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RESEARCH ARTICLE

Compound valence is conserved in binary odor mixtures in *Drosophila melanogaster*

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ABSTRACT

Most naturally occurring olfactory signals do not consist of monomolecular odorants but, rather, are mixtures whose composition and concentration ratios vary. While there is ample evidence for the relevance of complex odor blends in ecological interactions and for interactions of chemicals in both peripheral and central neuronal processing, a fine-scale analysis of rules governing the innate behavioral responses of *Drosophila melanogaster* towards odor mixtures is lacking. In this study we examine whether the innate valence of odors is conserved in binary odor mixtures. We show that binary mixtures of attractants are more attractive than individual mixture constituents. In contrast, mixing attractants with repellents elicits responses that are lower than the responses towards the corresponding attractants. This decrease in attraction is repellent-specific, independent of the identity of the attractant and more stereotyped across individuals than responses towards the repellent alone. Mixtures of repellents are either less attractive than the individual mixture constituents or these mixtures represent an intermediate. Within the limits of our data set, most mixture responses are quantitatively predictable on the basis of constituent responses. In summary, the valence of binary odor mixtures is predictable on the basis of valences of mixture constituents. Our findings will further our understanding of innate behavior towards ecologically relevant odor blends and will serve as a powerful tool for deciphering the olfactory valence code.

KEY WORDS: *Drosophila*, Insect, Mixture, Olfaction

INTRODUCTION

The sense of smell plays a pivotal role in an insect's life, enabling it to locate mating partners, food sources and oviposition sites and to avoid potential threats. In their natural environment, insects are usually exposed to a large number of odorants at any time. Therefore, most, if not all, odors that have to be evaluated by the insect's nervous system do not consist of monomolecular compounds but, rather, are mixtures of many chemicals varying in composition and concentration ratios.

The vinegar fly *Drosophila melanogaster* Meigen 1830 detects airborne volatiles with olfactory sensory neurons (OSNs) located in hair-like structures – olfactory sensilla – on two types of head appendages, the antennae and the maxillary palps (Stocker, 1994; Vosshall and Stocker, 2007). The majority of OSNs express one type of olfactory chemoreceptor, which defines the receptive range of the neuron (Benton et al., 2009; Couto et al., 2005; Fishilevich and Vosshall, 2005). One way to deal with the complexity of the olfactory environment is to evolve highly specific chemoreceptors and downstream information processing channels for signature chemicals, which is realized in the pheromone system of vinegar flies (Kurtovic et al., 2007; Schlief and Wilson, 2007; van der Goes van Naters and Carlson, 2007), but also for other chemicals of ecological significance (Stensmyr et al., 2012; Suh et al., 2004). With this labeled-line layout, the insect can extract unambiguous information irrespective of the complexity of the blend these signature chemicals are part of.

However, the majority of *D. melanogaster* OSNs do not display such a high degree of ligand specificity (de Bruyne et al., 1999; de Bruyne et al., 2001; Hallem and Carlson, 2006; Hallem et al., 2004; Pelz et al., 2006; Silbering et al., 2011). Therefore, odor identity is thought to be extracted from the combined activity of multiple OSN classes whose odor response profiles differ (Malnic et al., 1999). Nevertheless, accumulating evidence suggests that innate odor-guided behavior can be correlated to the activity of single processing channels (Ai et al., 2010; Dweck et al., 2013; Knaden et al., 2012; Min et al., 2013; Ronderos et al., 2014; Semmelhack and Wang, 2009).

Components of odor mixtures have been shown to influence each other's reception on the level of OSNs (Deisig et al., 2012; Hillier and Vickers, 2011; Münch et al., 2013; Pregitzer et al., 2012; Schuckel et al., 2009; Su et al., 2011; Su et al., 2012). Furthermore, olfactory information is significantly modulated by a dense network of local neurons in the first olfactory center of the insect brain, the antennal lobe (AL) (reviewed in Galizia and Rössler, 2010; Masse et al., 2009; Wilson, 2013; Wilson and Mainen, 2006). Nevertheless, physiological odor mixture responses in the AL are qualitatively predictable on the basis of mixture constituent responses in *Drosophila* (Olsen et al., 2010; Silbering and Galizia, 2007) and other insects (Carlsson et al., 2007; Deisig et al., 2006; Deisig et al., 2010; Fernandez et al., 2009; Joerges et al., 1997; Stierle et al., 2013; but see Anton and Hansson, 1996; Kuebler et al., 2011; Kuebler et al., 2012; Meyer and Galizia, 2012). Therefore, if single OSN class responses are already behaviorally meaningful, the innate hedonic valence of odor mixtures may in fact be predictable on the basis of the valences of mixture constituents.

In order to test this prediction, we performed experiments in the behavioral paradigm Flywalk (Steck et al., 2012), which allows us to assess behavioral responses towards pulses of monomolecular odorants and mixtures in the same set of individual flies at high temporal resolution. We examined behavioral responses of vinegar flies to a set of attractants and repellents and all possible binary mixtures thereof, and observed that mixture valences are indeed predictable. Our results suggest that even detailed predictions regarding response latency, intensity and duration can be made for binary mixtures based on the response characteristics towards the mixture constituents.

Although the results presented here cover only a small part of the enormous amount of chemical stimuli vinegar flies may encounter

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List of abbreviations

1Oct	1-octanol
ACV	apple cider vinegar
AL	antennal lobe
BEA	benzaldehyde
BEDN	2,3-butanedione
EtA	ethyl acetate
EtB	ethyl butyrate
LH	lateral horn
LN	local neuron
IAA	isopentyl acetate
MB	mushroom body
MeSa	methyl salicylate
Oct3ol	1-octen-3-ol
OSN	olfactory sensory neuron
PN	projection neuron
yBtl	γ-butyrolactone

in their natural environment, they suggest that behavioral responses are remarkably predictable on the basis of constituent valence and will serve as a framework for examining naturally occurring odor blends of higher complexity and ecological significance.

RESULTS

We used the behavioral paradigm Flywalk (Steck et al., 2012) to investigate whether the innate valence of single compounds is conserved in binary odor mixtures. In Flywalk, individual vinegar flies are placed in small wind tunnels, tracked automatically and repeatedly presented with olfactory stimuli. The odor pulses travel along the glass tubes at a constant speed. It is therefore possible to calculate the time of encounter with the odor for every fly and every stimulation cycle (Fig. 1A). Flies usually display no or only weak responses to the solvent mineral oil and robust upwind movement after encountering an attractive odor (Fig. 1B).

Dose-response characteristics of attractants

As a starting point for studying mixture interactions, we selected four monomolecular compounds, all of which elicit attraction at intermediate concentrations, and examined dose–response characteristics for three concentrations. Attractants were chosen on the basis of their consistent attractivity in pilot experiments and because they are commonly used in behavioral and physiological studies (e.g. Ayyub et al., 1990; Bhandawat et al., 2010; Borst and Heisenberg, 1982; Krishnan et al., 2011; Störtkuhl et al., 1999; Woodard et al., 1989). Responses were odorant- and concentration-specific and strongly stereotyped in both amplitude and duration across individuals (Fig. 1C–E).

Although 2,3-butanedione (BEDN) failed to elicit significant upwind movement at the 10^{-5} dilution, flies were strongly attracted by higher concentrations (Fig. 1C–E; supplementary material Fig. S1). For both the intermediate and the high concentrations (10^{-3} and 10^{-1}, respectively), flies reached a mean maximum upwind speed of approximately 0.4 cm s^{-1}, but responses differed in duration. At the intermediate concentration, statistically significant upwind movement was observed until 6 s after the odor pulse was presented, whereas responses towards the high concentration of BEDN stayed significant throughout the whole interval considered for analysis (up to 7 s after the odor pulse). Ethyl acetate (EtA) elicited odor-induced upwind movement at all concentrations tested with mean amplitudes of 0.4 cm s^{-1} at low and high concentrations (Fig. 1C–E; supplementary material Fig. S1). Upwind speed was highest for the intermediate 10^{-3} dilution, reaching a maximum of 0.6 cm s^{-1}. While responses were similarly brief (~2 s) in the low and intermediate concentrations, the high concentration induced a prolonged response lasting approximately 5 s after the encounter with the odor pulse. Ethyl butyrate (EtB) displayed a narrow response profile, with significant upwind movement observed only at the intermediate concentration (Fig. 1C–E; supplementary material Fig. S1). On average, response time courses reached a maximum of 0.4 cm s^{-1} and otherwise displayed temporal dynamics similar to those of EtA at the intermediate concentration. Isopentyl acetate (IAA) induced significant upwind movement at all concentrations tested and similar response dynamics at the two lower concentrations, reaching a maximum of 0.3–0.4 cm s^{-1} (Fig. 1C–E; supplementary material Fig. S1). At the highest concentration, responses were less strong than those at lower concentrations, reaching a maximum of only 0.2 cm s^{-1}.

To assess immediate responses towards different odorants in a single metric and allow for direct statistical comparison, we calculated the mean upwind displacement for each fly and odor within 4 s of odor encounter. Results of this analysis mirror the observations described above regarding the intensity of the response to the different odorant concentrations (Fig. 1F). However, using this metric we did not identify significant attraction towards the intermediate and high concentrations of IAA (P=0.1 and 0.09, respectively). In these cases, significant upwind trajectories were masked by the previously mentioned weak responses to the negative control and the general anemotactic behavior of vinegar flies (Fig. 1D,E) (Budick and Dickinson, 2006). This general tendency to move in the upwind direction occluded weak upwind surges, when averaging over 4 s after the odor pulse. Shortening the temporal window to 2 s after the odor pulse restored statistical significance in both cases (P<0.01). Because we wanted our metric to reflect a large part of the odor response including the prolonged duration of the BEDN response, we nevertheless used the 4 s window throughout the study.

Binary mixtures of attractants

Based on the results of the dose–response characteristics described in the previous section, we chose the intermediate 10^{-3} dilutions to examine how behavioral responses towards binary mixtures of attractants relate to the responses towards mixture constituents.

In addition to the attractants described in the previous section, we included a 10^{-3} dilution of γ-butyrolactone (yBtl) in these experiments; yBtl was highly attractive in another behavioral assay (Knaden et al., 2012) and also in the Flywalk paradigm at high concentrations, but neutral at intermediate and low concentrations (Steck et al., 2012) (supplementary material Fig. S2). We hypothesized that even though a 10^{-3} dilution of yBtl is behaviorally neutral by itself, it may nevertheless affect behavioral responses when mixed with another attractant. However, none of the responses towards binary mixtures containing yBtl differed from the responses towards the other attractive mixture constituent (supplementary material Fig. S2), which is why we will not expand on mixtures containing yBtl. In total, binary mixtures of attractants were examined in 10 experiments, each containing three of the five single compounds and mixtures thereof in all possible combinations (supplementary material Table S1).

Mixtures of attractants were always more attractive than mixture constituents (Fig. 2A). For every given mixture, the mixture response was significantly higher than the response to the less attractive constituent, and in some cases it was significantly higher than both constituent responses (Wilcoxon signed rank test; Fig. 2B). The reason for the increased strength of the responses to binary mixtures lies in the temporal dynamics of mixture responses; these dynamics follow an optimum time course of both response time

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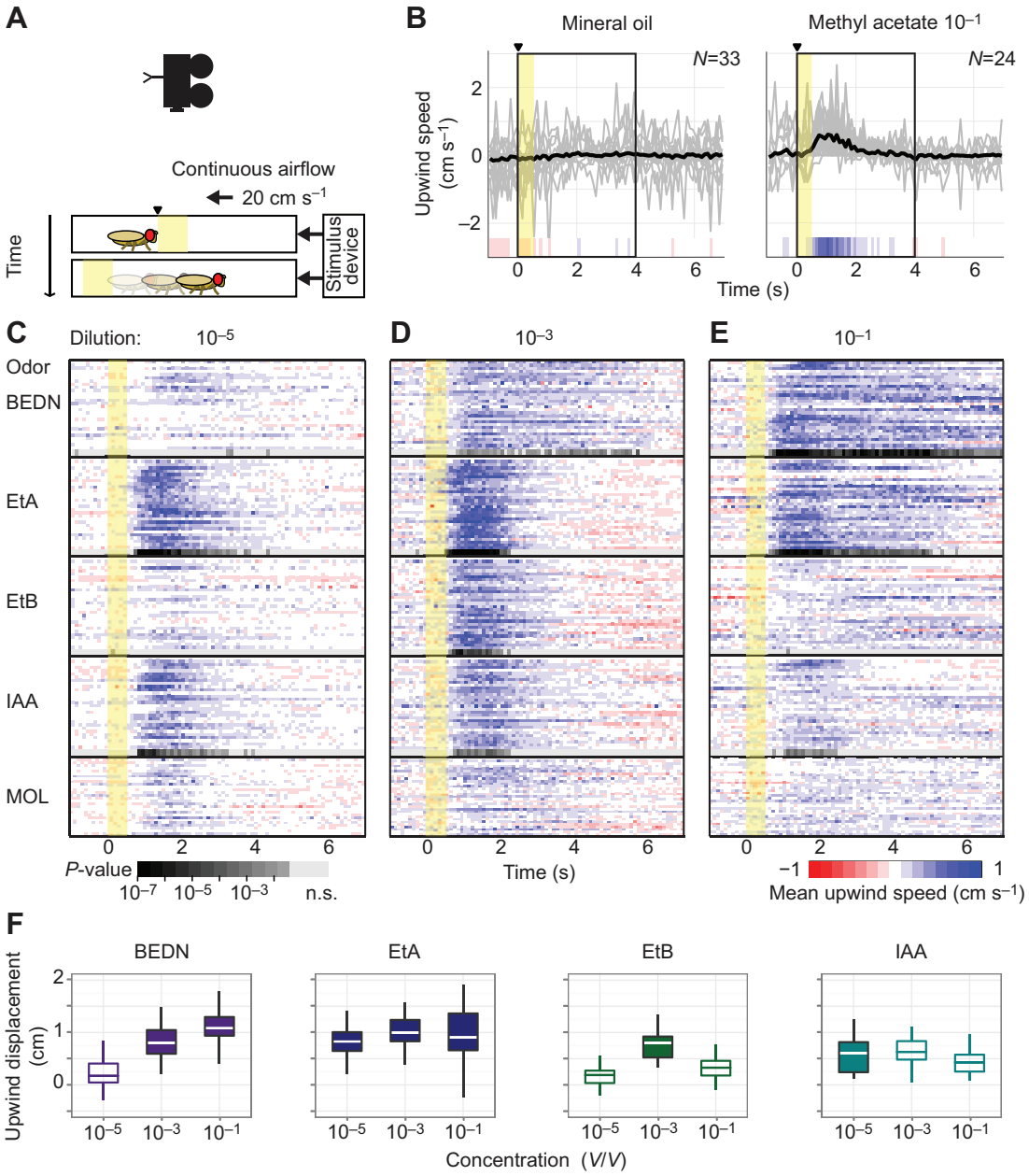

Fig. 1. Dose–response characteristics of attractive odors. (A) Schematic of the behavioral paradigm Flywalk. Individual *Drosophila melanogaster* are situated in small wind tunnels and exposed to a constant airflow of ~20 cm s^{-1}. Odor pulses are added to the airflow, and fly positions before and after each encounter with the odor pulse (arrowhead) are tracked automatically at a temporal resolution of 100 ms. (B) Representative responses of an individual fly to repeated presentations of the solvent mineral oil (left) and the attractant methyl acetate (right). Gray, single trajectories; black, mean trajectories. As flies are allowed to distribute freely within their glass tube and may leave the region of interest of the tracking system, within-fly sample sizes differ between odorants. Black rectangle shows the temporal interval used for the calculation of net displacement. Color-coded rows at the bottom of the panels exemplify the data in the same way it is embedded in C–E. Same scale as C–E. (C–E) Color-coded mean response trajectories towards different concentrations of the attractive odors 2,3-butanedione (BEDN), ethyl acetate (EtA), ethyl butyrate (EtB) and isopentyl acetate (IAA), and the solvent mineral oil (MOL). Each row shows the mean trajectory of a single individual. Yellow bar represents the odor pulse. Grayscale rows represent the time-resolved *P*-values of a one-sided Wilcoxon signed rank test against the negative control, mineral oil (n=28–30 flies; note logarithmic scaling of *P*-values). (F) Net upwind displacement within 4 s of encounter with pulses of different concentrations of attractants (n=28–30 flies). Same data as in C–E. Filled boxes indicate significantly higher upwind displacement compared with the negative control mineral oil (P<0.05; Wilcoxon signed rank test).

courses towards mixture components. This effect is strikingly apparent for the mixture of EtB and BEDN, which follows the sharp onset of the EtB response and the prolonged duration of the BEDN response (Fig. 2C); this observation could be confirmed in every single case in which the optimum time course was not identical to one of the constituent response time courses (see supplementary material Fig. S2).

To analyze this observation quantitatively, we defined predicted optimum response time courses from the mean response time courses towards mixture constituents by creating a new speed vector of the same length using the higher of the two compound speed values at any given point in time (Fig. 2D). We then examined the correlation of response time courses of mixture constituents and the mixture, and of this optimum time course and the mixture time

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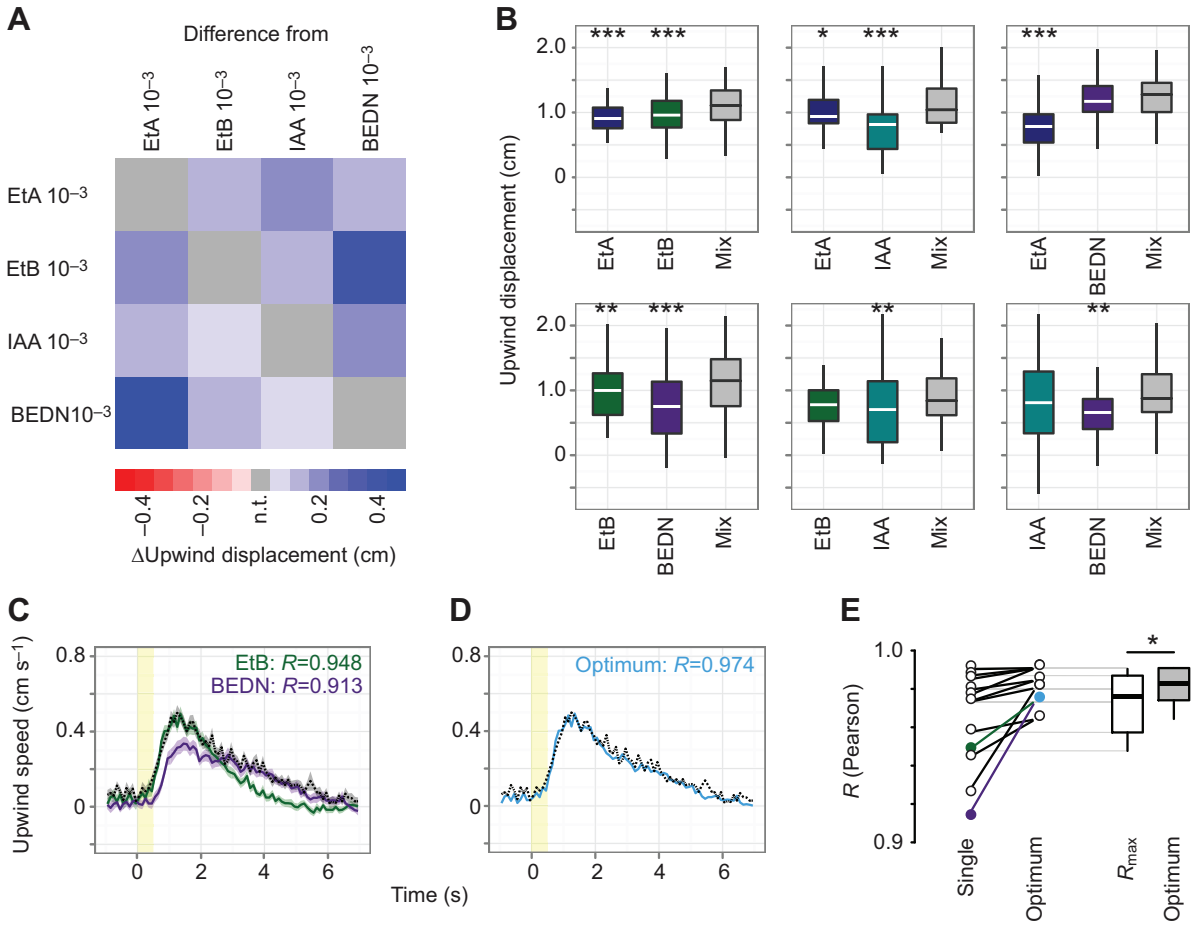

Fig. 2. Binary mixtures of attractants. (A) Color-coded difference in medians between responses towards mixtures and mixture constituents (n.t., not tested). For every mixture there are two comparisons, one for each mixture constituent, and those account for the asymmetry. Color code represents the difference between the mixture and the constituent indicated on top of the figure. Note that all differences are positive, i.e. responses evoked by mixtures are always stronger than responses elicited by mixture constituents. (B) Statistical analysis of mixture responses versus constituent responses (mixtures shown in gray). Asterisks indicate significant differences from the mixture response (*P<0.05; **P<0.01; ***P<0.001; Wilcoxon signed rank test; n=43–45 flies). (C) Comparison of trajectories evoked by BEDN (purple), EtB (green) and the mixture of BEDN and EtB (dotted black; mean ± s.e.m.; n=45 flies) and Pearson's correlation coefficients between single compound and mixture response time course (top right). Yellow bar represents the odor pulse. (D) Comparison between the optimum time course extracted from EtB and BEDN time courses (blue; see Results) and the mixture trajectory (dotted black), and correlation coefficient. (E) Left: correlation coefficients between compound and mixture time courses (single) and between corresponding optimum and mixture time courses (optimum). Connected data points are extracted from the same mixtures; colored data points and connections represent the data presented in C and D. Right: statistical comparison between the higher of the two compound correlation coefficients (R_{max}) and the correlation coefficients between optimum and the mixture (optimum) (P=0.03125; Wilcoxon signed rank test).

course. While correlation coefficients between single compounds and mixtures were already high because of the general kinetics of attractant responses (R>0.9 in all cases), the correlation between mixture responses and optimum responses was higher and displayed less variability (Fig. 2E, left). A similar result may be obtained if the mixture response is simply dominated by the more attractive compound, without any contribution of the second odor in the mixture. However, the correlation between optimum and mixture was also significantly higher than the correlation between the more similar of the two constituents and the mixture (P<0.05, Wilcoxon signed rank test; Fig. 2E, right). In other words, both compounds contribute to the mixture response and the mixture response represents the optimum of both compound responses.

Binary mixtures containing repellents

Having shown that positive valence is conserved in binary odor mixtures, we next asked how mixing in a repellent affects attractant responses. It is important to note here that we use the term 'repellent' for compounds of negative hedonic valence for reasons of linguistic simplicity. Even reported *Drosophila* repellents such as geosmin provoke rather inconsistent behavior when tested alone in the Flywalk assay (Stensmyr et al., 2012). Therefore, the choice of repellents was based on previous reports on the hedonic valence of these compounds. We used a 10^{-1} dilution of benzaldehyde (BEA), because it was previously shown to induce downwind movement in Flywalk (Steck et al., 2012), and 10^{-3} dilutions of methyl salicylate (MeSa), 1-octanol (1Oct) and 1-octen-3-ol (Oct3ol). 1Oct and Oct3ol were chosen because they were shown to repel vinegar flies in a different assay (Knaden et al., 2012). MeSa strongly activates Or10a-expressing OSNs, which innervate the DL1 glomerulus, which in turn was predicted to exhibit a negative valence in the aforementioned study.

In our initial experiments, BEA, MeSa and 1Oct elicited significant downwind displacement within 4 s of an odor encounter when presented alone (P<0.05, one-sample Wilcoxon signed rank test; Fig. 3A). The median response across individuals for Oct3ol

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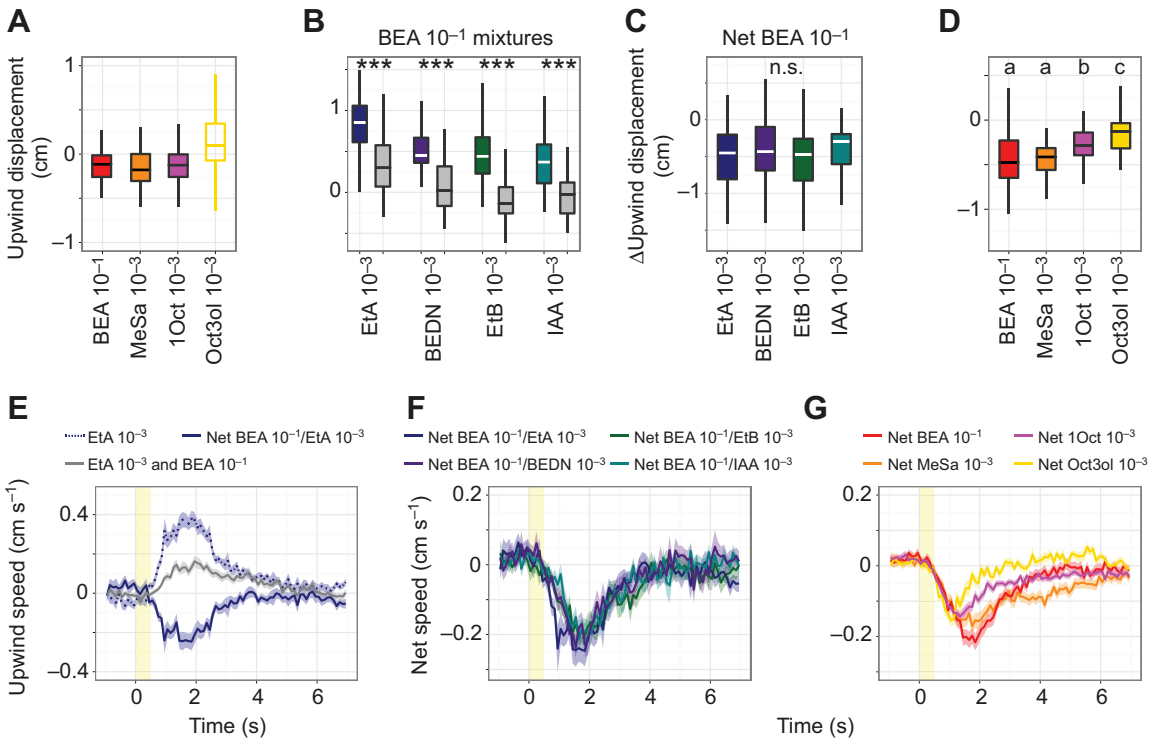

Fig. 3. Mixtures of attractants and repellents. (A) Responses towards repellent compounds (n=51–60 flies). BEA, benzaldehyde; MeSa, methyl salicylate; 1Oct, 1-octanol; Oct3ol, 1-octen-3-ol. Filled boxes indicate statistically significant downwind movement (P<0.05; one-sample Wilcoxon signed rank test). (B) Comparison of attractant responses and binary mixtures (gray) of attractants with BEA (n=37–39 flies; ***P<0.001; Wilcoxon signed rank test). (C) BEA net responses for different attractive mixture constituents extracted by subtracting responses to attractants from responses to the corresponding mixtures. Note that there is no significant difference between net responses extracted from mixtures with different attractants (P>0.1; Kruskal–Wallis rank sum test; n=37–39 flies). (D) Consensus net responses for repellents (see Results for details). All net responses are significantly less than zero (P<0.001; one sample Wilcoxon signed rank test; n=37–39 flies). Different letters indicate statistically significant differences between consensus net responses (P<0.01; Kruskal–Wallis rank sum test and *post hoc* Wilcoxon rank sum test). (E) Example of the extraction of repellent net trajectories (mean ± s.e.m.; n=39 flies). Dotted blue line, EtA trajectory; gray line, mixture of EtA and BEA; solid blue line, BEA net trajectory from mixtures with EtA. (F) BEA net trajectories calculated from mixtures with different attractants. Color code as in B and C; blue line corresponds to the trajectory shown in E; n=37–39 flies. (G) Consensus net trajectories for the four repellents (see Results for details). Color code as in A and D; n=51–60 flies.

was slightly positive because of a late, insignificant upwind deflection in the response time course (Fig. 3A; supplementary material Fig. S2). However, compared with the fast and stereotyped upwind trajectories evoked by attractive odors, the response trajectories evoked by repellents were less stereotyped across individuals, lower in amplitude and displayed greater latency (supplementary material Fig. S3A). Moreover, repellent responses were also less reproducible than attractant responses in different data sets (supplementary material Fig. S2, see binary mixtures of the repellents BEA, MeSa, 1Oct and Oct3ol), suggesting that what we observe when presenting repellents on their own does not accurately represent their true hedonic valence.

To gain insight into the impact of these compounds of reported negative valence on innate attraction behavior, we tested responses towards the four previously described attractants and compared those with responses towards mixtures of attractants and repellents. For each of the four repellents, we performed four experiments containing three of the four attractants and all possible mixtures of attractants with the repellent (supplementary material Table S1). In 15 out of 16 cases, responses to mixtures were significantly lower than responses to the corresponding attractants (Fig. 3B as an example for mixtures with BEA, supplementary material Fig. S4 for other repellents). Even in the single case in which the difference between attractant and mixture response was not statistically significant – the mixture of IAA and Oct3ol – a clear trend towards a similar reduction was apparent (supplementary material Fig. S4).

Intriguingly, the degree of response reduction conferred by a given repellent appeared to be similar irrespective of the attractant in the mixture, suggesting that the contribution of a repellent to the mixture response is independent of the identity of the attractive mixture constituent. To test for this possibility, we calculated the net responses of individual flies to the repellents by subtracting the responses to attractants from the responses to corresponding mixtures. This way we obtained four different net responses per repellent, each one corresponding to the net contribution of the respective repellent to the mixture with a different attractant. We did not observe any statistically significant differences between net responses derived from mixtures with different attractants for any of the repellents (P>0.1, Kruskal–Wallis rank sum test; Fig. 3C for BEA, supplementary material Fig. S4 for other repellents).

Because the contribution of the repellents to mixture responses is independent of the attractant in the mixture, we were able to obtain general net responses for each of the repellents. As we tested mixtures with three different attractants in every experiment, simple averaging of all net responses for a given repellent would lead to an artificial triplication of the dataset. We therefore calculated consensus net responses by averaging the three net responses per repellent for every individual fly and used these for further analysis. For all repellents, this net response was significantly lower than zero

($P<0.001$; one-sample Wilcoxon signed rank test), and net responses for different repellents differed significantly from each other ($P<0.01$; Kruskal–Wallis rank sum test and *post hoc* Wilcoxon signed rank test; Fig. 3D). BEA and MeSa were equally potent in reducing attractant responses, 1Oct was intermediate and Oct3ol was the weakest of our repellents.

In order to gain deeper insight into the contribution of repellents to the mixture response time course, we calculated repellent net trajectories by subtracting the mean attractant trajectories from the mean mixture trajectories within individuals (Fig. 3E). This way we obtained four net trajectories for every repellent, each one deduced from mixing the repellent with a different attractant. As already observed for net displacement (see net responses, above), contribution of the repellent to the mixture response time course was largely independent of the identity of the attractant in the mixture (Fig. 3F for BEA, supplementary material Fig. S4 for other repellents). Because the net trajectories of the repellents are independent of the identity of the attractive mixture constituents, we could obtain consensus net trajectories for each repellent, again by averaging the three different net trajectories of every individual fly (see above). Like attractant response time courses, repellent net time courses were repellent-specific in amplitude and duration (Fig. 3G). Importantly, these repellent net trajectories display a shorter latency – as repellents already reduce the very beginning of the attractant response – and are more stereotyped across individuals than are the trajectories obtained for repellent compounds when presented alone (supplementary material Fig. S3B,C).

As previously described, the response trajectories towards repellents presented alone were generally less stereotyped than were the attractant trajectories, and in many cases repellents would be classified as behaviorally neutral in the Flywalk paradigm because of the lack of fast downwind movement upon odor encounter. Nevertheless, we also examined mixtures of repellents to test the hypothesis that compound valence is conserved in binary odor mixtures. Mixtures of repellents were less attractive than or in between both compounds, which further supports our hypothesis (supplementary material Fig. S2) that the valence of individual constituents is conserved in a binary mixture. However, as the responses to the individual repellents as well as to the mixtures of two repellents were rather small and inconsistent, we cannot draw clear conclusions on mixture interactions on the time-course level from these experiments.

DISCUSSION

We show that constituent valence is conserved in binary odor mixtures in *D. melanogaster*. All mixtures of attractants in our dataset are at least as attractive as the more attractive mixture constituent. When we mix attractants with repellents, the response to the mixture is between the two responses to the compounds. Responses towards mixtures of repellents are either intermediate between the two responses or more negative than both constituent responses. Importantly, none of the 32 (38 including supplementary material, see below) binary mixtures tested elicited responses that were entirely unpredictable. We did not observe response reduction when we mixed attractants; adding a repellent never increased an attractant response; and responses towards mixtures of repellents were never more attractive than responses towards both repellents in the mixture. These findings lend strong support to the hypothesis that innate hedonic valence is already encoded in the identity of the OSN–projection neuron (PN) channels a given odor activates and that behavioral output is dictated by the summed weights of all activated channels (Knaden et al., 2012; Semmelhack and Wang, 2009; Wilson, 2013). Because we did not adjust mixtures to achieve the same absolute number of molecules in the headspace as in single compounds, there is a formal possibility that the increase in total input intensity may have an effect on overall valence, as attractive compounds often become repellent or neutral at high concentrations. This possibility would predict that compounds with high vapor pressures or compounds inducing stronger total OSN input would be more likely to reduce attractant responses. In contrast to this prediction, our repellents are consistently lower in vapor pressure and on average induce lower summed OSN activity than our attractants do (supplementary material Table S2) (Hallem and Carlson, 2006; Kreher et al., 2008). This observation further supports the notion that odor valence is determined by the identity of activated OSN populations rather than total OSN input.

The valence of odor mixtures

The rules governing responses to mixtures appear to be, at least within the limits of our dataset, very simple: for mixtures of attractants, the response is an optimum of both constituent responses (Fig. 2C–E). A repellent adds a negative component to the mixture and the response is the linear sum of the attractant response plus this negative component (Fig. 3; supplementary material Fig. S4). Because immediate responses towards single repellents are comparably weak, we cannot draw a clear conclusion on rules governing mixtures of repellents. However, we did observe a trend that response trajectories of repellent mixtures tend to follow the lower of the two constituent trajectories (supplementary material Fig. S2).

Our findings seem to stand in contrast to recent studies on odor-guided flight behavior towards odor blends in vinegar flies (Becher et al., 2010) and the hawkmoth *Manduca sexta* (Riffell et al., 2009), both of which reported synergistic effects of initially neutral blend constituents on attraction behavior. There are several possible explanations for this discrepancy. In both studies, attraction was scored as the completion of a sequence of behavioral decisions, i.e. take-off, upwind flight and feeding (Riffell et al., 2009), and take-off, upwind flight and landing at the odor source (Becher et al., 2010). In contrast, our approach tests immediate responses towards short pulses of odorized air and is therefore probably comparable to odor-induced upwind surges observed directly after flies enter an attractive odor plume (Budick and Dickinson, 2006; van Breugel and Dickinson, 2014). Although single compounds may not be sufficient to induce the full behavioral program from take-off to landing (i.e. appear to be neutral in the wind tunnel studies), they may nevertheless induce upwind surges upon plume encounter (i.e. become identified as attractive in the Flywalk assay). This hypothesis is supported by the fact that in addition to acetic acid, two of the compounds reported to be behaviorally neutral by Becher and co-workers, i.e. ethyl acetate and acetoin, are attractive in our as well as in other behavioral paradigms (supplementary material Fig. S5) (Larsson et al., 2004; Stensmyr et al., 2003), and binary mixtures of these compounds largely follow the rules we derived with our odor set. Alternatively, the difference may arise from the fact that flies are walking in our paradigm, while they are flying in the wind tunnel, a difference that has been suggested to influence the evaluation of CO_2 (Wasserman et al., 2013). A fine-scale analysis of flight behavior in the wind tunnel particularly focusing on instantaneous responses upon plume encounter will be needed to resolve the reason for the differences between our results and the results provided by Becher and co-workers.

Additionally, both Becher and co-workers and Riffell and co-workers tested blends of ecological relevance for which privileged

coding strategies may exist. The notion that ecologically relevant blends may be specially processed is supported by a recent study on host detection in the black bean aphid *Aphis fabae*; this aphid is repelled by single constituents of its host blend but strongly attracted by the full blend (Webster et al., 2010). In contrast to these studies, we did not attempt to mimic naturally occurring odor blends, but, rather, tested ecologically meaningless mixtures; this was done to empirically derive a mechanistic zero-hypothesis against which responses towards ecologically relevant blends may be tested in the future.

Nevertheless, the predictability of the innate valence of odor mixtures may be surprising especially in light of the complexity of the information that must be integrated to lead to the behavioral output, but it is not unprecedented. Binary mixtures of attractants are more attractive than mixture constituents to the spiny lobster *Panulirus argus* (Derby et al., 1996). In a psychophysical study on human odor perception, Lapid and co-workers were able to establish a model that could predict with astonishing accuracy the pleasantness of binary odor mixtures on the basis of the pleasantness of mixture constituents (Lapid et al., 2008). However, in contrast to our results and the observations in *P. argus*, human subjects rate the pleasantness of an odor mixture as intermediate between the pleasantness of mixture constituents even when both constituents are pleasant. This discrepancy may theoretically be explained by the fact that experience-based evaluation cannot be entirely excluded when test subjects are adult humans, while rearing conditions are highly controlled in laboratory organisms.

However, the discrepancy may also reflect a fundamental difference in the innate evaluation of chemical signals between arthropods and vertebrates or between organisms with chemoreceptor repertoires of different sizes. The adult *Drosophila* olfactory system detects volatile chemicals via ~45 olfactory receptors (reviewed in Touhara and Vosshall, 2009; Vosshall and Stocker, 2007) and ~15 ionotropic receptors (Benton et al., 2009). Although information on olfactory chemoreceptors in crustaceans is sparse, available data suggest a comparably low number (Corey et al., 2013; Groh et al., 2013). In contrast, humans possess ~400 functional olfactory receptors (reviewed in Ache and Young, 2005) with which to sample olfactory space, which should theoretically increase the resolution of the human olfactory system compared with those of the aforementioned arthropods (Bushdid et al., 2014; Keller and Vosshall, 2007). This higher resolution may allow for a graded analysis of chemical information already in the innate situation, while a lower resolution may favor a binary valence code. Further studies of innate behavior towards odor mixtures in several organisms are needed to test this hypothesis. The sizes of olfactory receptor repertoires vary greatly in both vertebrates [e.g. zebrafish: 98; mice: 1200 (reviewed in Ache and Young, 2005)] and insects [e.g. vinegar flies: ~60; Hymenoptera: 200–400 (reviewed in Hansson and Stensmyr, 2011)], a fact that provides an excellent opportunity to test whether the difference in innate mixture evaluation strategies is conferred by evolutionary history or the resolution of the respective olfactory systems.

Response strategy towards repellents

We chose our repellents mainly on the basis of previous reports on their behavioral activity or – in the case of methyl salicylate – on predictions on the basis of the OSNs they activate and not on the basis of their activity in Flywalk (Knaden et al., 2012; Steck et al., 2012; Wasserman et al., 2012).

In our experiments, presenting the repellents alone led to weak downwind walks in some cases, which occurred much later after odor encounter than attractant responses (supplementary material Fig. S3A). This initially, and counterintuitively, suggested that responses towards repellents are generally slower and less stereotyped across individuals than responses towards attractants. Moreover, we also observed delayed downwind walks after fast upwind surges when flies were exposed to attractants (Fig. 1D), and therefore cannot unambiguously interpret them as indicating negative valence. While the upwind surges in this case can clearly be directly linked to odor onset because of their short latency, the late downwind walks may also be induced by odor offset, as has been shown for casting behavior (van Breugel and Dickinson, 2014). Because we have access only to one-dimensional movement along the wind direction, we cannot exclude the possibility that the late downwind walks we observe both for attractants after upwind surges and for repellents may in fact be a downwind-biased form of crosswind movement.

However, mixture experiments revealed that immediate repellent responses are at least as fast as attractant responses, as repellents stereotypically and strongly reduced the very beginning of attractant responses (e.g. supplementary material Fig. S3B,C). This is in agreement with the results of Stensmyr and co-workers, who recently identified a dedicated neuronal circuit for the detection of geosmin (Stensmyr et al., 2012), which is the strongest vinegar fly repellent currently known. Similar to our observations, encountering geosmin alone did not induce downwind movement in Flywalk, but it strongly reduced attraction towards balsamic vinegar. The only indication of a fast response induced by geosmin alone in these experiments was a decrease in activity upon odor encounter. This ‘freezing’ behavior has also been observed by Steck and co-workers for benzaldehyde and at a higher concentration also for Oct3ol, and has been proposed to be an indicator of negative valence (Steck et al., 2012). Because flies in our experiments generally displayed a lower baseline level of activity compared with levels in the aforementioned studies – probably as a result of different rearing conditions and a lower temperature during experiments – we were not able to observe significant odor-induced freezing. Nevertheless, this reduction in a fly’s activity after encountering a repellent may be the reason for the strongly reduced attractant responses in our study.

Insights into the hedonic valence code

At the concentrations we used for our experiments, most compounds in our odor set activate several OSN classes of partially unknown innate valence (de Bruyne et al., 1999; de Bruyne et al., 2001; Galizia et al., 2010; Hallem and Carlson, 2006; Hallem et al., 2004; Kreher et al., 2008; Pelz et al., 2006). Therefore, the responses we observe for single compounds already are the result of the integration of the information from several OSN–PN channels, and what we observe as the final behavioral output is the net valence of all active channels. Nevertheless, we can speculate on the major players involved in the odor valences observed in our dataset. Although activity ratios between different OSN–PN channels are likely to influence information processing in the olfactory system and therefore also behavioral output, we will focus on a binary all-or-nothing logic of activity/inactivity of certain channels (Koulakov et al., 2007) for these speculations.

All attractants in this study have been reported to activate *Or42b*, which has been shown to be necessary and sufficient for attraction to apple cider vinegar (ACV) in a four-field olfactometer (Semmelhack and Wang, 2009). However, whereas our results support this general observation, the diversity of responses towards our attractants in both dose–response and mixture experiments

suggests that *Or42b* is not the only olfactory receptor determining the behavioral output. Apart from *Or42b*, EtA also strongly activates OSNs expressing both *Or59b*, which is preferably activated by attractive odors (Knaden et al., 2012), and *Or42a*. As EtA elicits the highest amplitude in response trajectories in our dataset and is attractive throughout the whole concentration range we tested, we assume that most, if not all, receptors activated by this compound exhibit a positive hedonic valence or are neutral in attraction behavior. We therefore also assume a positive valence or neutrality for *Or42a*. In addition to *Or42b*, BEDN also strongly activates OSNs expressing *Or92a*, which has also been suggested to contribute to ACV attraction (Semmelhack and Wang, 2009) and may be responsible for the late phase of the BEDN response.

Although the discussion about the valence of *Or85a* is still ongoing, the lower attraction towards EtB in comparison to the attraction towards EtA may be explained by the additional activity of *Or85a*, which reduces attraction towards ACV and EtB at high concentrations in a different paradigm (Semmelhack and Wang, 2009). BEA strongly activates *Or7a*, which preferentially responds to odors of negative valence (Knaden et al., 2012) and therefore is the most likely candidate for conferring the negative valence of this compound. In adult flies, MeSa almost exclusively activates *Or10a*, and our results support the previously suggested negative valence of OSNs expressing this receptor (Knaden et al., 2012). Both 1Oct and Oct3ol strongly activate *Or35a* (among others), which may be a candidate for conferring the negative valence of those compounds. In addition, both odors also activate *Or22a*, which is also activated by the attractants IAA and EtB and is alleged to exhibit a positive valence (Knaden et al., 2012). This may offer an explanation for the reduction of BEA repulsion in mixtures with 1Oct and Oct3ol.

Neural correlates of innate odor-guided behavior

Taken together, our results suggest that attractants induce upwind movement while repellents suppress it. The easiest explanation for our observations would be that positive-valence channels promote forward/upwind movement through mainly excitatory connections, whereas repellents interfere with this positive information by inhibition at one of several possible processing levels of the olfactory system (Galizia, 2014). If this simple model is true, where does negative information interfere?

In principle, peripheral mechanisms such as ligand-induced OSN inhibition at the receptor level (de Bruyne et al., 1999; de Bruyne et al., 2001; Hallem and Carlson, 2006; Hallem et al., 2004; Su et al., 2011), ephaptic interactions within the sensillum (Su et al., 2012) or syntopic interactions (Münch et al., 2013) may already contribute to shaping the input signal in a valence-specific way. This would require a system layout in which positive receptors are consistently inhibited (or weakly activated) by compounds of negative valence, or that positive and negative OSNs are co-localized within the same sensilla to allow for valence-specific bilateral inhibition. However, we do not see a general pattern of olfactory receptors activated by our attractants being inhibited by the repellents we used (Hallem and Carlson, 2006; Kreher et al., 2008). Moreover, while some examples do exist in which OSNs with putatively opposing valences are co-localized within the same sensillum type, e.g. *Or42b* (e.g. ethyl acetate) and *Gr21a/Gr63a* (CO_2) in the ab1 sensillum (Su et al., 2012), this does not seem to be a general principle [e.g. *Or7a* (e.g. benzaldehyde) as a putatively negative receptor and *Or56a* (geosmin) in ab4]. If the effects we observe were caused by a reduced activation of positive valence OSNs in mixtures because of competition for the ligand binding site, we would expect odors having higher headspace concentrations to dominate mixture responses. Because we used all odors except for BEA at the same liquid phase dilution and the compounds vary widely in vapor pressure, estimated headspace concentrations also vary over almost five orders of magnitude (supplementary material Table S2) [headspace concentrations estimated after previous publications (Cometto-Muñiz et al., 2003; Münch et al., 2013; Pelz et al., 2006)], with EtA (est. 1226.3 ppm) and MeSa (est. 0.013 ppm) forming the extremes. Despite this difference in headspace concentrations, MeSa reduced EtA responses as strongly as it reduced IAA responses (est. 14.3 ppm), arguing against a major contribution of syntopic interactions to the main effects we observe. In any case, if peripheral interactions were the main determinants for the valence of binary odor mixtures, we would expect to find more odor-pair-specific interactions especially for mixtures of attractants and repellents, because a given repellent may inhibit one OSN type while not affecting another.

By the same argument, valence-specific coding in the AL – although it may undoubtedly exist – is unlikely to be the primary determinant of our observations. In the AL, olfactory information is modulated by both excitatory and inhibitory lateral interactions conferred by a dense array of local neurons (LNs) (Wilson, 2013). Part of the inhibition in the AL is dedicated to normalizing the population code via a pre-synaptic gain control mechanism (Olsen and Wilson, 2008; Olsen et al., 2010; Root et al., 2008), which is probably global and unlikely to be critically involved in valence-specific modulations. However, a large proportion of AL LNs are morphologically and physiologically diverse (Chou et al., 2010; Seki et al., 2010; Silbering et al., 2008) and may be involved in blend-specific computations. Nevertheless, if such blend-specific LNs exist, we would expect to observe their contribution to innate behavior mainly for blends of ecological significance to *Drosophila*. Moreover, we would again expect the resulting behavior towards binary odor mixtures to be more odor-pair specific.

From the AL, olfactory information is relayed to two higher brain centers, the mushroom body (MB) and the lateral horn (LH). The MB is generally considered the brain center for odor identification and learning, while the LH has been implicated to be involved in innate behavior (de Belle and Heisenberg, 1994; Heimbeck et al., 2001; Jefferis et al., 2007). MB output has recently been described to be important for CO_2 avoidance in the context of starvation (Bräcker et al., 2013) and therefore does play a role in innate behavior under certain circumstances. However, blocking MB output during an odor discrimination task in trained flies restores innate preferences (Parnas et al., 2013), suggesting that these preferences are hard-wired in the LH circuitry. In contrast to the apparently random connectivity of PNs with their postsynaptic partners in the MB (Caron et al., 2013; Gruntman and Turner, 2013; Murthy et al., 2008), neuronal connectivity seems to be strongly stereotyped across individuals in the LH (Fişek and Wilson, 2014), a fact that matches the inter-individual stereotypy we observe in our behavioral experiments. More information on individual third-order neurons and inhibitory local neurons in the LH in combination with a detailed analysis of valence weights of individual processing channels are needed to substantiate the hypothesis that these neurons carry hedonic valence information, but these first observations lead us to suggest that the neural correlate of our results might be found in the LH.

Interestingly, the consistency of repellent net responses derived from mixtures with different attractants suggests that the effect of repellents follows an all-or-nothing logic and that the absolute repellent concentration may be more predictive for the behavioral output than the ratio between attractant and repellent mixture

constituent. If this is the case, the repellent net response at a given concentration should be identical in mixtures with different concentrations of the same attractant. Also, it is not clear from our experiments whether net responses are dependent on the concentration of the repellent mixture constituent. However, to conclusively answer these questions and to further dissect the neural mechanisms underlying the evaluation of chemical signals, it will be crucial to assign valence weights to the physiological activity of individual processing channels. This goal can be achieved by artificial activation of individual OSN types through targeted expression of light-gated ion channels (Bellmann et al., 2010) or by the use of low odor concentrations (Mathew et al., 2013), i.e. concentrations at which only single OSN channels become activated. Simultaneous activation of two individual OSN channels will then be a powerful tool to identify negative valence.

MATERIALS AND METHODS

Flies

All experiments in this study were performed using female Canton-S wild-type flies. Flies were reared on standard cornmeal medium under a 12 h:12 h light:dark regime at 23°C and 70% relative humidity.

Chemicals

All chemicals used in this study were acquired from Sigma-Aldrich (http://www.sigmaaldrich.com) in the highest purity available. Oct3ol was a racemic mixture of R- and S-enantiomers. Fresh dilutions in mineral oil were prepared once a week.

Flywalk experiments

Behavioral experiments were performed in the Flywalk paradigm as previously described (Steck et al., 2012), with 4- to 6-day-old mated female flies starved for 24 h before the start of the experiments. In short, 15 individual flies were placed in glass tubes (inner diameter 0.8 cm). Glass tubes were aligned in parallel, and flies were continuously monitored by an overhead camera (SONY EVI, Sony Corporation, Japan) under red-light conditions (λ>630 nm). During the experiment, flies were continuously exposed to a humidified airflow of 20 cm s^{-1} (70% relative humidity, 20°C). Flies were repeatedly presented with pulses of different olfactory stimuli at an interstimulus interval of 90 s. Stimuli were added to the continuous airstream and thus traveled through the glass tubes at a constant speed.

Odor stimulation was performed with a multicomponent stimulus device described elsewhere (Olsson et al., 2011; Steck et al., 2012). In summary, 100 µl of odor dilution was prepared in 200 µl PCR tubes, which were placed in odor vials made of polyetheretherketone. Odor vials were tightly sealed and connected to the stimulus device via ball-stop check valves; these valves only allowed uni-directional airflow through the odor-saturated headspace. Odor stimulation was achieved by switching an airflow otherwise passing through an empty vial (compensatory airflow) to the odor-containing vial. Odor pulses were 500 ms in duration at an interstimulus interval of 90 s. Binary mixtures were presented by simultaneously opening the two vials used to present the corresponding single compounds. As mixtures were established in the central mixing chamber of the stimulus device from the same vials as individual compounds, headspace concentrations of mixture constituents were identical in mixtures as when they were presented alone. Different stimuli were presented in pseudo-randomized order to avoid odor-sequence artifacts.

Data analysis

Tracking data were analyzed using custom-written routines programmed in MATLAB (MathWorks, Natick, MA, USA) and R (www.r-project.org) and the distributed packages gplots (Warnes, 2010) and ggplot2 (Wickham, 2009). In a first step, flies were assigned to individual glass tubes using the *y*-coordinates and could thus be unambiguously identified throughout the whole experiment. As flies are allowed to distribute freely within their glass tubes, they may encounter the odor pulse at different times. This is compensated for by calculating the time of odor encounter for every individual tracking event based on the *x*-position of the fly, system-intrinsic delay and airspeed. Time of encounter was set to 0 and speed of movement was interpolated in the interval between 10 s before and 10 s after encounter at 10 Hz.

As the tracking system does not capture the whole length of the glass tubes, not every fly is tracked for every stimulation cycle and some enter or leave the region of interest during the tracking event. We therefore decided to only consider complete trajectories in the interval between 1 s before and 7 s after odor encounter for further analysis. If a fly lacked complete trajectories in this interval for any of the tested odors, it was excluded from further analysis; this policy led to 20 out 540 flies being excluded (96.3% of flies tested were included in the analysis). In total, after the aforementioned criteria were applied, ~60,000 single trajectories and a mean of 15.3 trajectories per fly and odor were considered for analysis.

A fly's response time course was calculated as the arithmetic mean of all complete trajectories of this fly to a specific odor. In a further step, mean time courses across flies were calculated as the arithmetic mean of single fly mean trajectories. To extract a single metric of a fly's response to a given odor for statistical comparison, we calculated the net displacement along the glass tube in a temporal window of 4 s after encounter with the odor pulse for every single tracking event and the fly's response as the arithmetic mean of these single-event responses per odor. Positive values indicate a net displacement in the upwind direction.

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Competing interests

The authors declare no competing financial interests.

Author contributions

M.T., M.K. and B.S.H. conceived the study and designed experiments. M.T. performed and analyzed experiments. M.T., M.K. and B.S.H. wrote and commented on the manuscript.

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Supplementary material

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