h-ACP (attraction) to aversion. In contrast, the response to d3-ACP was not significantly different (P = 0.012) from that of h-ACP. (B) Files discriminate against d8-ACP if presented with equal amount (1:1) of h-ACP (75 μl odorant/925 μl IPM). However, a 50% reduction in the amount of deuterated odorant yielded an equal distribution of the flies in the arms (% excess flies not significantly different from zero), defined as a balanced maze. (C) Similarly equal amounts (200 μl) of h-1-octanol (OCT) and d1-1-octanol yielded strong discrimination against the deuterated odorant, which was eliminated upon reducing it by 75% (1:0.25). (D) In contrast, equal amounts of normal and deuterated benzaldehyde (90 μl) did not result in differential discrimination. In accord, decreasing the amount of h-BZA resulted in differential avoidance of d1-BZA. (E) The preferential discrimination against d8-ACP was eliminated in Or83b1 and Or83b2 mutants (P < 0.002 and P < 0.001, respectively, Dunnett test). (F) Similarly, discrimination against d1-1-octanol was eliminated in Or83b1 and Or83b2 mutants (P < 0.001 for both, Dunnett test).

Generalization of Conditioned Isotope Discrimination Across Odorant Pairs. The results so far are consistent with an unambiguous difference between deuterated and normal odorants for all three pairs tested. Is the difference associated with the majority (≥99%) compound or with impurities? Although the odorants used were of the highest purity available (Figs. S3 and S4), they

balanced distribution of the flies in the maze arms, indicative of equal response to the two isopes (i.e., balanced maze). Similarly, d1-1-octanol was preferentially discriminated against versus equal concentration of the normal odorant. In this case, however, the deuterated isotope appeared even more aversive, as 75% reduction in its concentration equalized aversion with h-1-octanol (Fig. 1C). In contrast, flies did not exhibit spontaneous differential avoidance of d3-benzaldehyde, which occurred only upon reducing the concentration of the normal odorant (Fig. 1D). These differences cannot result from reduced evaporation of the slightly heavier deuterated odorants because two of the three elicited increased aversion, known to be proportional to the concentration of airborne odorants in this T-maze system (14, 20, 21).

If the differential response to the deuterated odorants relied solely on olfaction and not any other sensory modality, it should be completely eliminated in anosmic mutants. To that end, we obtained two anosmic Drosophila strains carrying the null alleles Or83b1 and Or83b2 of the gene encoding the common subunit of the dimeric Drosophila olfactory receptor (13, 22, 23). Clearly, the spontaneous avoidance of deuterated d3-ACP and d1-1-octanol were eliminated and the mutant flies distributed equally in the maze arms as expected (Fig. 1 E and F). Therefore, Drosophila use olfaction alone to discriminate between deuterated and normal odorants. Furthermore, the spontaneous discrimination against deuterated odorants indicates that they likely present to the flies recognizable, salient features distinct from their hydrogen counterparts.

Conditioned Discrimination of Isotopes. If the deuterated and normal isotypes of a pair exhibit salient odor character differences, then Drosophila should be able to associate either odorant with a punishing stimulus (20). To eliminate bias, the amounts of each odorant in a pair were adjusted as shown in Fig. 1 B–D to yield spontaneous preference as near zero as possible (i.e., balanced maze), so any postconditioning distribution changes would be a consequence of training. Flies were conditioned to associate electric foot-shock punishment with the presence of the deuterated or normal odorant of a pair. Drosophila successfully associated either odorant with punishment, demonstrated by the conditioned selective aversion of the shock-associated odor upon testing (Fig. 2). Flies shocked in the presence of the normal odorant distributed preferentially in the arm carrying its deuterated counterpart and vice versa. The shock-associated learning extended to h-benzaldehyde and d2-benzaldehyde, for which the flies exhibited no spontaneous preference, suggesting, as expected (24), that conditioned discrimination is independent from spontaneous preference.

This was not an exclusive property of the w1118 control strain (14), as experiments were repeated with Canton-S, a different WT strain, with identical results (Fig. S2 A and B). We also reversed the order of stimulus presentation, such that the shock-associated odor was delivered immediately before testing. Flies continued to selectively avoid the shock-associated odor (Fig. 2C), eliminating the possibility that they were simply attracted to the odor presented last in the absence of the shock reinforcer. Thus, deuterated and normal odorants present salient differences to the fly olfactory system, which can be used to predict punishment or its absence by association with either isotope.
nevertheless may contain small amounts of impurities that, in principle, could account for the spontaneous preferences and the shock-associated learning. To examine this possibility, we asked whether Drosophila could recognize deuterium as a salient feature from one odorant pair to another (i.e., generalize). If different impurities were present, salient features(s) of the training odors allowing their discrimination would be absent if testing with a different odorant pair. Hence, the flies would be unable to generalize (25) and thus distribute equally in the maze arms, as if faced with novel odors. In contrast, if the deuterium odors had a common salient feature or odor character, then flies could, in principle, generalize between pairs.

To address this hypothesis, Drosophila were trained to selectively discriminate against deuterated or normal 1-octanol or deuterated versus normal ACP, but tested against the novel pair h-benzaldehyde versus d5-benzaldehyde. We found that flies trained to avoid h-1-octanol avoided h-benzaldehyde selectively when tested with the h-benzaldehyde/d5-benzaldehyde pair. Conversely, conditioning to discriminate against d5-1-octanol resulted in selective avoidance of d5-benzaldehyde (Fig. 3A). Similarly, flies trained to selectively avoid h-ACP discriminated preferentially against benzaldehyde and those trained against d5-ACP avoided the deuterated isotope selectively (Fig. 3B). Because h-ACP is attractive, the task of discriminating against the aversive h-benzaldehyde was expectedly more difficult than the converse choice, as indicated by the modest selective discrimination (Fig. 3B).

To further substantiate these findings, we also trained with the h-benzaldehyde/d5-benzaldehyde pair, which do not elicit differential naive discrimination, and tested against normal and deuterated ACP. Again, flies exhibited differential avoidance of d5-ACP if conditioned with d5-benzaldehyde and of h-ACP if trained with h-benzaldehyde (Fig. 3C). Therefore, generalization occurred irrespective of the odorant pairs used for training and testing, suggesting that salient differences independent of their chemical identity and shape allow learned selective aversion to be transferred from the training to the testing pair.

Significantly, even if impurities were present, it is not expected that they would be similar in all three odorant pairs and account for differential conditioning, because the syntheses of the six compounds are different. Furthermore, the graded switch from attraction to aversion for a single molecule, ACP, clearly depended on the number of deuterium atoms it carried (Fig. 1A). Hence, it would be remarkable for an aversive impurity to fortuitously follow the number of deuterium atoms in that odorant. Nevertheless, could a single aversive impurity present only in every deuterated compound conceivably account for these results? If this were the case, it is difficult to explain how this impurity is ineffective in d5-benzaldehyde, consistent with the observed lack of spontaneous avoidance (Fig. 1D), but renders d5-ACP and d5-1-octanol aversive. Furthermore, it is possible that benzaldehyde masks the putative impurity, a potential explanation for the equivocal selection of its two isotopes. However, if the putative impurity was masked, then, in contrast to our results (Fig. 3A and B), the benzaldehyde isotopes would not elicit differential discrimination in flies conditioned with 1-octanol/d5-1-octanol or ACP/d5-ACP. Collectively, then, impurities cannot account for the observed across odorant differential conditioned discrimination. In contrast, the generalization results are explained if we hypothesize that the salient cue(s) is a common property that differentiates the isotopes of each pair and distinguishes all deuterated odors from their normal counterparts. As this cannot be molecular shape, differential evaporation, or impurities, it is likely that the common salient cue is the presence or absence of deuterium.

Odorant Molecular Vibrations Are Salient Cues for Conditioning. The doubling of hydrogen mass by deuteration will affect every molecular vibration in which movement of the hydrogen atoms occurs. When the vibrational mode involves mostly heavy atoms (i.e., C, N, O), the change in frequency is modest because of the relatively small change in mass of the deuterated versus normal compounds. In contrast, when most of the mode motion is in the hydrogen atoms themselves, as is the case for C-H stretch, wag, and scissor modes, the effect of deuteration on mode frequency will be large. A plausible biophysical mechanism for detection of molecular vibrations has been proposed previously (6), and a detailed study of the underlying physics was recently presented.
Fig. 3. Drosophila can be conditioned to selectively avoid deuterium. The mean relative distribution of flies in the arms of the maze (% excess flies) carrying the indicated odorants ± SEM is shown and the total number of flies in each group is denoted. The mean was derived from at least six repetitions per group. Drosophila was conditioned with the indicated pairs of isotopes, but tested against a different pair as indicated. The test odorants were added such that avoidance of naive animals was as near zero as possible (open bar) and was used to compare the distribution of conditioned animals. (A) Animals conditioned with h-1-octanol, but tested after training with d5-BZA versus d5-BZA exhibited significantly different distribution than that of naive animals (P < 0.001 vs. naive, Dunnett test). Animals trained with d17-1-octanol selectively avoided the deuterated test odorant and vice versa. (B) Animals punished to h-ACP discriminated selectively against h-BZA, albeit not as robustly as previously described (P = 0.004 vs. naive, Dunnett test), but ones trained to avoid d5-ACP avoided the deuterated BZA efficiently (P < 0.001 vs. naive, Dunnett test). (C) Flies conditioned to avoid d5-BZA exhibited efficient selective avoidance of d5-ACP and h-ACP, respectively (P < 0.001 vs. naive, Dunnett test).

Discussion

Flies, like humans, perceive odor quality and intensity and can be conditioned to discriminate differences in its concentration (28, 29), and at sufficiently different concentrations, the same odorant may appear as distinct qualities (30). Therefore, it can be argued that flies discriminate d5-ACP and d17-1-octanol from their normal isotopes based on putative (small) concentration differences we observe are caused by a property of isotopes unrelated to their vibrational modes.
selectively avoid the molecular vibrations. Deuterated compounds are more aversive than their non-deuterated counterparts. This is also consistent with independent recent evidence suggesting contributions of the vibrational spectra of odorants in the electrophysiological response of isolated Drosophila olfactory receptors (31).

In the past there have been four main objections to the notion that olfaction detects molecular vibrations. First, that molecular shape is an adequate predictor of smell (2, 30, 32), which currently seems unlikely (33, 34). In fact, Drosophila has only 62 olfactory receptors (35), suggesting that a single receptor must generally respond to multiple odorants, and in addition that a simple odorant can activate multiple receptors (10, 15). Clearly, therefore, the structural recognition mechanism alone does not suffice to explain odorant recognition, suggesting that additional properties of these volatile molecules likely contribute to the process. The second objection was to the main assertion of vibrational theory that odorants with similar spectra should produce similar olfactory responses and, conversely, molecules of identical molecular shape but distinct spectra should smell different. Our data are consistent with this hypothesis, at least in flies, and suggest that with gas chromatography-pure odorants, and perhaps with the use of aversive conditioning, similar effects may be revealed for vertebrates—even humans—as shown for perceptual discrimination of enantiomers (36). The third objection has been that no known biological mechanism could behave as the equivalent of a vibrational spectroscopy. However, the proposal (6) that olfaction uses inelastic electron tunneling spectroscopy (IETS) has made the idea physically plausible. IETS is a quantum mechanism whereby electrons move from a donor to an acceptor at constant total energy, although the acceptor is energetically lower than the donor (37). To satisfy conservation of energy, tunneling occurs only if a molecule is present between donor and acceptor, possessing vibrational mode(s) at or near this excess energy, absorbing it, and becoming excited. A modified IETS mechanism appropriate to proteins was recently described (26), suggesting a testable model applicable to olfactory receptors. Finally, it was objected that enantiomers with identical vibrations should always smell the same, whereas some smell different (38). Given that proteins are chiral, a shape-only theory cannot account for the identical odors of most enantiomer pairs. In contrast, this objection is easily answered by IETS because it exhibits the pronounced polarization effects expected when sensing molecules in fixed orientations such as enantiomers (6).

Individual olfactory receptors in Drosophila and other species can mediate excitatory and inhibitory responses to different odorants (10). Our data suggest that the vibrational mode and frequency of particular atoms and active groups of odorant molecules may also provide discriminatory cues that could broaden the recognition repertoire of odorant receptors, but still retain specificity. Currently, we do not know whether the same or different olfactory receptors are recruited to sense the normal and deuterated versions of the odorant molecules, and this question is currently under investigation broadly guided by the following considerations. A single receptor may recognize, by broad odotope features, a given odorant whose particular vibrational resonance may contribute to the odor-specific activity patterns of odorant receptor neurons (39), potentially modifying its particular odor character. This hypothesis predicts that receptors with distinct shape selectivities must also recognize similar vibrational modes and frequencies to explain our data of selective avoidance of citronellyl nitrile after conditioning with d17-1-octanol and vice versa. By analogy to color vision, it is possible that the multiple olfactory receptors may be divided into spectral classes, the members of each class sensing the same vibrational range and differing in their affinity for molecules of different shapes and physical properties.
For example, we would expect to find several receptors in the 2,150-cm⁻¹ band, able to sense deuterated molecules but not their undeteriated counterparts.

If the proposed vibrational sensing component is arranged in broad frequency bands of 100 to 200 cm⁻¹ wide, 10 to 20 receptors would suffice to cover the entire vibrational range. However, why then do flies (and mammals) have many more receptors (2, 3)? A possible answer may be that the olfactory system has evolved to reconcile conflicting requirements, namely, to sense a wide range of odors, thus being nonspecific, but also to possess high affinity to detect them in small concentrations. Therefore, multiple receptors may serve to ensure that one or more will always bind with adequate affinity to any molecule to provide broad olfactory recognition through the combined activities of multiple receptors. Testing these and other predictions is well within reach of available behavioral, imaging, and genetic techniques.

Materials and Methods

_Drosophila_ were cultured as described previously (40) under a 12-h dark, 12-h light cycle. Mixed-sex groups of 2- to 3-old flies were lightly anesthetized and segregated in groups of 50 to 70 animals in vials containing food. Twenty-four hours later they were changed to fresh vials and placed in the dark. Olfactory behavior experiments commenced. Details of the behavioral procedures can be found in SI Materials and Methods.

All behavioral experiments were performed at 25 °C and 80% to 85% relative humidity. Training and testing were performed in complete darkness, with dim red light used only during manipulations not requiring exposure to odors. The standard T-maze was modified by replacing the odorant holding cups (20) with glass cylinders of 2.25 cm diameter and 14 cm height as described previously (19, 40). An air stream of 600 mL/min passing over the meniscus carried the odors to the maze arms via silicon rubber tubing. The meniscus area was kept constant by maintaining the volume of the odorant and the solvent, isopropyl myristate (Fluka), at a total of 1 mL in all experiments. Deuterated compounds were from CDN Isotopes. The amounts of odorants added to isopropyl myristate to yield equivalent avoidances were 200 μL of 1-octanol (Fluka) and 50 μL of the fully deuterated d₅-1-octanol, 90 μL of benzaldehyde (Fluka) and 90 μL of perdeuterated benzaldehyde-d₃ 150 μL of ACP (Fluka) and 75 μL of the perdeuterated d₅-ACP, 100 μL of citronellol and 100 μL of citronellol nitrile (gift of International Flavors and Fragrances). As for d₅-ACP (CDSCD3C3), 75 μL d₅-ACP (CDSHSOC3D3), d₅-ACP (CDSDOCH3), and normal odorant were also used for discrimination experiments. Different sets of silicone rubber tubing holding cylinders and maze arms were used for each odor. Complete descriptions of the behavioral procedures are presented in SI Materials and Methods.

Computation of IR Intensity Spectra. Spectra were computed by using the Amsterdam Densit Functional software package (www.scm.com) at D2PBE level of theory. Frequencies were computed numerically by differentiation of energy gradients in small displaced geometries. The force constants and hence the frequencies were computed by comparison of the gradients (in the harmonic approximation of the energy surface). Under these conditions, intensities are proportional to the change in dipole occurring during atom movements in a given vibrational mode (41). Accuracy of mode calculations is typically fewer than 10 wave numbers for molecules comprising C, O, N, and H atoms.

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