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Minute Impurities Contribute Significantly to Olfactory Receptor Ligand Studies: tales from Testing the Vibration Theory.

Minute impurities distort olfactory receptor responses

M. Paoli[1,2,*], D. Münch[1,*], A. Haase[2], E. Skoulakis[3], L. Turin[3] and C. G. Galizia[1]

[1]*Neurobiology, University of Konstanz, Konstanz, 78457, Germany*

[2]*Department of Physics and Center for Mind/Brain Sciences, University of Trento, Povo, TN 38123, Italy*

[3]*Division of Neuroscience, Biomedical Sciences Research Centre "Alexander Fleming, Vari, 16672, Greece"*

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*M.P. and D.M. contributed equally to this work

Correspondence should be addressed to C. G. Galizia, E-mail: galizia@uni.kn

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List all Author Names and Affiliations in order as they would appear in the published article

Paoli M*[1,2], Münch D*[1], Haase A[2], Skoulakis E[3], Turin L[3], CG Galizia[1, 4]

* contributed equally to this work

[1]: Neurobiology, University of Konstanz, 78457 Konstanz, Germany.

[2]: Department of Physics and Center for Mind/Brain Sciences, University of Trento, Povo (TN), 38123, Italy

[3]: Division of Neuroscience, Biomedical Sciences Research Centre "Alexander Fleming," 16672 Vari, Greece

[4]: correspondence to: galizia@uni.kn

Author Contributions:

All authors designed research. MP and DM performed research. MP, DM and CGG analyzed data. CGG wrote the paper, all authors edited the paper.

Correspondence should be addressed to (include email address)

CGG, galizia@uni.kn

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Abstract

Several studies have attempted to test the vibrational hypothesis of odorant receptor activation in behavioral and physiological studies using deuterated compounds as odorants. The results have been mixed. Here we attempted to test how deuterated compounds activate odorant receptors using calcium imaging of the fruit fly antennal lobe. We found specific activation of one area of the AL corresponding to inputs from a specific receptor. However, upon more detailed analysis, we discovered that an impurity of 0.0006% ethyl acetate in a chemical sample of benzaldehyde-d_5 was entirely responsible for a sizable odorant-evoked response in *Drosophila melanogaster* olfactory receptor cells expressing dOr42b. Without gas chromatographic purification within the experimental setup, this impurity would have created a difference in the responses of deuterated and non-deuterated benzaldehyde, suggesting that dOr42b be a vibration sensitive receptor, which we show here not to be the case. Our results point to a broad problem in the literature on use of non GC-pure compounds to test receptor selectivity, and we suggest how the limitations can be overcome in future studies.

Significance statement

How exactly odorant receptors create selectivity for some odorants against the vast number of alternatives remains as yet unclear, and is generally probed by measuring responses to different substances. Chemical senses are highly sensitive to minute amounts of odorants in the environment. Therefore, when testing the responses of olfactory receptors, substances of highest purity are used, generally 95% or 99%, i.e. with impurities of 5% or 1%. The authors report a case where an impurity of 0.0006% was sufficient to explain the full response of an olfactory receptor in a test situation. The

authors demostrate why all experiments investigating the selectivity of odor receptors have to be performed with gas-chromatography-purified odors to eliminated potential impurity artifacts.

Introduction

How odorants interact with receptors remains elusive: a key-lock system has been proposed early on (Amoore, 1963), but this does not yet explain how a transduction cascade is activated (i.e. how the fitting key is turned inside the lock). Different mechanisms have been proposed, including the involvement of metal ions creating metalloproteins (Turin, 1996; Wang et al., 2003; Duan et al., 2012), and electron tunneling in resonance with molecular vibrations (Turin, 1996).

Crystallography is the most direct approach to studying receptor-ligand interaction, but only few examples exist, including the cholinergic receptor (Warne et al., 2008) and photoreceptors (Palczewski et al., 2000; Standfuss et al., 2011). No olfactory receptor has been analyzed in this way yet. An alternative approach relies on modeling the binding pocket (Guo & Kim, 2010). Here, large sets of odor-response data are necessary, ideally recorded in a hypothesis-free approach. However, in both cases, the result consists in an estimate for the shape of the binding pocket, but not yet in a mechanism of how the receptor is activated. Dedicated, hypothesis-driven studies are better suited to this end: if vibrations are to be tested, the task would be to find a receptor that does respond to one vibration frequency, and not to another.

Deuterated substances offer an ideal possibility to test whether molecular vibrations contribute to activating olfactory receptors. When hydrogen (H) is replaced by deuterium (D) in a molecule, the chemical properties do not change, but a new vibration range is added. For example, the C-D bond has a vibration at around 2150 cm^{-1}, which is not present in a molecule lacking deuterium. Deuterium can also add other vibrations: the ring in benzaldehyde-d_5 creates a collective out-of-plane vibration around 550 cm^{-1} (Klika, 2013; Paoli et al., 2016). The logic of these experiments is that, if an animal can differentiate between a deuterated and a non-deuterated substance that otherwise are equal (say, between benzaldehyde and its deuterated form, which smell almond-like to humans), vibrations

must play a role, since that is the main physical factor that differentiates the two odorant stimuli. This hypothesis has been tested in a variety of studies, using humans, fruit-flies, honeybees and other animals, and using paradigms including behavior and physiology (Haffenden et al., 2001; Keller and Vosshall, 2004; Franco et al., 2011; Bittner et al., 2012; Gane et al., 2013; Gronenberg et al., 2014; Paoli et al., 2016). However, the results are contradictory, since some studies argue for and others against vibrations, leading to controversial discussions (Solov'yov et al., 2012; Block et al., 2015).

Another aspect to be considered is that olfactory receptor gene families are highly divergent. Even within single species, there are several unrelated families of olfactory receptors: in mammals, at least 6 different families have been reported (Fleischer et al., 2009; Greer et al., 2016), in insects IRs and ORs are two distinct families (Silbering et al., 2011). A hypothesis would be that a single family, or even a particular receptor, could use one or more activation mechanisms – e.g. vibration detection, size, etc. – while others could respond to different odorant properties. Therefore, studying how responses to deuterated substances differ from non-deuterated substances is best done on single receptor types, rather than the whole olfactory system.

Receptors have broad or narrow response profiles (Galizia et al., 2010; Münch and Galizia, 2016), but even the latter respond to minor ligands when presented at a sufficiently high concentration. Optimal concentrations for eliciting responses in receptors can span many orders of magnitude. For example, Or22a in *Drosophila* has an EC50 (effective concentration/dilution for half-maximal response) of $10^{-6.9}$ for methyl hexanoate, and an EC50 of $10^{-4.2}$ for isoamyl acetate, and both dilutions create concentrations that *Drosophila* is easily exposed to in a natural environment (Pelz et al., 2006). The difference of several orders of magnitude between these two stimuli means that small amounts of impurities can have a strong effect on odor responses. Examples of single sensillum recordings where the responses were entirely due to impurities in commercial odorant sources have been published for moths (Stranden et al., 2003).

116 In this study, we combined these thoughts in an attempt to test the vibration theory of olfaction.
117 First, we searched for a single receptor type that would show differential responses between
118 deuterated and non-deuterated substances, and found one with an apparent difference. Results such
119 as these have been published as evidence in favor of the vibrational theory. Next, we recorded the
120 odorants' responses via a gas chromatograph, and found that in our case the difference was due to a
121 minute contaminant (0.0006%, or 6 ppm). Finally, we show that adding the contaminant to the non-
122 deuterated substances elicits a response similar to the one seen for for the deuterated substance.
123 We conclude that the results do not support the vibrational theory. Importantly, however, they do
124 not disprove it either – rather, they show how important it is not only to use substances of highest
125 purity, but indeed to purify substances on the spot using gas chromatography. As a corollary, the
126 validity of data in studies on receptor-ligand interaction in general that have not used appropriate
127 purification techniques needs to be reconsidered.

128 Materials and methods

129 Animals

130 All recordings were performed on female *Drosophila melanogaster* fruit flies expressing either the
131 calcium reporter G-CaMP5 (Akerboom et al., 2012) under the control of the olfactory co-receptor
132 Orco (Orco-Gal4>UAS-GCaMP5), or expressing the reporter GCaMP6m (Chen et al., 2013) in Or42b
133 olfactory receptor neurons (Or42b-Gal4>UAS-GCaMP6m). Calcium reporter driver lines were
134 obtained from the Bloomington Stockcenter (Bloomington, USA; RRID:BDSC_42038 and
135 RRID:BDSC_42748), Or42b-Gal4 (likely RRID:BDSC_9972) and Orco-Gal4 (likely RRID:BDSC_26818)
136 flies were kindly provided by Veith Grabe and Silke Sachse, MPI for Chemical Ecology, Jena, Germany.
137 Flies were kept at 25°C in a 12/12 light/dark cycle at 60-70% RH. Animals were reared on standard
138 medium (100 ml contain: 2.2 g yeast, 11.8 g of sugar beet syrup, 0.9 g of agar, 5.5 g of cornmeal, 1 g
139 of coarse cornmeal and 0.5 ml of propionic acid).

140 ## Animal preparation

141 For antennal lobe recordings flies were anesthetized on ice and placed into a custom-made holder.
142 The head was fixed to the holder with low-melting wax, the antennae were gently pulled forward
143 with a thin copper wire, and a polyethylene foil was placed on the head and sealed with
144 bicomponent silicon (Kwik-Sil, WPI). A small window was cut through the foil and head cuticle, and
145 the exposed brain was covered in saline solution (130 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$,
146 36 mM sucrose, 5 mM Hepes, pH 7.3, all chemicals from Sigma-Aldrich). Glands and tracheae were
147 removed to allow optical access to the antennal lobe. For antenna recordings flies were mounted in
148 custom-made holders. The head was fixed to the holder with a drop of low-melting wax. A half
149 electron-microscopy grid was placed on top of the head, stabilizing the antenna by touching the 2nd,
150 but not the 3rd antennal segment. For details on the antennal lobe preparation, see (Silbering and
151 Galizia, 2007; Silbering et al., 2008). For details on the antennal preparation, see (Münch and Galizia,
152 2016).

153 ## Odorant preparation

154 Benzaldehyde-2,3,4,5,6-d_5 was purchased at CDN isotopes (CAS: 14132-51-5, Lot #: I240P14, isotopic
155 enrichment 99%). All other odorants were purchased at Sigma-Aldrich in the highest purity available.
156 Odorants used were: benzaldehyde (CAS: 100-52-7, Lot #: STBD7798V, ≥99.5%), E2-hexenal (CAS:
157 6728-26-3, Lot #: S28442V, 98%), ethyl acetate (CAS: 141-78-6, Lot #: BCBR9070V, ≥99.9%), ethyl
158 propionate (CAS: 105-37-3, Lot #: BCBL5952V, ≥99.7%), ethyl butyrate (CAS: 105-54-4, Lot #:
159 BCBR7796V, ≥99.5%), propyl acetate (CAS: 109-60-4, Lot #: BCBL5998V, ≥99.7%), ethyl (S)-(+)-3-
160 hydroxybutyrate (CAS: 56816-01-4, Lot #: BCBM4473V, 99%), 3-hexanone (CAS: 589-38-8, Lot #:
161 BCBJ8237V, 98%), beta-butyrolactone (CAS: 3068-88-0, Lot #: MKBJ3709V, 98%), (±)-2-Hexanol (CAS:
162 626-93-7, Lot #: MKBJ5626V, ≥ 98%), methyl acetate (CAS: 79-20-9 , Lot #: BCBN9450V, ≥99.9%), 3-
163 penten-2-one (CAS: 625-33-2, Lot #: SHBC5346V, ≥70%). Pure substances were diluted in mineral oil
164 (Sigma-Aldrich) at the indicated dilutions, and covered with Argon (Sauerstoffwerk Friedrichshafen
165 GmbH, Germany) to avoid oxidation. Dilutions were prepared in 5 ml mineral oil (CAS: 8042-47-5;

166 Acros Organics, Belgium) in 20ml head space vials covered with pure nitrogen to avoid oxidation
167 (Sauerstoffwerk Friedrichshafen GmbH, Germany) and immediately sealed with a Teflon septum
168 (Axel Semrau, Germany).

169 Odorant delivery

170 A GC-FID system (TRACE GC Ultra, Thermo Fisher Scientific, USA) in conjunction with an autosampler
171 (PAL, CTC Switzerland) was used for odorant delivery. The autosampler was used to either inject
172 headspace samples into the GC, or directly to the antenna, bypassing the GC system. For GC-coupled
173 antenna measurements, 1 ml of headspace was injected into the GC at split mode with the injector
174 temperature set to 200°C, the split flow to 15 ml/min and the split ratio to 10. The GC was equipped
175 with an Optima® 5 MS 30 m × 0.25 mm × 0.25 µm column (Macherey-Nagel, Germany). The flow of
176 the carrier gas helium was set to 1.5 ml/min. The oven was held at 60°C for 1 min, then the
177 temperature was increased to 200°C at 20°C/min, the final temperature was again held for 1 min.
178 One half of the eluate was directed to the FID detector (set to 200°C) and the other half to the
179 animal's antenna via an olfactory detection port (either ODP3, Gerstel, Germany, or Semrau,
180 Germany). GC-FID trace and antennal trace alignment was calibrated using the response peak to
181 ethyl acetate. FID data was recorded using Xcalibur software (Thermo Fisher Scientific,
182 Massachusetts, USA). After each injection the syringe was washed with n-pentane (Merk KgaA,
183 Germany), heated and flushed with clean air. For direct stimulations (bypassing the GC) a head space
184 of 2 ml was injected in two 1 ml portions at time points 6 s and 8.5 s with an injection speed of 1 ml/s
185 into a continuous flow (60 ml/min) of purified air (two one-second stimuli with 1.5 s gap). Stimuli
186 arrived at the antenna with ~750 ms delay due to delays in the autosampler and the flow. Therefore,
187 stimulus onset was determined as 6.75 s and 9.25 s. In the Figures, $t = 0$ was set to correspond to the
188 first stimulus onset. The stimulus was directed at the antenna of the animal via a Teflon tube (inner
189 diameter 2 mm, length 39.5 cm, with the exit positioned ~2 mm from the antenna). Between
190 successive stimuli, the syringe was flushed with clean air. The inter-trial interval was approximately 2

191 min. For each animal, prior to odor delivery, responses to clean air and to mineral oil only were
192 tested as controls.

193 ## Calcium imaging

194 Calcium imaging of antenna (dendrites and somata of olfactory sensory neurons) and antenna lobes
195 (axon terminals of olfactory sensory neurons) was performed on a setup consisting of a fluorescence
196 microscope (BX51WI, Olympus, Japan) equipped with a 20× water immersion objective for antennal
197 lobe recordings (Olympus XLUM Plan FI 20×/0.95) or with a 50× air lens without cover slip correction
198 for antenna recordings (Olympus LM Plan FI 50×/0.5). Images were recorded with a CCD camera
199 (SensiCam, PCO, Germany) with 4 × 4 pixel on-chip binning, which resulted in 160 × 120 pixel sized
200 images for AL recordings or with 8 × 8 pixel on-chip binning, which resulted in 80 × 60 pixel sized
201 images for antenna recordings. For AL measurements we recorded each stimulus for 20 s at a rate of
202 4 Hz using TILLvisION (TILL Photonics, Germany), GC-coupled antenna imaging was performed at 1 Hz
203 for 9 min. A monochromator (Polychrome V, TILL Photonics, Germany) produced excitation light at a
204 wavelength of 470 nm which was directed onto the antenna via a 500 nm low-pass filter and a 495
205 nm dichroic mirror. Emission light was filtered through a 505 nm high-pass emission filter.

206 Benzaldehyde-h/d_5 antennal lobe measurements were performed in a total of *N*=6 animals
207 expressing Orco>GCaMP5 (Fig. 1A & C), and *N*=3 animals expressing Or42b>GCaMP6m (Fig. 1 D). GC-
208 coupled antenna recordings of benzaldehyde-h/d_5 were performed in a total of *N*=3 animals
209 expressing Or42b>GCaMP6m (Fig. 1E & Fig. 3C). Responses to blended benzaldehyde-h with
210 increasing concentrations of "contaminant" were measured in *N*=3 animals expressing
211 Or42b>GCaMP6m (Fig. 4). The GC-coupled antenna recordings in Fig. 2 are based on data from *N*=5
212 animals expressing Or42b>GCaMP6m, dose-response data in Fig. 3A & B are based on data from *N*=5
213 animals expressing Or42b>GCaMP6m.

Data analysis

Custom made R and Python scripts were used for data analysis. The Python-based ILTIS software (Raiser et al., unpublished; https://github.com/grg2rsr/ILTIS) was used for calcium imaging visualization, baseline subtraction and normalization. Relative fluorescence change was calculated as $\Delta F/F = (F_i - F_0)/F_0$ with F_i being the fluorescence at frame i and F_0 being the mean fluorescence before stimulus onset. GC-antenna recordings were corrected for dye bleaching by fitting an exponential decay function of the form $A * e^{-x/B} + C$ to each response trace, leaving out the parts of the trace where activity was recorded. Responses were calibrated across animals to the first response peak of ethyl butyrate, most likely $10^{-5.6}$ ethyl acetate (Fig. 3).

Dose-response curve (Fig. 3B) was obtained by least-squares fitting responses R at concentrations c with a sigmoidal logistic function of the form $R = R_{max} * \frac{1}{1+e^{-h*(c-EC50)}}$, with R_{max} corresponding to maximum response asymptote, $EC50$ the half-effective dilution, and h the steepness (reminiscent of the Hill-coefficient).

Results

We used calcium imaging of the antennal lobe in the fruit fly *Drosophila melanogaster* to record odorant evoked activity patterns. Specifically, we were interested in differences between the responses to benzaldehyde-h (normal benzaldehyde), and benzaldehyde-d_5, where the hydrogen atoms of the benzene ring were replaced by deuterium. We expressed the calcium sensor GCaMP5 (Akerboom et al., 2012) under the control of the olfactory coreceptor Orco (GAL4-Orco > UAS-GCaMP5), and stimulated with two 1 s stimuli with a 1.5 s gap in between. Both normal and deuterated benzaldehyde elicited similar responses throughout the antennal lobe, with the strongest response in the dorsolateral area (area R1 in Fig. 1A).

However, we also noted a dorsomedial area with clearly different responses to the two isotopomers, with apparent odorant elicited responses to benzaldehyde-d_5, and no apparent responses to benzaldehyde-h (area R2 in Fig. 1A). Therefore, we focused on this area because it could provide an

239 important, clear test of the vibrational hypothesis. Using the antennal lobe atlas for *Drosophila*
240 (Grabe et al., 2015), we identified two potential candidates for this area: glomerulus DM1,
241 innervated by Or42b, and glomerulus DL5, innervated by Or7A. To confirm the identity of the
242 putative isotope-sensitive area, we screened the DoOR database (Münch and Galizia, 2016) for two
243 odorants that induced a strong response in either the DL5 or the DM1 glomerulus. For this purpose,
244 we selected E2-hexenal (Fig. 1B) and ethyl butyrate (Fig. 1B'). E2-hexenal gave a strong response in
245 the dorsolateral area, corresponding to glomerulus DL5, which is innervated by axons from ORs
246 expressing Or7A (Fig. 1C). Ethyl butyrate elicited responses more medially, corresponding to the area
247 innervated by Or42b and Or22a (Fig. 1C). A comparison between the response patterns induced by
248 the four odorants indicated a clear overlap between the dorsomedial area of the ethyl butyrate-
249 induced signal – corresponding to glomerulus DM1 – and the benzaldehyde-d_5 responsive region
250 (dotted line in Fig. 1C). Thus, we confirmed this area to be glomerulus DM1, innervated by Or42b. We
251 then expressed the calcium sensor GCaMP6m (Chen et al., 2013) specifically in the Or42b receptor
252 neurons (Or42b-GAL4>UAS-GCaMP6m), and confirmed that Or42b responded to ethyl butyrate as
253 well as to benzaldehyde-d_5 (Fig. 1D). Responses to benzaldehyde-h, however, were inhibitory (blue
254 trace in Fig. 1D).

255 In order to show more conclusively that the response of this glomerulus was due to benzaldehyde-d_5,
256 and to exclude that minor impurities could cause this difference between the two isotopomers, we
257 coupled the imaging setup to a gas chromatograph outlet. With this experimental setup, response to
258 either benzaldehyde-h or benzaldehyde-d_5 was inhibitory at the elution time of benzaldehyde.
259 However, we found a strong excitatory response to benzaldehyde-d_5 at an earlier elution time, which
260 was not present in the benzaldehyde-h recording (Fig. 1E). These results indicated that the apparent
261 response to benzaldehyde-d_5 in Or42b was due to some contaminating trace molecules. These data
262 also suggested that the inhibitory response to benzaldehyde-d_5 (as seen in Fig. 1D) was masked by
263 the contaminating substance.

264 Next, we sought to identify the impurity. Using the DoOR database (Münch and Galizia, 2016), we
265 selected a set of best ligands for Or42b, purchased them at highest available purity, and measured
266 their chemical purity using GC-FID (red traces in Fig. 2). With the exception of 3-penten-2-one, where
267 we saw two peaks, all other substances only had a single detectable peak in the FID trace, with all
268 minor peaks in the noise range. Next, we recorded the calcium responses in Or42b to the GC eluates.
269 We found a strong response to ethyl acetate that decayed progressively after the stimulus, indicating
270 receptor saturation. Similarly, ethyl propionate, propyl acetate and ethyl (S)-(+)-3-hydroxybutyrate all
271 elicited responses that decayed slowly after the stimulus had terminated, indicating some degree of
272 saturation. Most importantly, however, we noted that ethyl propionate, ethyl butyrate, propyl
273 acetate and ethyl (S)-(+)-3-hydroxybutyrate all also elicited responses at the elution time of ethyl
274 acetate (Fig. 2). These responses indicated that ethyl acetate might have been a trace impurity in
275 these stimuli. The responses were quite different in size for the different stimuli, indicating that the
276 contamination differed in concentration. Indeed, other stimuli that we tested did not elicit any
277 response at the elution time of ethyl acetate (see, for example, the response to methyl acetate or to
278 3-hexanone in Fig. 2), indicating that these responses must have been generated by a specific
279 impurity. Other impurities also elicited responses: ethyl butyrate elicited four response peaks in
280 Or42b (Fig. 2), one with retention time corresponding to ethyl acetate, one with retention time
281 corresponding to ethyl propionate or propyl acetate, one unknown, and one corresponding to ethyl
282 butyrate itself.

283 What was the concentration of the ethyl acetate contamination in the benzaldehyde-d_5 sample? We
284 recorded a dose-response curve of ethyl acetate calcium responses in Or42b from the purified GC
285 eluate. At very low concentration, no response could be detected. With increasing concentration, the
286 response size increased, and at very high concentration the response formed a tail, with calcium
287 decreasing only slowly (red traces in Fig. 3A). Across concentrations, this yielded a sigmoidal dose
288 response curve, with half-maximal response at a dilution of $10^{-5.0}$ (Fig. 3B). We normalized these
289 responses to the ethyl acetate peak in the response to ethyl butyrate (gray trace in Fig. 3A). The

290 responses to the benzaldehyde-d_5 concentration were weaker (green traces in Fig. 3C, corresponding
291 to benzaldehyde-d_5 dilutions of 10^{-2}, light trace, and 10^{-1}, dark trace). These responses corresponded
292 to the values for ethyl acetate of $10^{-7.4}$ and $10^{-6.1}$, in good approximation of a single decadic dilution
293 step. Thus, we could quantify that a 10^{-1} dilution of benzaldehyde-d_5 contained $10^{-6.1}$ ethyl acetate,
294 while a 10^{-2} dilution contained $10^{-7.4}$ ethyl acetate, on average a $10^{-5.2}$ contamination. This
295 corresponded to an impurity of 6 ppm, or 0.0006%, which is at the low end of the detection limit of
296 gas chromatography using flame ionization detectors.

297 Could the heat in the GC cause unexpected artefacts, such as conformational changes in the
298 molecules? To exclude this possibility, and to test whether artificially adding an impurity of ethyl
299 acetate to benzaldehyde-h is sufficient to generate a response as the one that we found for
300 benzaldehyde-d_5, we generated synthetic mixtures of benzaldehyde-h with the impurity. We
301 recorded the calcium responses in Or42b>GCaMP6m antennae. Responses to benzaldehyde-h were
302 again inhibitory (trace "0" in Fig. 4). Adding increasing concentrations of ethyl acetate ranging from
303 10^{-10} to 10^{-2} led to a dose-dependent shift from the inhibitory response (traces 10^{-10} to 10^{-7} in Fig. 4)
304 to an increasingly excitatory response (traces 10^{-6} to 10^{-2} in Fig. 4), confirming that adding minute
305 amounts of ethyl acetate was sufficient to mimic the response induced by benzaldehyde-d_5.

306 Discussion

307 Many olfactory receptors have a broad response profile, with sensitivities ranging over many log-
308 decade concentrations. For example, the *Drosophila* receptor Or22a has a half-maximal response to
309 methyl hexanoate at a dilution of $10^{-6.9}$, and to isoamyl acetate at a dilution of $10^{-4.2}$ (Pelz et al., 2006)
310 (note that quantitative indications of concentrations depend on experiment specific settings,
311 therefore absolute values are difficult to compare between experiments. Relative values, however,
312 are comparable). Both are substances and concentrations that occur in the environment of the fruit
313 fly, therefore both are ecologically relevant. This gives an interesting twist to analyzing odorant

314 responses in a natural environment, where most stimuli are mixtures of several chemicals: a
315 response might derive from a major component, from a trace element, or both (Münch et al., 2013).

316 Here, we give an example where an impurity of 0.0006% (6 ppm) explains the full response of a
317 single receptor cell type. Given that for most substances the highest commercially available purity is
318 95% or 99%, these results are important for our interpretation of many odorant-response studies,
319 and not limited to investigating the vibrational theory. The headspace of the benzaldehyde-d_5 batch
320 that we used in our experiments had been analyzed chemically in great detail, resulting in 99.85%
321 purity, with a 0.1% impurity due to an individual contaminant, but no evidence for ethyl acetate
322 (data not shown), since the GC analysis did not reach the 0.0006% sensitivity that the natural
323 *Drosophila* receptor has. Another study used benzaldehyde-d_6, and the chemical analysis revealed
324 eight contaminants, all of which at a concentration higher than 0.0006% (Drimyli et al., 2016). Under
325 such circumstances, the contribution of ethyl acetate can easily go undetected when testing
326 deuterated benzaldehyde. Furthermore, ethyl acetate is not used in the synthesizing process of
327 benzaldehyde-d_5 (personal communication from the manufacturers), adding the additional caveat
328 that post-production impurities could be any chemical. We do not claim that any particular study
329 about the effect of deuterated substances can be explained by trace impurities. For example,
330 experiments showing learning transfer between deuterated compounds and nitriles (Franco et al.,
331 2011) are less likely to suffer from an impurity problem. We can only add a note of caution, and
332 substantiate the need for on-the-spot purification. Furthermore, trace compounds, even if they are
333 good ligands when given alone, do not always dominate the response of a receptor in a mixture: a
334 "secondary" ligand given simultaneously in a mixture could be able to obscure the response to the
335 primary ligand due to syntopic interactions (Münch et al., 2013). In such a case, the response to the
336 trace component would be visible when purified (e.g., as done here, with the GC), but it would not
337 contribute significantly to the response when given in a mixture, as contaminant.

338 Examples of highly sensitive olfactory receptors have been published previously: several moth
339 species have receptors highly sensitive and selective for (-)-germacrene-D, and give responses to

340 stimulation down to 1 ng, and 10-fold less sensitive (10 ng) to the enantiomer. In these recordings,
341 tiny amounts of (-)-germacrene-D among other substances created false positive results in
342 physiological recordings in moths (Stranden et al., 2003). In order to ensure purity of the delivered
343 stimulus, it is necessary to record from the olfactory receptor at the exit of a gas chromatographic
344 column (Stranden et al., 2003; Schubert et al., 2014). This technique has been used to identify other
345 highly selective and sensitive receptors (Stensmyr et al., 2012; Dweck et al., 2013; Ebrahim et al.,
346 2015).

347 Odors are encoded as combinatorial patterns of activated olfactory receptors (Galizia, 2014).
348 Therefore, it is necessary to measure the responses of many receptor neurons to many chemical
349 substances, an approach that has been performed in a series of screening experiments, many of
350 them in *Drosophila* (Hallem and Carlson, 2006; Kreher et al., 2008; Montague et al., 2011; Silbering et
351 al., 2011). These have been collected in a consensus data-base (Münch and Galizia, 2016) that allows
352 for computational analyses of odor coding (Boyle et al., 2013; Saberi and Seyed-allaei, 2016).
353 However, the results here add a note of caution to the reliability of large odor-response screens. Out
354 of the ten substances tested in Fig. 2 for Or42b, four (ethyl propionate, ethyl butyrate, propyl acetate
355 and ethyl(S)-(+)-3-hydroxybutyrate) gave responses not only to the main component, but also to a
356 (small) contamination with ethyl acetate. Importantly, ethyl acetate was not the only trace impurity
357 to elicit responses (see responses to 3-penten-2-one and responses to ethyl butyrate, that had two
358 more effective impurities, one putatively propyl acetate). These minute contaminations create a
359 distortion in large screening studies that is difficult to correct without reassessing all measurements
360 in a GC-coupled mode. In the specific case of Fig. 2, for example, we tested the ten best ligands
361 according to the consensus database in DoOR (Münch and Galizia, 2016). The best ligand in our data
362 was ethyl acetate (see Fig. 2). In the DoOR database ethyl acetate does not rank first, since not all
363 studies of Or42b reported ethyl acetate as the strongest ligand, and the merging algorithm in DoOR
364 is agnostic about the reliability of each study. Some of the differences, e.g. in the case of ethyl (S)-(+)-
365 3-hydroxybutyrate, may be due to differences in concentrations used across studies (most screening

studies do not include full concentration series). However, some "best ligands" in the database may have been overvalued due to the contribution of a contaminant in the chemical sample.

We started this study searching for a receptor that would respond differently to a deuterated substance than to the hydrogenated substance – in the case of a positive result, this would have indicated that that receptor might have been sensitive to a vibration around 550 cm^{-1} or around 2150 cm^{-1}. While we found a receptor that responded differently to our two stimuli, we could show that this difference was due not to the deuteration, but rather to a minute impurity of 0.0006%, while the response to deuterated benzaldehyde was identical to the response to hydrogenated benzaldehyde (Fig. 1E). By adding the impurity to benzaldehyde-h we obtained the same response as for the contaminated benzaldehyde-d_5, confirming that the contamination was sufficient to overcome the inhibitory effect of benzaldehyde-h and induce an excitatory response (Fig. 4). We can show that Or42b is not responding to a vibration of 550 or 2150 cm^{-1}, and it is unlikely that any of the ORs labeled in an Orco line are responding to that vibration in benzaldehyde-d_5 either, because such a difference would have been seen in our measurements of the antennal lobe (Fig. 1C). These results do not exclude that there might be receptors in *Drosophila* (or other species) that have evolved a mechanism for using molecular vibration to support response selectivity.

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Figure captions

Fig. 1

Apparent differential responses to deuterated and non-deuterated benzaldehyde. (A) Example of responses to benzaldehyde-d_5 (green traces) and benzaldehyde-h (blue traces) at two different dilutions (dashed: 10^{-3}, continuous 10^{-2}) in two areas of the antennal lobe (R1 and R2). The left

487 photograph indicates the position of R1 and R2 in the antennal lobe stained with the calcium sensor
488 GCaMP5, the middle graph depicts the response time-traces in area R1, the right graph R2. Gray bars
489 indicate stimulation times. Scale bar: 20 µm. (B, B') Spatial activity maps of the *Drosophila* antennal
490 lobe for the odorants E2-hexenal and ethyl butyrate, taken from the DoOR database,
491 http://neuro.uni.kn/door. (C) Spatial response patterns in the antennal lobe (false color)
492 superimposed onto the morphological view of the brain (grayscale). Responses to E2-hexenal, ethyl
493 butyrate, and the two benzaldehydes (BZA-h and BZA-d_5). Glomerulus DM1 innervated by dOr42b is
494 circled with a dotted line. The mid-line of the brain is to the left, and the orientation of the brain
495 corresponds to B. Scale bar: 20 µm. (D) Calcium recording from neurons expressing Or42b in the
496 DM1 glomerulus of the antennal lobe using the calcium sensor GCaMP6m. Stimuli were diluted to 10^{-}
497 ^{2}. Ethyl butyrate elicited long-lasting responses, that did not resolve the temporal structure of the
498 double stimulus. Benzaldehyde-d_5 elicited clear excitatory responses (calcium increases), while
499 benzaldehyde-h elicited clear inhibitory responses (calcium concentration decreases) to each of the
500 two odor pulses. Mean $\pm$ s.e.m. (*N*=3 animals). (E): Coupled GC-antennal lobe recordings in
501 Or42b>GCaMP6m flies. The two bottom traces show the FID signal for the two benzaldehydes used,
502 the top panel shows the mean response $\pm$ s.e.m to benzaldehyde-h (concentration 10^{-2}, blue trace)
503 and benzaldehyde-d_5 (concentration 10^{-2}, green trace, *N*=3 animals). Both benzaldehydes show a
504 clear calcium decrease in glomerulus DM1 at the elution time of benzaldehyde (approx. 240 s), but
505 only benzaldehyde-d_5 shows a strong calcium increase at elution time around 100 s.

506 Fig. 2

507 **GC-Imaging recordings reveal minute impurities in commercial odorant sources.** Each panel shows a
508 GC-FID recording (red trace) and a simultaneous antenna calcium imaging trace from
509 Or42b>GCaMP6 flies (black trace). All odors were injected as headspace samples at 10^{-2} dilution. The
510 yellow bar indicates the elution time for ethyl acetate (100 s). A response in Or42b at that elution
511 time is present in several samples (left column), but other impurities were also found (see response

512 to ethyl butyrate). All traces: *N*=4-5, average ± s.e.m. For GC-FID traces, the error is smaller than the
513 line width. Bottom right: schematic of the experimental setup.

514 Fig. 3

515 **The impurity in benzaldehyde-d_5 is 0.0006% ethyl acetate.** (A): responses to different
516 concentrations of ethyl acetate in GC-Imaging of Or42b>GCaMP6 antennae (dilutions 10^{-10} to 10^{-2}).
517 Increasing concentrations are given in colors from orange-yellow to red. With increasing
518 concentration, the response increases in size, but remains at the same elution time of approx. 100 s.
519 At the highest concentrations, responses tail to the right. In gray, the response to 10^{-2} ethyl butyrate,
520 which gives four response peaks, the first peak likely due to presence of ethyl acetate. All responses
521 are normalized to the first response peak in ethyl butyrate. (B) Dose response curve to ethyl acetate
522 in GC-Imaging recordings. Peak responses are taken from panel A (dotted lines from the left).
523 Responses have been fitted with a sigmoidal dose-response curve, half-maximal response (EC50) is
524 reached at a dilution of $10^{-5.0}$. Green lines from panel C indicate the response intensities found there,
525 gray line the value of the first peak in the ethyl butyrate response. (C): GC-Imaging responses to our
526 samples of benzaldehyde-d_5 at a dilution of 10^{-2} (bright green) and 10^{-1} (dark green). At the elution
527 time of benzaldehyde (approx. 240 s) both samples elicit a prominent concentration dependent
528 calcium decrease. At the elution time of ethyl acetate (approx. 100 s) both samples elicit a strong,
529 concentration dependent calcium increase. Traces have been normalized to the response to ethyl
530 butyrate (gray trace). The concentration of the impurity can be extracted from the dose-response
531 curve in B (green lines) as $10^{-7.4}$ and $10^{-6.1}$, for 10^{-2} and 10^{-1} dilution, respectively. (*N*=3 animals).

532 Fig. 4

533 **A minute impurity of ethyl acetate is sufficient to elicit a positive response to its mixture with**
534 **benzaldehyde.** We recorded antennal calcium responses in Or42b>GCaMP6 flies. Responses to
535 benzaldehyde-h 10^{-2} were inhibitory. Gradually adding ethyl acetate in concentrations from 10^{-10} to
536 10^{-2} led to increasingly excitatory responses, in a dose dependent manner (color-scale, see inset; for
537 example, "0" in the legend means benzaldehyde-h at a dilution of 10^{-2}, "10^{-7}" in the legend means

538 that ethyl acetate at a dilution of 10^{-7} was added to benzaldehyde-h at dilution 10^{-2}, i.e. the relative

539 concentration was 10^{-5}). Gray: response to ethyl butyrate 10^{-2}, for calibration. Odors were pre-mixed

540 in mineral oil to mimic the contamination situation, and delivered with a PAL multisampler. All

541 traces: *N*=3, average $\pm$ s.e.m. Gray bars indicate stimulation times.

542