

**Research Article: New Research / Sensory and Motor Systems**

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

Minute impurities distort olfactory receptor responses

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DOI: 10.1523/ENEURO.0070-17.2017

Received: 4 March 2017

Revised: 18 May 2017

Accepted: 19 May 2017

Published: 5 June 2017

**Funding:** EMBO short term fellowship  
ASTF 108-2015

**Funding:** DFG SPP1392  
SPP1392

**Conflict of Interest:** Authors report no conflict of interest.

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

We gratefully acknowledge that M.P. was supported by an EMBO short-term fellowship (ASTF 108-2015). Part of this research was funded by DFG grants (Deutsche Forschungsgemeinschaft) and SPP1392 ("Integrative Analysis of Olfaction").

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**Cite as:** eNeuro 2017; 10.1523/ENEURO.0070-17.2017

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**Manuscript Title (50 word maximum)**

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**Abbreviated Title (50 character maximum)**

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

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Number of Figures:	4
Number of Tables:	0
Number of Multimedia:	0
Number of words for Abstract:	##
Number of words for Significance Statement:	##
Number of words for Introduction:	##
Number of words for Discussion:	##

**Acknowledgements**

Thanks to members of the Galizia lab for comments on the manuscript.

**Conflict of Interest**

Authors report no conflict of interest

**Funding sources**

We gratefully acknowledge that M.P. was supported by an EMBO short-term fellowship (ASTF 108-2015). Part of this research was funded by DFG grants (Deutsche Forschungsgemeinschaft) and SPP1392 ("Integrative Analysis of Olfaction").

43 Minute impurities contribute significantly to olfactory receptor ligand  
44 studies: tales from testing the vibration theory.

## 45 Abstract

46 Several studies have attempted to test the vibrational hypothesis of odorant receptor activation in  
47 behavioral and physiological studies using deuterated compounds as odorants. The results have been  
48 mixed. Here we attempted to test how deuterated compounds activate odorant receptors using  
49 calcium imaging of the fruit fly antennal lobe. We found specific activation of one area of the AL  
50 corresponding to inputs from a specific receptor. However, upon more detailed analysis, we  
51 discovered that an impurity of 0.0006% ethyl acetate in a chemical sample of benzaldehyde-d<sub>5</sub> was  
52 entirely responsible for a sizable odorant-evoked response in *Drosophila melanogaster* olfactory  
53 receptor cells expressing dOr42b. Without gas chromatographic purification within the experimental  
54 setup, this impurity would have created a difference in the responses of deuterated and non-  
55 deuterated benzaldehyde, suggesting that dOr42b be a vibration sensitive receptor, which we show  
56 here not to be the case. Our results point to a broad problem in the literature on use of non GC-pure  
57 compounds to test receptor selectivity, and we suggest how the limitations can be overcome in  
58 future studies.

## 59 Significance statement

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

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

## 68 Introduction

69 How odorants interact with receptors remains elusive: a key-lock system has been proposed early on  
 70 (Amoore, 1963), but this does not yet explain how a transduction cascade is activated (i.e. how the  
 71 fitting key is turned inside the lock). Different mechanisms have been proposed, including the  
 72 involvement of metal ions creating metalloproteins (Turin, 1996; Wang et al., 2003; Duan et al.,  
 73 2012), and electron tunneling in resonance with molecular vibrations (Turin, 1996).

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

83 Deuterated substances offer an ideal possibility to test whether molecular vibrations contribute to  
 84 activating olfactory receptors. When hydrogen (H) is replaced by deuterium (D) in a molecule, the  
 85 chemical properties do not change, but a new vibration range is added. For example, the C-D bond  
 86 has a vibration at around  $2150\text{ cm}^{-1}$ , which is not present in a molecule lacking deuterium. Deuterium  
 87 can also add other vibrations: the ring in benzaldehyde- $\text{d}_5$  creates a collective out-of-plane vibration  
 88 around  $550\text{ cm}^{-1}$  (Klika, 2013; Paoli et al., 2016). The logic of these experiments is that, if an animal  
 89 can differentiate between a deuterated and a non-deuterated substance that otherwise are equal  
 90 (say, between benzaldehyde and its deuterated form, which smell almond-like to humans), vibrations

91 must play a role, since that is the main physical factor that differentiates the two odorant stimuli.

92 This hypothesis has been tested in a variety of studies, using humans, fruit-flies, honeybees and  
93 other animals, and using paradigms including behavior and physiology (Haffenden et al., 2001; Keller  
94 and Vosshall, 2004; Franco et al., 2011; Bittner et al., 2012; Gane et al., 2013; Gronenberg et al.,  
95 2014; Paoli et al., 2016). However, the results are contradictory, since some studies argue for and  
96 others against vibrations, leading to controversial discussions (Solov'yov et al., 2012; Block et al.,  
97 2015).

98 Another aspect to be considered is that olfactory receptor gene families are highly divergent. Even  
99 within single species, there are several unrelated families of olfactory receptors: in mammals, at least  
100 6 different families have been reported (Fleischer et al., 2009; Greer et al., 2016), in insects IRs and  
101 ORs are two distinct families (Silbering et al., 2011). A hypothesis would be that a single family, or  
102 even a particular receptor, could use one or more activation mechanisms – e.g. vibration detection,  
103 size, etc. – while others could respond to different odorant properties. Therefore, studying how  
104 responses to deuterated substances differ from non-deuterated substances is best done on single  
105 receptor types, rather than the whole olfactory system.

106 Receptors have broad or narrow response profiles (Galizia et al., 2010; Münch and Galizia, 2016), but  
107 even the latter respond to minor ligands when presented at a sufficiently high concentration.  
108 Optimal concentrations for eliciting responses in receptors can span many orders of magnitude. For  
109 example, Or22a in *Drosophila* has an EC50 (effective concentration/dilution for half-maximal  
110 response) of  $10^{-6.9}$  for methyl hexanoate, and an EC50 of  $10^{-4.2}$  for isoamyl acetate, and both dilutions  
111 create concentrations that *Drosophila* is easily exposed to in a natural environment (Pelz et al.,  
112 2006). The difference of several orders of magnitude between these two stimuli means that small  
113 amounts of impurities can have a strong effect on odor responses. Examples of single sensillum  
114 recordings where the responses were entirely due to impurities in commercial odorant sources have  
115 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).

## Animal preparation

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

## Odorant preparation

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

## 214 Data analysis

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

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

## 227 Results

228 We used calcium imaging of the antennal lobe in the fruit fly *Drosophila melanogaster* to record  
 229 odorant evoked activity patterns. Specifically, we were interested in differences between the  
 230 responses to benzaldehyde-h (normal benzaldehyde), and benzaldehyde-d<sub>5</sub>, where the hydrogen  
 231 atoms of the benzene ring were replaced by deuterium. We expressed the calcium sensor GCaMP5  
 232 (Akerboom et al., 2012) under the control of the olfactory coreceptor Orco (GAL4-Orco > UAS-  
 233 GCaMP5), and stimulated with two 1 s stimuli with a 1.5 s gap in between. Both normal and  
 234 deuterated benzaldehyde elicited similar responses throughout the antennal lobe, with the strongest  
 235 response in the dorsolateral area (area R1 in Fig. 1A).

236 However, we also noted a dorsomedial area with clearly different responses to the two isotopomers,  
 237 with apparent odorant elicited responses to benzaldehyde-d<sub>5</sub>, and no apparent responses to  
 238 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<sub>5</sub> 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<sub>5</sub> (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<sub>5</sub>,  
 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<sub>5</sub> was inhibitory at the elution time of benzaldehyde.  
 259 However, we found a strong excitatory response to benzaldehyde-d<sub>5</sub> 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<sub>5</sub> in Or42b was due to some contaminating trace molecules. These data  
 262 also suggested that the inhibitory response to benzaldehyde-d<sub>5</sub> (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<sub>5</sub> 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<sup>-5.0</sup> (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<sub>5</sub> concentration were weaker (green traces in Fig. 3C, corresponding  
 291 to benzaldehyde-d<sub>5</sub> dilutions of 10<sup>-2</sup>, light trace, and 10<sup>-1</sup>, dark trace). These responses corresponded  
 292 to the values for ethyl acetate of 10<sup>-7.4</sup> and 10<sup>-6.1</sup>, in good approximation of a single decadic dilution  
 293 step. Thus, we could quantify that a 10<sup>-1</sup> dilution of benzaldehyde-d<sub>5</sub> contained 10<sup>-6.1</sup> ethyl acetate,  
 294 while a 10<sup>-2</sup> dilution contained 10<sup>-7.4</sup> ethyl acetate, on average a 10<sup>-5.2</sup> 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<sub>5</sub>, 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<sup>-10</sup> to 10<sup>-2</sup> led to a dose-dependent shift from the inhibitory response (traces 10<sup>-10</sup> to 10<sup>-7</sup> in Fig. 4)  
 304 to an increasingly excitatory response (traces 10<sup>-6</sup> to 10<sup>-2</sup> in Fig. 4), confirming that adding minute  
 305 amounts of ethyl acetate was sufficient to mimic the response induced by benzaldehyde-d<sub>5</sub>.

## 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<sup>-6.9</sup>, and to isoamyl acetate at a dilution of 10<sup>-4.2</sup> (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<sub>5</sub> 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<sub>6</sub>, and the chemical analysis revealed  
 324 eight contaminants, all of which at a concentration higher than 0.0006% (Drimyly 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<sub>5</sub> (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

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

Odors are encoded as combinatorial patterns of activated olfactory receptors (Galizia, 2014). Therefore, it is necessary to measure the responses of many receptor neurons to many chemical substances, an approach that has been performed in a series of screening experiments, many of them in *Drosophila* (Hallem and Carlson, 2006; Kreher et al., 2008; Montague et al., 2011; Silbering et al., 2011). These have been collected in a consensus data-base (Münch and Galizia, 2016) that allows for computational analyses of odor coding (Boyle et al., 2013; Saberi and Seyed-allaei, 2016).

However, the results here add a note of caution to the reliability of large odor-response screens. Out of the ten substances tested in Fig. 2 for Or42b, four (ethyl propionate, ethyl butyrate, propyl acetate and ethyl(S)-(+)-3-hydroxybutyrate) gave responses not only to the main component, but also to a (small) contamination with ethyl acetate. Importantly, ethyl acetate was not the only trace impurity to elicit responses (see responses to 3-penten-2-one and responses to ethyl butyrate, that had two more effective impurities, one putatively propyl acetate). These minute contaminations create a distortion in large screening studies that is difficult to correct without reassessing all measurements in a GC-coupled mode. In the specific case of Fig. 2, for example, we tested the ten best ligands according to the consensus database in DoOR (Münch and Galizia, 2016). The best ligand in our data was ethyl acetate (see Fig. 2). In the DoOR database ethyl acetate does not rank first, since not all studies of Or42b reported ethyl acetate as the strongest ligand, and the merging algorithm in DoOR is agnostic about the reliability of each study. Some of the differences, e.g. in the case of ethyl (S)-(+)-3-hydroxybutyrate, may be due to differences in concentrations used across studies (most screening

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

368 We started this study searching for a receptor that would respond differently to a deuterated  
 369 substance than to the hydrogenated substance – in the case of a positive result, this would have  
 370 indicated that that receptor might have been sensitive to a vibration around  $550\text{ cm}^{-1}$  or around  $2150$   
 371  $\text{cm}^{-1}$ . While we found a receptor that responded differently to our two stimuli, we could show that  
 372 this difference was due not to the deuteration, but rather to a minute impurity of 0.0006%, while the  
 373 response to deuterated benzaldehyde was identical to the response to hydrogenated benzaldehyde  
 374 (Fig. 1E). By adding the impurity to benzaldehyde-h we obtained the same response as for the  
 375 contaminated benzaldehyde-d<sub>5</sub>, confirming that the contamination was sufficient to overcome the  
 376 inhibitory effect of benzaldehyde-h and induce an excitatory response (Fig. 4). We can show that  
 377 Or42b is not responding to a vibration of  $550$  or  $2150\text{ cm}^{-1}$ , and it is unlikely that any of the ORs  
 378 labeled in an Orco line are responding to that vibration in benzaldehyde-d<sub>5</sub> either, because such a  
 379 difference would have been seen in our measurements of the antennal lobe (Fig. 1C). These results  
 380 do not exclude that there might be receptors in *Drosophila* (or other species) that have evolved a  
 381 mechanism for using molecular vibration to support response selectivity.

## 382 Acknowledgements

383 We gratefully acknowledge that M.P. was supported by an EMBO short-term fellowship (ASTF 108-  
 384 2015). Part of this research was funded by DFG grants (Deutsche Forschungsgemeinschaft) and  
 385 SPP1392 ("Integrative Analysis of Olfaction"). Thanks to Hanna Schnell for help with the experiments.  
 386 Thanks to members of the Galizia lab for comments on the manuscript.

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## Figure captions

### Fig. 1

**Apparent differential responses to deuterated and non-deuterated benzaldehyde.** (A) Example of responses to benzaldehyde-d<sub>5</sub> (green traces) and benzaldehyde-h (blue traces) at two different dilutions (dashed: 10<sup>-3</sup>, continuous 10<sup>-2</sup>) 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  $\mu$ m. (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<sub>5</sub>). 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  $\mu$ m. (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<sup>-2</sup>.  
 497 Ethyl butyrate elicited long-lasting responses, that did not resolve the temporal structure of the  
 498 double stimulus. Benzaldehyde-d<sub>5</sub> 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<sup>-2</sup>, blue trace)  
 503 and benzaldehyde-d<sub>5</sub> (concentration 10<sup>-2</sup>, 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<sub>5</sub> 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<sup>-2</sup> 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  $\pm$  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<sub>5</sub> 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<sub>5</sub> 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.

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