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Synergism and combinatorial coding for the binary odor mixture perception in *Drosophila*

Synergism, Odor Coding, Receptor Specificity

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Dedication: This paper is dedicated to Late Professor Obaid Siddiqi who died on 26th July 2013.

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1 **Synergism and combinatorial coding for the binary odor mixture perception in *Drosophila***

2

3 **Abbreviated Title-** Synergism, Odor Coding, Receptor Specificity

4

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47

48 **Abstract**

49

50 Most odors in the natural environment are mixtures of several compounds. Olfactory receptors
51 housed in the olfactory sensory neurons (OSNs) detect these odors and transmit the information
52 to the brain, leading to decision making. But whether the olfactory system detects the ingredients
53 of a mixture separately or treat mixtures as different entities is not well understood. Using
54 *Drosophila melanogaster* as a model system we have demonstrated that fruit flies perceive
55 binary odor mixtures in a manner which is heavily dependent on both the proportion and the
56 degree of dilution of the components, suggesting a combinatorial coding at the peripheral level.
57 This coding strategy appears to be receptor specific and is independent of inter-neuronal
58 interactions.

59

60 **Significance Statement**

61 Insects rely on olfaction to successfully identify and distinguish between volatile chemical cues
62 that are essential for reproduction and survival. Most naturally occurring olfactory signals are
63 complex mixtures of many chemicals in varying composition and proportion. In the present
64 study, using *Drosophila melanogaster*, we have shown that the olfactory system can encode the
65 information for binary odor mixtures by exhibiting a strong response towards specific
66 combinatorial concentrations independent of the individual odor intensities. We further found
67 that the ratio coding is receptor specific and is independent of ephaptic interactions. The
68 particular combinations could be relevant in terms of the fly's ecology. This study can also pave
69 way towards better understanding of the mechanism of host-tracking by insect pests and vectors.

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77 **Introduction**

78 Most odors that animals encounter in their natural environment are complex mixtures of
79 many chemicals. Yet ironically most studies in olfaction have been directed towards sensing
80 single odors and different aspects of sensing odor mixtures have remained unexplored. Previous
81 studies suggested that the identity of individual components is lost in the blend and, as a result,
82 odor mixtures are perceived differently. (Freitag et al., 1998; Syed and Guerin, 2004; Rebora et
83 al., 2012; Faucher et al., 2013; Rebora et al., 2013; Roussel et al., 2014; Schutze et al., 2014).
84 The measured response to an odor mixture is often inconsistent with predictions based on
85 measured responses to the individual components contained in the mixture (Deisig et al., 2006;
86 Eschbach et al., 2011; Barth et al., 2014). For example, in rats, the magnitude of the response of
87 the olfactory sensory neurons (OSNs) to odor mixtures is different from the simple summation of
88 the individual components (Duchamp-Viret et al., 2003). Among various types of interaction
89 observed with the odor mixture, the most frequent interaction is suppression where one of the
90 odors cancel the response to the other. However OSNs are also capable of exhibiting synergism,
91 a less documented phenomenon where the response of a binary mixture is greater than the simple
92 summation of the individual components present (Deisig et al., 2006). Synergistic interaction
93 among the components of an odor blend have been proposed to contribute to the attraction of
94 some species of insects to their host plants (Visser, 1986). Empirical evidence for synergism
95 between plant derived volatiles and specific aromatic compounds or pheromones have been
96 documented in the oriental fruit moth (Piñero and Dorn, 2007), and in the males of codling moth
97 (Z. Yang et al., 2004) and corn earthworm (S.A. Ochieng et al., 2002). In this study, we
98 investigate whether synergized responses are encoded in the OSNs, using *in vivo* extracellular
99 single unit recording and behavioral studies in the relatively simple and well defined *Drosophila*
100 *melanogaster* olfactory system.

101 Odors are first recognized by a large repertoire of olfactory receptors, each of which is
102 expressed in a specific class of OSNs (Gao et al., 2000; Vosshall et al., 2000). OSNs expressing
103 a specific odorant/class of odorant receptor project their axons to the antennal lobe, where they
104 synapse onto the dendrites of the corresponding classes of projection neurons (PNs) (Stocker et
105 al., 1990; Jefferis et al., 2001). Both insect and mammalian olfactory system shares many of

106 these organization principles, indicating a common solution to odor mixture representation in the
107 higher brain centers (Davis, 2004; Silbering and Galizia, 2007; Su et al., 2009; Chou et al., 2010;
108 Wilson, 2013). In honey bees, it has been suggested that the peripheral olfactory neuronal layout
109 hardly contributes towards the mixture perception (Deisig et al., 2006; Huang et al., 2009; Deisig
110 et al., 2010). However, accumulating evidence suggests that the odor evoked responses at the
111 OSN level is also important, as it transmits key information about odor quality to the brain (Den
112 Otter, 1977; Nikonov et al., 2002; Su et al., 2012). Few recent studies have reported that odor-
113 guided behavior can be correlated to the activity of sensory neurons (Knaden et al., 2012;
114 Semmelhack and Wang, 2009). Hence in order to understand a fly's behavior towards odor
115 mixtures a detailed understanding of the response patterns in the OSNs and the underlying
116 mechanisms is crucial.

117 We focused our study on type I and type II sensilla basiconica (ab1 and ab2) (Stensmyr et
118 al., 2003) of *Drosophila melanogaster* housing four and two OSNs respectively. They mostly
119 detect food odors including esters, alcohols and aldehyde (de Bruyne et.al, 2001; Chakraborty TS
120 et.al, 2009). We tested a set of odorants (and their binary mixtures) representing the ligands for
121 these two sensilla type as reported by previous studies (de Bruyne et al., 2001; Hallem and
122 Carlson, 2004). We observed a synergistic effect in response to the binary mixtures in the OSNs.
123 We further noticed that these sensory neural responses to binary odor mixtures were also
124 reflected in the fly's behavior i.e. synergistic interaction between two odorants significantly
125 increases the fly's attraction towards their binary mixture. Although the results presented here
126 cover only a small part of an enormous amount of chemical stimuli that a fruit fly encounters in
127 the natural environment, this finding suggests that the enhanced behavioral response to the odor
128 mixture is at least in part due to the increased firing of OSNs.

129

130 **Material and methods**

131 **Flies:** 2-3 days old adult female *Drosophila melanogaster* (Canton-S Benzer - CsBz) were used
132 for the experiments. Flies were reared on standard cornmeal medium at 24°C and 40-50%
133 relative humidity under laboratory photoperiod regime (~14 hrs light: 10 hrs dark). These flies
134 were used through all the experiments unless specified otherwise.

135 For the UAS-reaper experiments, ~7 day old flies were used for recording. The flies were
136 exposed to 29° for 4-5 days to enable a higher expression of reaper so as to ensure cell death.

137 **Preparation of flies:** Female flies were anesthetized by cold shock for 20 seconds on ice and
138 mounted in a 1.6 mm diameter glass capillary (Harvard Apparatus Inc, USA). The protruding
139 head was immobilized with a low melting point myristic acid (Himedia, India). Extra care was
140 taken while mounting the fly in order to protect the sensilla from heating. The proboscis was also
141 fixed side way so as to prevent any movement

142 **Odor preparation and Odor delivery:** A custom made five port olfactometer made up of a 3
143 way open solenoid valve (Mod: LFAA1200118H, Lee Company, Connecticut USA) was used to
144 deliver the odor pulse. 20ml scintillation vials were used as odor reservoirs and 2 ml dilutions of
145 the desired odor (Sigma-Aldrich, St. Louis MO USA) were placed in each vial. All the odorants
146 were prepared by serial dilution in liquid paraffin oil (SD fine Chemical) Odor mixtures were
147 pre-mixed just before delivery. The purity of the diluted chemicals was further checked by a Gas
148 Chromatography Mass selective Detector (Hewlett Packard Gas Chromatography, model 6890
149 and Mass selective detector model 5973). A small volume of constant air was injected through
150 the inlet reservoir into the chemical scintillation vials, which eventually forced the odor-saturated
151 air to come out through the outlet of the vial. Odor pulses were delivered through a 2.00 mm
152 glass tube directed towards the fly antennae at a rate of 9ml/min. Each odor stimulation duration
153 was 500 msec and was controlled electronically and back fed to Labview 7.1 (National
154 Instruments, USA) acquisition software. Time taken by the odor to travel from the outlet to the
155 antennae, delay between valve opening, and onset of the response were standardized before the
156 experiments started and were maintained throughout the experiments.

157 ***In vivo* single unit extracellular recording:** Large sensilla basiconica on the third antennal
158 segment were identified by their position using a standard map of antennal surface ((de Bruyne
159 et al., 2001). Glass microelectrodes of 5-10 M Ω were prepared by pulling glass capillaries
160 (Kimble chase, catalogue No.121411) on a vertical electrode puller (Narishige Scientific
161 Instrument Laboratory, Tokyo, Japan) and filling them with 0.8% NaCl solution. Chloridised
162 silver wire was used for making electrical connection with the electrode. The ground electrode
163 was placed inside the third antennal segment and the recording electrode was placed on the base
164 of a type I/type II basiconic sensillum and connected to a high impedance unit gain pre amplifier

165 (Electro 705, WPI) as shown in figure 1A. Signals were amplified 1000X (Model 750, World
166 Precision Instruments, New Haven CONN, USA) and viewed on a two channel oscilloscope
167 (Tektronix 2216, Holland NL) linked to an audio amplifier and fed into computer via a 16-bit
168 analogue to digital converter (NI-DAQ BNC 2110) to be analysed off line with LabVIEW 7.1
169 software (National Instruments Software, USA) or Clampfit.

170 **Single fly behavioral Assay:** 2-3 days old female flies were separated in vials containing a
171 moist bed of tissue paper. For behavioral experiments, five flies were tested per set in five
172 separate glass tubes. At least five sets were done for each experiment. Each glass tube was closed
173 on both ends using an apparatus made up of a microfuge tube (Tarson) with the tip cut off to a
174 diameter of 4mm and replaced with a micropipette tip so that odor could pass from the microfuge
175 tube into the glass tube. Clean plastic mesh with a diameter of 11 mm and a pore size of
176 approximately 1mm was attached to the mouth of the apparatus to prevent the flies from entering
177 the mouth of the trap. The odor was applied on a filter paper disc placed on the cap of the
178 microfuge tube at one of the arms (odor arm) and paraffin oil was applied on the other arm
179 (control arm). An overhead camera (Watec Monochrome Video Camera) was used to record the
180 movement of the flies for 2 minutes at 25 frames per sec. Fly tracking analysis was done using a
181 custom written program in MATLAB 6.5 (R2007b) (Mathworks Inc. USA) which tracks the path
182 of the each individual fly in each tube. A fly covers 623 ± 48 mm in 2 minutes in the absence of
183 odor. The total time spent by the fly at the odor arm was calculated. Response Index (RI) was
184 calculated using the following formula: (time spent in odor arm - background)/ total time of the
185 analysis (2 minutes).

186 **Data analysis:** All the graphs and the statistical analysis mentioned in the experiments were
187 done using Origin9.1 software. For figure 2, spike sorting was carried out using plexon offline
188 sorter and frequency histograms were plotted in Neuroexplorer with 500 msec binwidth. We
189 calculated the average basal firing rate prior to the stimulation for 2 sec. Starting at the zero point
190 of the stimulation we determined the point where the evoked response came down to the same
191 basal firing rate and were marked as green and considered as response duration. The linear
192 regression (blue solid line) plots of the response duration were drawn using origin 9.1 software.
193 The slopes of the decay plot for 2-3 butanedione (10^{-3}) and the binary mixture of 2-3 butanedione
194 (10^{-8}) and acetone (10^{-4}) were then compared using paired t-test for significant analysis.

195 **Results**

196 Flies extract biologically relevant information from the environment via chemical signals
197 detected by a large array of olfactory receptors (ORs) present in the sensilla of each antenna. In
198 the present study, we focused our attention on Or42b-expressing neurons and Or59b-expressing
199 neurons olfactory receptors, housed in ab1 and ab2 respectively. We tested all the odorants to
200 which Or59b-expressing neurons (Figure 1B) receptors are sensitive, as identified in previous
201 studies (de Bruyne et al., 2001).

202 Extracellular single unit recordings were acquired from ab1 and ab2 (antennal sensilla
203 basiconica) sensilla with glass electrodes. For ab2 sensillum, the larger amplitude spike marked
204 “A” and the smaller amplitude spike marked “B” (Figure 1A) represented responses obtained
205 from ORNs with Or59b-expressing neurons and Or85a-expressing neurons receptors,
206 respectively (Pellegrino et al., 2010). Firing frequency of “A” spikes was measured throughout
207 the experiment. For ab1 sensillum, all four (A, B, C, D) amplitude spikes (Figure 3D) were
208 recorded, but only spike ‘B’ was used for experimental consideration. Firing rates were obtained
209 from OSNs from 10 flies (3 OSNs from each fly per odorant) using *in vivo* extracellular
210 recording in the presence of varying odorant concentrations. Appropriate controlled conditions
211 for the olfactometer were maintained throughout the experiment (see methods section). A dose
212 response curve for each odorant was then generated. For complete quantitative analysis of the
213 dose response curve, two criteria were used. First, for all ten odorants, data points encompassing
214 the entire dynamic range were included, up to the dilution close to the saturation vapor (dilutions
215 ranged from 10^{-9} to 10^{-1}). Second, at least 3 trials for each dilution set were included. We
216 carefully measured the responses of the odorants across different concentrations. Our results,
217 showed in figure 1B, suggested that all ten odorants exhibited a sigmoidal relationship between
218 the concentration and firing rate, as already described. The ten tested odorants were grouped into
219 three classes based on the firing frequency of the OSN response, an indicator of the affinity of
220 Or59b-expressing neurons receptors towards the odorant. Class I odorants evoked highest firing
221 rates (>100 spikes/sec) at their dynamic range, class II evoked a moderate response (>50-100
222 spikes/sec) and class III evoked the lowest firing rate (< 50 spikes/sec). While selecting odorants
223 for preparing binary mixtures, no two odorants from class I were combined to avoid reaching
224 firing frequency saturation at very low concentrations.

225 For ab1 sensillum, four neurons can be easily identified from the spontaneous firing
226 (Figure 3D). In our study, it has been noted that the spontaneous firing of ab1 is much higher
227 than that of the ab2 sensillum. We focused on the Or42b-expressing neurons olfactory receptor,
228 housed in ab1B sensory neuron. The responses of 2-3 butanedione and 3-hydroxy ethyl butyrate
229 in ab1B were consistent with earlier studies (de Bruyne et.al, 2001).

230 **Single odor and binary odor mixture responses in ab1 and ab2**

231 Responses to each set of two individual odorants and their binary mixture were tested.
232 Single unit responses for acetone and 2-3 butanedione were measured from ab2, both
233 individually and mixed together (Figure 1C-E and Figure 2). Sub-threshold levels of acetone (10^{-3})
234 and 2-3 butanedione (10^{-7}) when mixed together evoked a stronger and more sustained
235 response than the individual components alone. The green area of figure 1C indicates that the
236 response extended beyond the length of the odor stimulus i.e., 500 ms (the blue box). Typical
237 excitatory responses returned to the spontaneous firing level within 1.1 sec and 0.76 sec, after
238 the end of the odor stimulus, as illustrated by the responses elicited by acetone (10^{-3}) and 2-3
239 butanedione (10^{-7}) respectively when presented alone (Figure 1C and 1D). In contrast, a binary
240 mixture of 2-3 butanedione (10^{-7}) and acetone (10^{-3}) elicited a more sustained response of 6.8
241 sec. The linear regression fittings (blue solid line in figure 1C) of the response durations of the
242 two individual odorants and their binary combination suggested that the binary mixture of these
243 two odorants elicited a super sustained response. To understand the synergistic effect of the odor
244 mixture we mixed different concentrations of 2-3 butanedione and acetone and tested them on
245 Or59b-expressing neurons. Each mixture set contained a fixed dilution of acetone (10^{-4} , 10^{-3} or
246 10^{-2}) with serial dilutions of 2-3 butanedione (10^{-9} to 10^{-2}) (Figure 1E). However, the dose
247 response curves for the binary mixtures of 2, 3 butanedione and acetone exhibited a dramatic
248 effect: each mixture set showed a sharp increase in firing frequency at lower concentrations of 2,
249 3 butanedione (Figure 1E) followed by a sharp peak at a certain dilution combination of 2, 3
250 butanedione and acetone, which attenuated at higher concentrations of 2, 3 butanedione. Each
251 peak was significantly (oneway ANOVA; $p=0.001$) greater than the peak witnessed in response
252 to 2, 3 butanedione alone of that corresponding dilution, which suggested synergism at that
253 dilution. When, 2, 3 butanedione was mixed with a 10^{-3} dilution of acetone, the response of the
254 binary mixture peak/synergism appeared at 10^{-7} dilution of 2-3 butanedione, which was

255 significantly (paired t-test; $p=0.001$) greater than the combined response of 2-3 butanedione at
256 10^{-7} dilution and 10^{-3} dilution of acetone shown in the bar plot of figure 1F. The maximum
257 frequency was documented only at particular proportions. The highest response synergism for
258 10^{-8} and 10^{-6} dilutions was found for the binary mixtures of 2, 3 butanedione when presented
259 with 10^{-4} and 10^{-2} dilutions of acetone respectively (Figure 1E). Responses to the individual
260 odorants and to their mixture were obtained from at least 30 neurons. Notably, comparing the
261 combinations of varying dilutions of acetone with a fixed concentration of 2, 3 butanedione, it
262 was found that the peak responses to the binary mixtures were obtained at different dilutions of
263 acetone, indicating that 2, 3 butanedione showed synergism with a specific dilution combination
264 of acetone. The particular dilution of 2, 3 butanedione in the binary mixture combination that
265 exhibits synergism was found to be always four times less than the dilution of acetone. The firing
266 frequency at a higher dilution (10^{-9}) of 2, 3 butanedione presented with acetone (10^{-4}) however
267 appeared to be slightly less than the firing response of 2, 3 butanedione alone at that specific
268 dilution in figure 1E. But this difference is statistically (1 way ANOVA) insignificant. The
269 representative traces of these two odorants and their combinatorial responses over the serial
270 dilutions have been presented in figure 2. The peri-stimulus histogram indicates that the
271 responses (firing frequency) to both the individual odorants gradually increased with their
272 increasing concentration (Fig 2 first and second columns). In contrast, the firing frequency for
273 the binary mixture of 2-3 butanedione and acetone (10^{-3}) was remarkably different. The PSTH
274 indicates the maximum firing frequency and longest sustainable response at the binary
275 combination of 2-3 butanedione (10^{-7}) and acetone (10^{-3}).

276 Figure 2B showed the PSTH of 2-3 butanedione (10^{-3}) when presented alone and the binary
277 mixture of 2-3 butanedione of dilution 10^{-8} and 10^{-4} dilution of acetone. Though the first 500
278 msec of the odor evoked responses were not different (92 Hz and 98 Hz respectively), however,
279 the response profiles of these two responses were notable different. The odor evoked response
280 duration for 2-3 butanedione (10^{-3}) alone was 4.5 sec, which was significantly (paired t-test
281 $p>0.001$; green area in figure 2B) shorter than the duration of the evoked response of the above
282 mentioned odor mixture (9 sec). The time when the firing frequency during 500 ms period reach
283 the basal firing rate was recorded as the response time. More importantly, the linear regression /
284 decay response slope, blue solid line in figure 2B of the mixture of 2-3 butanedione (10^{-8}) and
285 acetone (10^{-4}) was -4.86 which was significantly (paired t-test, $p>0.001$) slower than the response

286 decay slope of -17.42 for 2-3 butanedione (10^{-3}) alone. Taken together our observations strongly
287 suggest that the prolonged response of the binary mixture was not a result of vapor pressure
288 differences of individual components. The response profiles presented in figure 2 clearly
289 exhibited that the evoked response of the binary mixtures were not due to sequential responses of
290 the corresponding mixture components at any point.

291 Synergism was not found to be restricted to the binary mixtures of 2, 3 butanedione and
292 acetone for ab2. We could observe synergistic responses being elicited by binary mixtures of 2,3
293 butanedione and ethyl butyrate (Figure 3A) and also by acetone in combination with iso-amyl-
294 acetate in figure 3B. For the binary mixtures of 2, 3 butanedione and ethyl butyrate, the
295 synergism always appeared when the dilution of 2, 3 butanedione was five times less than the
296 dilution of ethyl butyrate. For the binary combinations of acetone and iso-amyl acetate synergism
297 was observed when the dilution of acetone was two times less than that of isoamyl acetate
298 (Figure 3B). Interestingly, iso-amyl acetate fails to activate ab2 neurons (de Bruyne et.al, 2001)
299 but when presented with acetone showed a synergistic response. We have also found several
300 other binary combinations (listed in figure 3C) for which ab2 sensilla basiconica exhibited
301 synergism.

302 In order to investigate whether the above-mentioned synergism is restricted to Or59b-
303 expressing neuron or is a general phenomenon across all the ORs, we decided to study Or42b-
304 expressing neurons, expressed in the ab1B neuron. We chose 2, 3-butanedione and ethyl
305 butyrate, as Or42b-expressing neuron is known to detect both (de Bruyne et al. 2001). We found
306 that their mixtures at certain combinations could evoke synergistic responses (Figure 3E, 3F).
307 We tested binary mixtures of a 10^{-6} dilution of 2,3-butanedione and a 10^{-1} dilution of Ethyl
308 butyrate (Figure 3E) (as they were known to be synergistic in case of Or59b-expressing neurons,
309 figure 3C) as well as the individual dilutions of each of the components. The mixture was found
310 to elicit a response higher than the summation of responses (paired t-test, $p=0.01$) evoked by the
311 binary mixture of 10^{-6} dilution of 2,3-butanedione and 10^{-1} dilution of ethyl butyrate when tested
312 individually (Figure 3F). These observations tempt us to hypothesize that the synergism is
313 probably a common coding mechanism used by the OSNs across fly's olfactory system in order
314 to perceive complex odor mixtures present in the environment. Quite interestingly, the

315 synergistic effect observed for the ab1B (Or42b-expressing neurons) neuron was not as dramatic
316 as that observed for ab2A (Or59b-expressing neurons).

317 **OSNs synergistic response conveys to fly's behavior**

318 In order to investigate whether the electrophysiological responses translate to a fly's
319 behavior, we conducted single fly behavioral assays using the set-up already described in the
320 materials and methods (Figure 4A). Consistent with the electrophysiology, flies exhibited an
321 enhanced response for the binary mixtures of 2, 3 butanedione (10^{-7}) and acetone (10^{-3}) over the
322 individual odors (Figure 4B). The response index of all 30 flies and their response path-tracks are
323 shown in figure 4C and 4D respectively. The flies exhibited enhanced attraction (ANOVA;
324 $p=0.001$, $R^2=0.72$) to the combination of 2, 3 butanedione (10^{-7}) and acetone (10^{-3}) compared to
325 2-3 butanedione (10^{-7}) alone. To exclude any potential behavioral effect towards the solvent
326 (paraffin oil), we tested the fly's behavior with paraffin oil on both the arms. Fly tracks in figure
327 4D looked scattered/evenly distributed and showed no preference towards paraffin oil (Figure
328 4C-D). We also measured the fly's response to the serial dilutions of 10^{-9} to 10^{-3} of 2, 3
329 butanedione in two sets, each set containing a specific dilution of acetone (10^{-4} or 10^{-3}) (Figure
330 4E). Surprisingly, consistent with the peripheral OSN responses in the single fly behavioral
331 response index curve in figure 4E, the sharp peaks (ANOVA; $p=0.001$, $R^2=0.42$ and $R^2=0.66$)
332 were observed for both sets of acetone (10^{-3} & 10^{-4}) and 2-3 butanedione mixture (10^{-7} & 10^{-8})
333 combinations. So the enhanced response towards the binary mixture was not only observed at the
334 OSN level, but the fly also exhibited increased attraction towards the binary mixture too, which
335 was significantly higher (paired t-test, $p=0.001$) than the additive response of the two individual
336 odorants, 2, 3 butanedione (10^{-7}) and acetone (10^{-3}) in figure 4F.

337 More significantly, the corresponding combinatorial dilutions of 2, 3 butanedione and
338 acetone at which the highest response peaks were obtained in single fly behavior (figure 4F)
339 were exactly the same as of the sensory neuronal level responses towards the same combinatorial
340 binary mixture. Enhanced attraction was obtained at 10^{-8} and 10^{-7} dilutions of 2-3 butanedione
341 when presented with 10^{-4} and 10^{-3} dilutions of acetone respectively. Enhanced attraction towards
342 2-3 butanedione was found with a specific dilution combination of acetone. The dilution of 2-3
343 butanedione required to exhibit synergism both at the neuronal level as well as in behavioral
344 level was four times less than the dilution of acetone in their binary combination.

345 **Synergism is receptor specific**

346 In order to determine if the synergistic interaction observed at the sensory neuronal level
347 was specific to the receptor, we used a Gal4-UAS system to drive either reaper (rpr) or Kir2.1 in
348 sensory neurons housed in the ab2 sensillum. Expression of reaper in ab2B sensory neurons led
349 to cell death and abolished spontaneous firing of Or85a-expressing neuron (Figure 5E) whereas
350 expression of an inward rectifying Kir2.1 channel caused voltage-dependent K⁺ ion channel
351 opening, which shunted the membrane voltage toward the equilibrium potential of K⁺, thereby
352 hyperpolarizing the neuronal membrane and making it refractory to synaptic activity (Baines et
353 al., 2001). The crossed flies of reaper and Kir2.1 were tested electrophysiologically (Figure 5F-G
354 and 5A-B). We also carried out behavioral experiments with flies expressing Kir2.1. We found
355 that flies failed to exhibit synergism toward the binary mixture of acetone (10⁻³) and 2, 3
356 butanedione (10⁻⁷) when Or59b-expressing neuron's excitability was suppressed by Kir2.1
357 (Figure 5A and 5B). In contrast, when Kir2.1 was expressed in Or85a-expressing neurons,
358 synergism remained unaffected (figure 5A and 5B). Control flies in figure 5B showed enhanced
359 (ANOVA, $p=0.001$, $R^2=0.6$) firing towards the binary mixture of butanedione (10⁻⁷) and acetone
360 (10⁻³). Similar results were obtained when reaper was expressed in ab2 sensillum. Expression of
361 reaper in ab2B sensilla using a Or85a- Gal4 driver completely silenced that neuron as no ab2B
362 spikes were seen when subjected to a puff of 10⁻³ Ethyl 3-hydroxy butyrate, a natural ligand for
363 Or85a-expressing neurons (Figure 5E). However, normal Or85a-expressing neurons spikes were
364 observed when the positive control fly lines (UAS and Gal4 lines) were puffed with 10⁻³ ethyl 3-
365 hydroxy butyrate (Figure 5E). Quite interestingly, the ab2A neuron was also seen to respond
366 slightly to ethyl 3-hydroxy butyrate (Figure 5E). The flies were then puffed with 10⁻³ dilution of
367 acetone and 10⁻⁷ dilution of 2,3-butanedione as well as their binary mixture. As expected, we saw
368 synergistic responses in both the Or85a-Gal4-rpr flies as well as the UAS-rpr control flies
369 (Figure 5F-G). The responses of the Or85a- Gal4 line were slightly lower than the UAS line as
370 well as the experimental reaper flies but nonetheless they showed a synergistic response to the
371 mixture. Taken together, these results strongly suggested that Or85a-expressing neurons did not
372 physiologically contribute towards synergism. In both the Or85a-expressing neurons silenced
373 and the control flies we observed a lower response to 2,3-butanedione compared to that in figure
374 1 which could probably be due to different age and growing conditions.

375 We further carried out a single fly behavioral assay with flies expressing Kir2.1 (Figure
376 5C and 5D). As expected, flies exhibited significantly less attraction towards binary mixture
377 (paired t-test, $p=0.001$) when Or59b-expressing neurons were silenced (Figure 5C and 5D). Flies
378 spent almost no time in the odor zone when Kir 2.1 was expressed in ab2A sensory neurons. In
379 contrast, control flies and flies with silenced ab2B sensory neurons (Figure 5D) exhibited
380 synergism. There was no significant difference between the response index of control flies and
381 that of ab2B silenced flies. These results strongly suggest that the Or59b-expressing neurons
382 receptor present in ab2 sensory neurons was solely responsible for the synergism witnessed.

383

384 Discussion

385 In natural conditions, chemical stimuli are primarily complex mixtures of many
386 chemicals. The mechanism by which the OSNs encode these complex mixtures is poorly
387 understood. A recent study in *Drosophila* showed that the integration of information from odor
388 mixtures begins in the OSNs in the form of modulation of response dynamics and response
389 magnitude (Turner and Ray, 2009, Su et.al. 2012). Mixture interaction can therefore be observed
390 prior to the higher brain centers at the sensory neuronal level in the periphery. It is tempting to
391 say that the mixture interaction at the OSN level could shape the sensory input that the brain
392 receives. Previous studies have shown that the most common interaction of odorants is
393 suppression, where addition of one odor attenuated the response magnitude for another odor
394 (Steullet and Derby, 1997; Su et al., 2012). Here we have demonstrated another kind of
395 interaction, where the presence of two odorants in a binary mixture significantly enhances both
396 the response duration and magnitude, beyond that which would be expected based on an additive
397 model of OSN response (Cometto-Muniz et al., 1997). We refer to this phenomenon as
398 synergism (Shown in figure 1). The dynamics of the odor-coding for mixtures at the sensory
399 neuronal level is highly selective. Presentation of mixtures of two odorants, such as acetone and
400 2-3 butanedione (at certain dilution combinations), elicited long lasting evoked activities in ab1B
401 and ab2A sensory neurons. The response extended beyond the length of the odor stimulus
402 (Figure 1C). The results we obtained in this study highlights the fact that the binary mixture
403 response of sensory neurons cannot be predicted based on the individual component responses.
404 The interaction appears complex even in the simplest case where two odorants are mixed

405 together. We speculate that the complexity would increase with the number of odorant molecules
406 forming a mixture. We focused our study on two olfactory receptors, Or42b-expressing neurons
407 and Or59b-expressing neurons, housed in the ab1B and ab2A OSNs. Both of these receptors
408 have a broad profile with strong responses to many fruity odors, increasing the likelihood that the
409 mixture interaction we recorded in this study could occur in the natural environment. In our
410 study we found that synergism also exists in other classes of OSNs, namely in ab1B (Or42b-
411 expressing neurons) sensory neurons where the synergism effect is not as severe as that in ab2A
412 (Or59b-expressing neurons). It is possible that synergism is restricted to very selective cells
413 responding in a context dependent manner to import behavioural cues.

414 In this study, we used chemicals which are by and large attractive to the fruit fly. We observed
415 that mixtures of these attractants (2,3 butanedione and acetone) were more attractive than each
416 constituent alone. The cause behind the increased strength of the behavioural responses to the
417 binary mixture probably lies in the temporal dynamics of the mixture responses. Our results
418 support the observation made by Thoma et.al. (2014) that compound valence is conserved in the
419 binary mixtures. We went one step further and showed that the strongest synergism always
420 occurs at a specific dilution combination within odorant pairs, independent of their individual
421 absolute concentrations (Figure 1, 2 and 3). With changes in one odorant in the combinational
422 pairs, the peak changes. Interestingly, the ratio coding observed is not uncommon; it appears to
423 characterize pheromonal communication in moths (Baker et.al., 1976; Lanier et.al., 1980; Toth
424 et.al., 1992) and beetles (Lanier et.al., 1980), suggesting that such a strategy may be a general
425 one for chemical sensing under natural conditions.

426 We also showed that the synergistic response was receptor specific. Expression of Kir2.1
427 in ab2A (Or59b-expressing neurons) sensory neurons abolished enhanced behavioral attraction
428 to binary mixtures of 2, 3 butanedione and acetone. We obtained similar results in extracellular
429 single unit recording from ab2 sensillum in figure 5A, 5E. However, we did not observe response
430 reduction when the ab2B (Or85a-expressing neurons) sensory neuron was silenced (Figure 5B).
431 In order to strengthen our findings, we also expressed a cell death gene, reaper (rpr), in Or85a-
432 expressing sensory neurons. Since there is a stereotyped pairing of OSNs in ab2 sensillum, the
433 identity of the OSN that expresses rpr could be deduced from the identity of its surviving
434 neighbor. We found that the surviving OSN in Or85a-Gal4, UAS-rpr flies had an odorant

435 response that matched that of Or59b-expressing neurons (ab2A). Flies bearing Or85a-Gal4 and
436 UAS-rpr showed no change in the response towards the binary mixture of 2-3 butanedione and
437 acetone (Figure 5F). Thus, from our results, we can strongly state that the ratio tuning we
438 observed is not mediated by Or59b-Or85a-expressing neurons interaction. Peripheral
439 mechanisms such as ligand induced receptor inhibition at the OSN level (de Bruyne et.al, 1999;
440 Hallem and Carlson, 2004; Su et.al, 2012), ephaptic interaction within the sensillum (Su et.al,
441 2012), or syntopic interaction (Munch et al., 2013) all require a system layout where receptors
442 are inhibited by compounds, or positive and negative OSNs are co-localized within the same
443 sensillum, to allow bilateral inhibition. We did not see such patterns in our study.

444 In general the dose response curve for an odor follows a sigmoidal shape. In our study,
445 with synergism interaction, the response curve for the mixture did not look like that of a single
446 compound, so evidently the brain processes the complex mixtures of multiple odorants
447 differently from the pure constituents. The activity evoked from the Or42b-expressing neurons
448 and Or59b-expressing neuronal receptors studied here is only a small part of the whole ensemble
449 of activity in the olfactory system. In the antennal lobe, the ensemble responses are further
450 shaped by both excitatory and inhibitory lateral interactions conferred by a dense array of local
451 interneurons (Chou YH et.al, 2010). Nevertheless, we found that OSNs are the primary
452 determinant for the fly's behavior towards the perception of binary mixtures like 2-3 butanedione
453 with acetone and many others. The exact mechanism by which the synergism occurs requires
454 further investigation. It is possible that the ORs have multiple binding sites for different odorants
455 and binding at multiple sites leads to an amplified response. However, this also requires further
456 study, such as expressing the Or59b-expressing neurons and Or42b-expressing neuronal
457 receptors in cell systems, which is beyond the scope of this study.

458 Taken together, our findings provide evidence that synergistic OSN responses are
459 characteristic to the neurons and not an outcome of bilateral interaction within the sensillum. Our
460 study provide a better understanding of the strategies undertaken by the fly's olfactory system in
461 the presence of multiple odorants. We show that the insect olfactory system could encode
462 information about chemicals at the olfactory receptor level as a separate entity and recognizes
463 them by segregating their components dilution combinations and not by their individual
464 intensity. While information about the concentration is important for odor guided behavior, it is

465 often irrelevant for the purpose of odor recognition. In the current study, we showed that the fly's
466 olfactory system can deduce odorant identity independent of concentration when the two
467 variables are intertwined at the level of receptor activity. The majority of odors consist of a
468 combination of many chemical substances. The scent of banana, for example, was reported to
469 contain 152 components (Jordan et al., 2001) and 26 substances in the headspace of fresh fruit.
470 Whether the absolute concentration of the 'main component' of banana is necessary, or whether
471 the banana scent is only created by the specific dilution combination is an enigma. Here we show
472 a case study where an effect similar to ratio coding happens in the receptor neuronal level.

473

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587

588 **Figure Legends**

589 **Figure 1: In vivo extracellular single unit recording from ab2** (A) Schematic diagram of the
590 recording set up. Trace shows two olfactory sensory neurons expressing Or59b-expressing
591 neurons and Or85a-expressing neurons labelled as “A” and “B” distinguished by larger and
592 smaller amplitude spikes respectively. (B) Dose response curves of individual ligands. (C)
593 Representative traces and corresponding PSTHs in response to 500 msec (marked as blue)
594 stimulation by acetone (10^{-3}), 2, 3 butanedione (10^{-7}) and their binary odor mixture. Binary
595 mixture of acetone (10^{-3}) and 2, 3 butanedione (10^{-7}) had enhanced response magnitude, i.e firing
596 frequency as well as prolonged response sustained for longer time. The total duration of the odor
597 response for the binary mixture is 6.8 sec (marked as green), sustained longer than its individual
598 component responses, 1.1 sec and 0.76 sec respectively for acetone and 2, 3 butanedione. (D)
599 The dose response curve of the binary mixture did not follow the typical sigmoidal pattern. For
600 each dilution set of acetone ($10^{-4}/10^{-3}/10^{-2}$), the odor mixture evoked firing rate steeply increases
601 up to a certain dilution of 2, 3 butanedione and formed a sharp peak at $10^{-8}/10^{-7}/10^{-6}$ respectively.

602 (E) Bar graph shows that the observed response of the binary mixture of acetone and 2, 3
603 butanedione was significantly higher than the expected additive response. Response were
604 compared in paired sample t-test. Error bars represent SEM; *** $p = 0.001$. (N=30)

605

606 **Figure 2: Extracellular recording and their corresponding PSTH in response to serial**
607 **dilution of acetone, 2-3 butanedione and their binary mixture from ab2.**

608 (A) Representative traces of Or59b-expressing neurons responses towards serial dilutions from
609 10^{-3} to 10^{-9} of acetone, 2, 3 butanedione and the binary mixture of serial dilution of 2, 3
610 butanedione with 10^{-3} dilution of acetone. Both acetone and 2, 3 butanedione alone showed
611 concentration dependent increased firing frequencies. The odor evoked responses of the binary
612 mixture was not linear. Maximum response magnitude observed at a 10^{-7} dilution of 2, 3
613 butanedione when presented with acetone (10^{-3}). (B) The linear regression of odor evoked
614 response of 2-3 butanedione (10^{-3}) alone is significantly ($p > 0.001$) faster than the binary odor
615 mixture of acetone (10^{-4}) and 2,3 butanedione (10^{-8}). The duration of odor stimulation was 500
616 ms (marked as blue) and the total response duration marked as green. Blue solid line indicates
617 the linear regression.

618

619 **Figure 3: Synergistic responses in ab2A and ab1B.** Extracellular recording from Or59b-
620 expressing neurons showed synergistic interaction between (A) 2,3 butanedione with ethyl
621 butyrate and (B) acetone with iso- amyl acetate. Iso-amyl acetate did not evoke any response to
622 Or59b-expressing neurons, but in a binary mixture with acetone, synergism observed (B). The
623 peak responses of these two binary odor combinations observed at $1+10^{-5}$ and $1+1$ dilution

624 combinations of 2, 3 butanedione and ethyl butyrate and acetone and iso amyl acetate
625 respectively (A and B). (C) The list of binary mixture combinations that exhibit synergism in
626 ab2. Paired t-test reveals that the observed synergistic peaks were always significantly greater
627 than the expected additive response of their corresponding mixture components. (N=30)

628 (D) Trace showing the spontaneous firing of a typical ab1 sensillum. Spikes obtained from
629 different neuronal types within the sensillum have been marked accordingly. (E) Representative
630 traces for the responses elicited by 2, 3 butanedione (10^{-8}), ethyl butyrate (10^{-1}) and the binary
631 mixture of the aforementioned chemicals recorded from ab1 sensillum. (F) Bar graphs showing
632 the quantification of average electrophysiological responses elicited from ab1B neuron by 2, 3
633 butanedione (10^{-8}), ethyl butyrate (10^{-1}) and a mixture of the aforementioned chemicals. (N=12)
634 Error bars represent SEM. * $p = 0.5$, ** $p = 0.01$, *** $p = 0.001$.

635

636 **Figure 4: The binary mixture of acetone and 2-3 butanedione evokes a synergistic response**
637 **in fly's behavior.** (A) The schematic representation of single fly behavioral setup.
638 Representative traces from a single fly response to acetone (10^{-3}), 2, 3 butanedione (10^{-7}) and the
639 binary mixture of 2, 3 butanedione (10^{-7}) and acetone (10^{-3}) (B and D). Fly's response towards
640 the binary mixture of acetone (10^{-3}) and 2, 3 butanedione (10^{-7}) was significantly higher
641 ($R^2=0.57$, oneway ANOVA test) than the individual odorants. (D) The population fly tracks of
642 30 flies indicate that flies spent more time at the odor arm of the binary mixture than their
643 corresponding mixture components. (E) Fly's behavior over serial dilution of 2, 3 butanedione
644 with acetone ($10^{-4}/10^{-3}$). At a specific dilution combinations of the two odorants fly exhibited
645 higher response ($R^2=0.62$ and $R^2=0.583$). (F) The peak response index observed for the binary
646 mixture of 2, 3 butanedione (10^{-7}) with acetone (10^{-3}) is significantly greater in paired t-test than

647 the expected additive response of acetone (10^{-3}) and 2, 3 butanedione (10^{-7}) alone. Error bars
648 represent SEM ; *** $p = 0.001$.

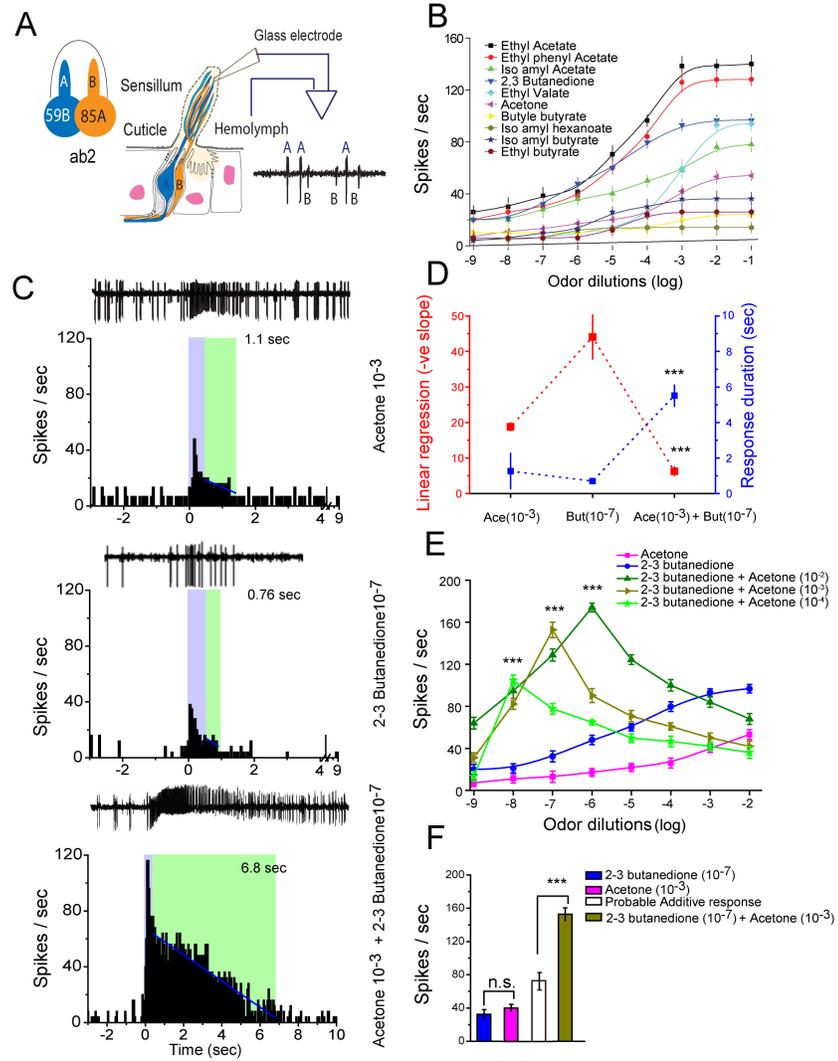
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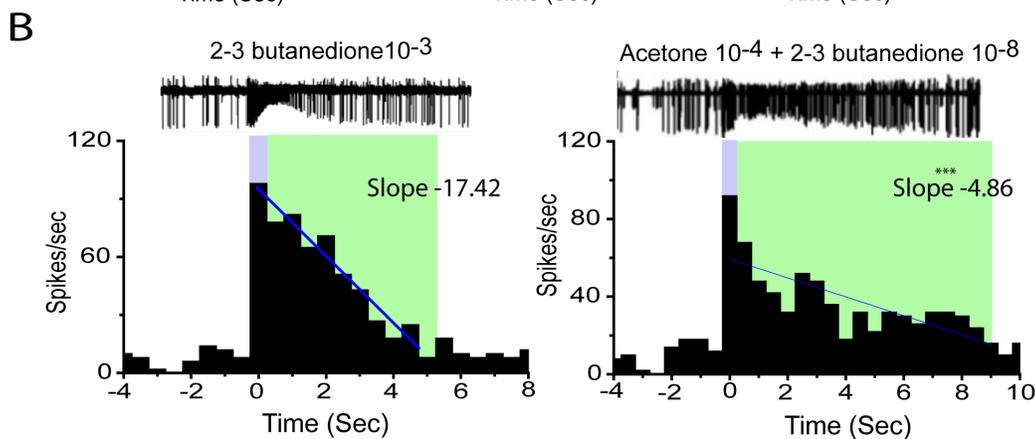
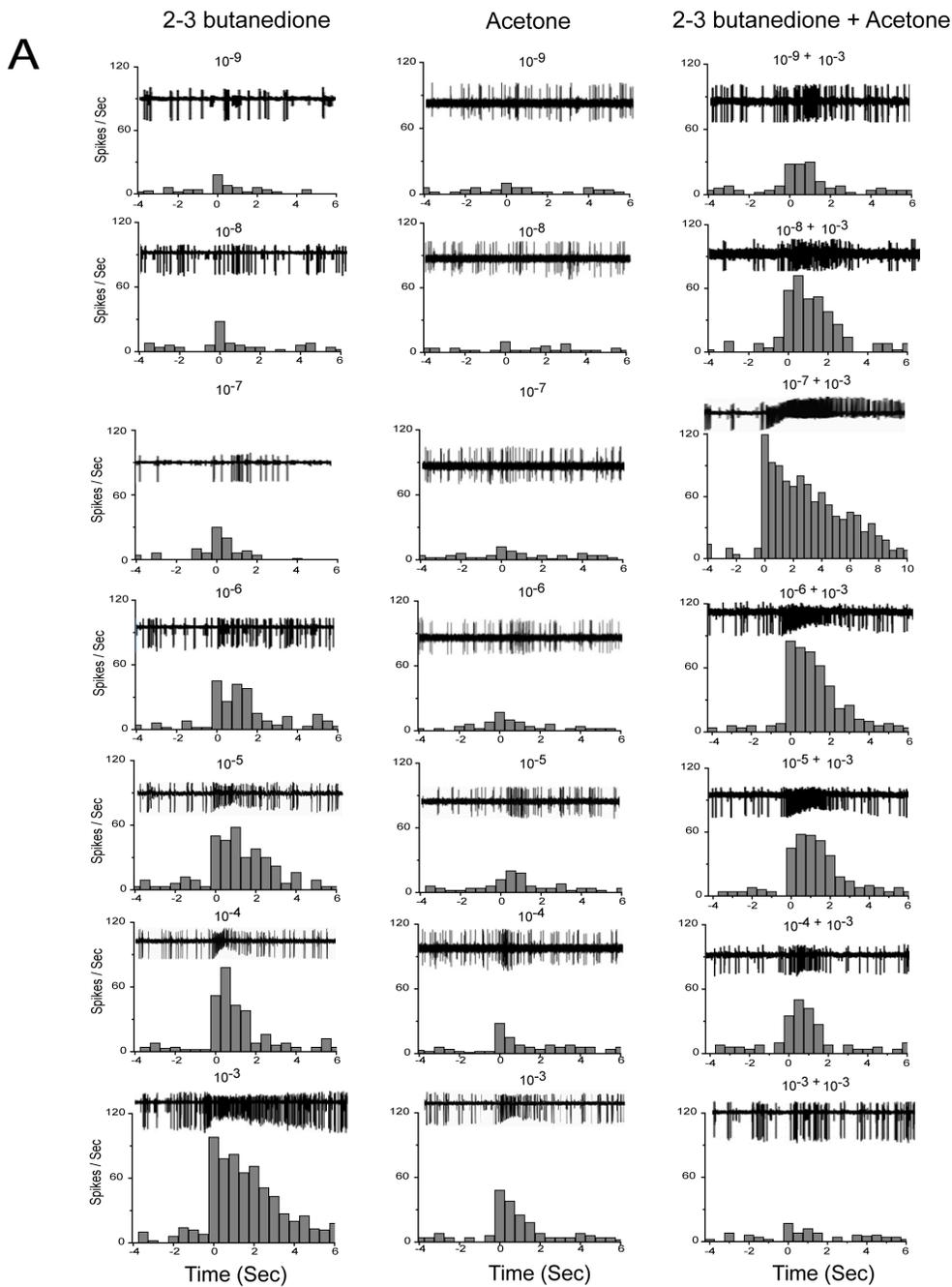
650 **Figure 5: Synergism response towards binary mixture is specific to the receptor.** (A-B)
651 Representative traces and dose response curves show the response profile towards the binary
652 mixture of acetone (10^{-3}) and 2, 3 butanedione (10^{-7}). Both the control and flies where Kir 2.1
653 was expressed in Or85a-expressing neurons exhibited a synergistic response to the binary
654 mixture. Expressing Kir 2.1 in Or59b-expressing neurons abolished synergism (C-D) (N=30).
655 (D) Flies with silenced Or59b-expressing neurons showed less response towards the binary
656 mixture (ANOVA, $R^2=0.725$). Gal4 and UAS-Kir2.1 controls maintain their synergistic
657 phenomenon. (E) Representative traces for the responses elicited by ethyl 3-hydroxy butyrate
658 from the ab2 sensilla from flies of three different genotypes viz. Or85aGal4,UAS-rpr and the
659 UAS and Gal4 controls (N=20). The larger spikes are from ab2A whereas the smaller spikes are
660 from ab2B. In Or85aGal4,UAS rpr the ab2B spikes are absent confirming cell death owing to
661 reaper expression. The Gal4 and UAS control lines show normal increase in ab2B spikes in
662 response (indicated by arrows) to ethyl 3-hydroxy butyrate confirming that there weren't any
663 background effects. (F) Representative traces obtained from ab2 sensillum of flies of two
664 different genotypes, Or85aGal4,UAS-rpr and the UAS control following stimulation by 2, 3
665 butanedione (10^{-8}), acetone(10^{-4}) and a mixture of these two odors. (G) Bar graphs showing the
666 quantification of average electrophysiological responses elicited from ab2A neuron of flies of
667 three different genotypes (Or85aGal4,UAS-rpr and the UAS and Gal4 controls) by 2,3
668 butanedione(10^{-7}), acetone(10^{-3}); a calculated summation of these two responses; and response

669 elicited by a mixture of these two odors. (N=6). Error bar represent SEM; ** $p = 0.01$, *** p
670 =0.001.

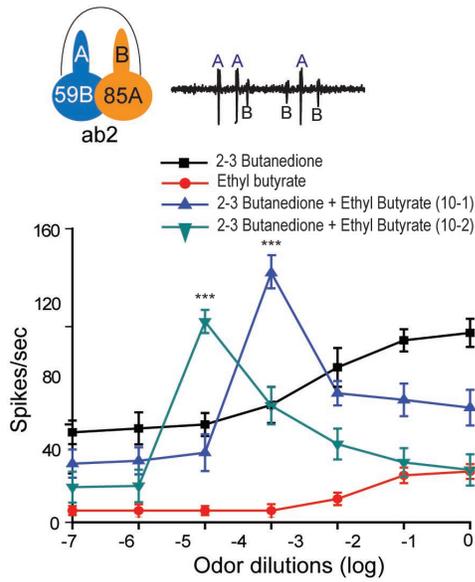
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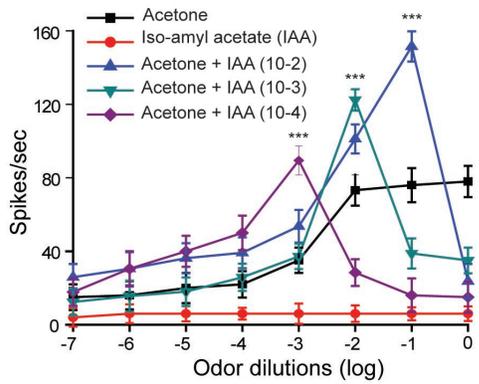




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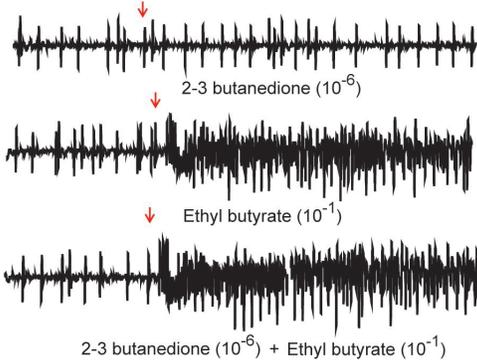
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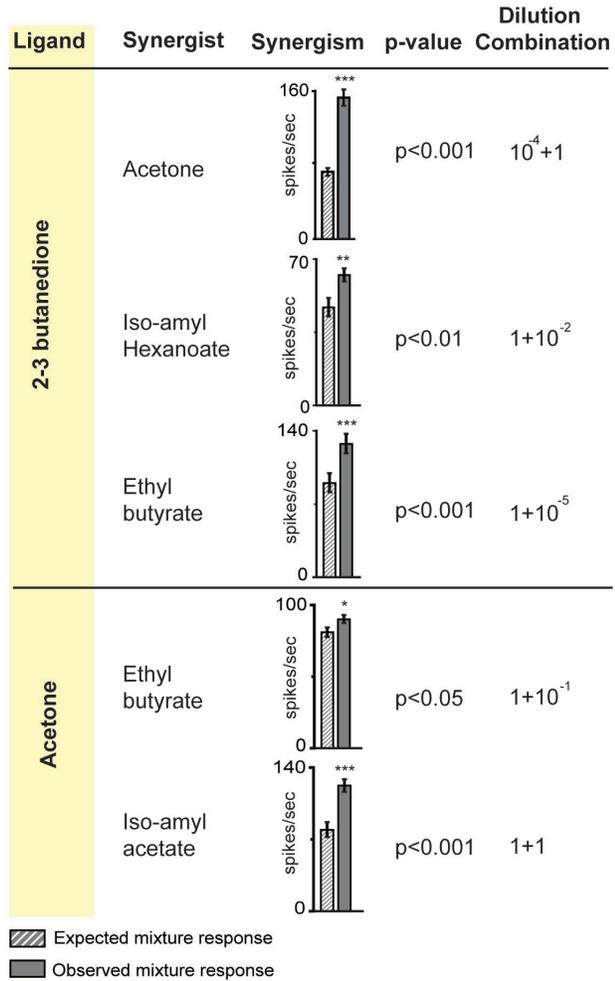
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C



F

