Electrical synapses mediate synergism between pheromone and food odors in *Drosophila melanogaster*

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In *Drosophila melanogaster*, the sex pheromone produced by males, *cis*-vaccenyl acetate (cVA), evokes a stereotypic gender-specific behavior in both males and females. As *Drosophila* adults feed, mate, and oviposit on food, they perceive the pheromone as a blend against a background of food odors. Previous studies have reported that food odors enhance flies' behavioral response to cVA, specifically in virgin females. However, how and where the different olfactory inputs interact has so far remained unknown. In this study, we elucidated the neuronal mechanism underlying the response at an anatomical, functional, and behavioral level. Our data show that in virgin females cVA and the complex food odor vinegar evoke a synergistic response in the cVA-responsive glomerulus DA1. This synergism, however, does not appear at the input level of the glomerulus, but is restricted to the projection neuron level only. Notably, it is abolished by a mutation in gap junctions in projection neurons and is found to be mediated by electrical synapses between excitatory local interneurons and projection neurons. As a behavioral consequence, we demonstrate that virgin females in the presence of vinegar become receptive more rapidly to courting males, while male courtship is not affected. Altogether, our results suggest that lateral excitation via gap junctions modulates odor tuning in the antennal lobe and drives synergistic interactions between two ecologically relevant odors, representing food and sex.

sex pheromone | mixture synergism | functional imaging | electrical synapse | courtship behavior

Synergism can be defined as the cooperation of two or more elements operating together to achieve an effect that is greater than the sum of the individual effects. It is a ubiquitous and crucial aspect of nature and has provided a functional basis for the evolution of complex systems (1). It has been observed, for example, that synergistic interactions between multilevel, multimodal circuits enhance selection for the fastest mode of escape behavior in *Drosophila melanogaster* (2). In the same way, synergistic effects between plant-emitted volatiles and specific aromatic compounds are known to modulate attraction behavior of several insect species (3–5). Like plant volatiles, animal-produced sex pheromones interact with habitats and food signals to enhance an animal's behavioral acuity (6, 7). Although such interaction between two chemosensory cues—namely, food and sex—is known to drive reproductive isolation and speciation (8, 9), the underlying neuronal mechanism has so far remained elusive. Hence, in this study, we aim to unravel the neural circuitry that leads to synergism between food and sex odors in *D. melanogaster*.

Most insects, including the vinegar fly *D. melanogaster*, heavily depend on their olfactory system when they perform elementary activities, such as feeding, mating, ovipositing, and avoiding predators. During mating, the sex pheromone *cis*-vaccenyl acetate (cVA), produced by males, plays a significant and sex-specific role in communication between males and females. Whereas cVA evokes aggressive behavior in males and suppresses courtship with other males (10, 11), it increases sexual receptivity in females (10). cVA perfuming of miR-124 mutants males, which generally produce less cVA, restored their ability to achieve copulation with females (12). cVA also acts as an aggregation-promoting pheromone, attracting both males and females to food (13–16). In nature, odors do not usually occur as single cues but, rather, are perceived as a blend, consisting of different odor components. Vinegar flies mostly aggregate, oviposit (17), and mate (18) on fermenting fruits. As pheromone communication and food odor reception naturally occur together, we hypothesized that these odors are also linked at the neuronal level. Recently, it has been shown that virgin fed *Drosophila* females are more attracted to the blend of cVA and vinegar than to vinegar alone in different behavioral assays, while males are not (19). Vinegar represents a complex blend and highly attractive food odor to *D. melanogaster* (20). Insulin signaling was reported to partially control cVA perception (depending on a fly's nutritional state) and to modulate sexual receptivity in virgin females (19).

The architecture of the *Drosophila* olfactory circuit has been nearly fully characterized. The antenna houses ∼40 different types of olfactory receptors (ORs), which are expressed in olfactory receptor neurons (ORNs). ORNs expressing the same ORs project onto the same glomerulus in the antennal lobe (AL) (21), the primary olfactory center of the fly brain. Furthermore, ORNs expressing the same ORs exhibit the same odor response properties (22). In each glomerulus, the axons of the ORNs synapse onto the dendrites of the corresponding projection neurons (PNs) (23, 24). In adult male and female flies, the sex pheromone cVA is perceived by ORNs expressing OR67d, and

Significance

We elucidated the neuronal mechanism underlying the interaction of two ecologically relevant odors in the fly brain. Our study demonstrates that exposure to the male-produced sex pheromone *cis*-vaccenyl acetate, in combination with the complex food odor vinegar, evokes an enhanced and synergistic functional response in the primary olfactory center of virgin female flies. This effect arises within the neuronal network of the antennal lobe and is mediated by electrical synapses. The synergistic response in virgin females leads to an increased sensitivity to the pheromone, and therefore an enhanced female receptivity during courtship. This mechanism is highly useful, since it promotes mating in females when food is present: that is, when the nutritional supply of the female and its offspring is guaranteed.

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these ORNs project onto the DA1 glomerulus in the AL (10). Gender-specific differences in behavioral response to cVA, which derive from sexually dimorphic third-order olfactory neurons, have been observed (25–28). In addition, gender-specific differences in response to food odors have also been reported: the ionotropic receptor IR84a in *Drosophila* detects food odors, such as phenyl acetic acid and phenyl acetaldehyde, and increases male courtship behavior without altering female receptivity (29). In addition, it has been recently shown that yeast increases the female's sexual receptivity through the interaction between its odorous fermentation product acetic acid, sensed by IR75a, and its nutritional content (30). Hence, by coupling the perception of food odors with the activation of the courtship circuitry, the specific sensory pathways coordinate both feeding and reproductive behaviors. However, how and where the different olfactory inputs interact has so far remained unknown. Therefore, in this study, we investigated the neuronal circuitry underlying the interaction between a sex pheromone and food odors in the *Drosophila* brain.

Results

The Food Odor Vinegar Enhances the Pheromone Response in the Glomerulus DA1. Vinegar in the presence of cVA has been reported to attract more virgin females than vinegar alone (19), suggesting that the perception of pheromone and food signals are modulated simultaneously. To scrutinize whether vinegar, a complex food odor (31), modulates the reception of the sex pheromone cVA, which is produced by males, we first focused on the primary olfactory center, the AL, and analyzed *Drosophila*'s functional response to the pheromone at the PN level. We performed functional imaging experiments using transgenic flies that genetically express the calcium sensor GCaMP3 under the control of the *GH146-Gal4* driver line to selectively monitor odor responses in uniglomerular PNs (Fig. 1*A*). We analyzed the odor-evoked responses of the cVA-responsive glomerulus DA1 during stimulation to cVA and vinegar, as well as the binary mixture of both at three different concentrations (10^{-3}, 10^{-2}, and 10^{-1}) (Fig. 1 *B* and *C*). As expected, cVA evoked a strong and clear response in the DA1 glomerulus in a dose-dependent manner, whereas vinegar did not elicit any activity in this glomerulus. Interestingly, in virgin females, the binary mixture of cVA and vinegar elicited a significantly higher response than cVA alone at concentrations of 10^{-2} and 10^{-1} (Fig. 1*C*). To examine whether the observed enhanced response is the result of an additive response of either odors or whether it represents the result of their interaction, we compared the sum of the individual responses to the measured mixture response (Fig. S1*A*). Since the measured response in the glomerulus DA1 to the binary mixture was significantly higher than the predicted additive response of both odors, the enhancement of the response we observed can be defined as synergism. Notably, we did not observe this synergistic effect in the glomerulus DA1 in virgin males (Fig. 1 *D–F* and Fig. S1*B*), which supports previous observations that behavioral interactions of cVA and food odors are restricted to females only (19). Interestingly, mated females also failed to show this phenomenon, since the response to the binary mixture equals the responses to the pheromone alone (Fig. 1 *G–I* and Fig. S1*C*). Moreover, the general PN response to cVA was very low in mated females compared with virgin females, which is well in line with previous results (32). To analyze whether the presence of vinegar enhances the sensitivity of virgin females to cVA in general, we established a dose–response curve to cVA at different concentrations against the background of a steady vinegar concentration (Fig. S1*D*). We observed that vinegar increases the sensitivity of virgin females to cVA in a ratio-dependent manner, meaning that only the 1:1 mixture induced a synergistic response. When we compare the responses between the binary mixture and the individual compounds across many glomeruli, we see that this synergistic effect occurs only in the pheromone-responsive glomerulus DA1 (all other glomeruli labeled by *GH146-Gal4* responded as predicted and did not show any kind of interactions) (Fig. S2). Unfortunately, the most responsive glomeruli to vinegar (i.e., glomeruli DL2d/v, DP1l, DC4) could not be monitored, since they were not labeled by *GH146-Gal4*.

To analyze whether the observed synergism is confined to the mixture of vinegar and cVA or whether it can be evoked by other combinations of odors, we measured the response of glomerulus DA1 to limonene [an oviposition cue (33)], to 1-hexanol (a neutral odor), to acetic acid [the major component of vinegar (34)], and to their binary mixture with cVA. However, neither limonene nor 1-hexanol elicited a significant increase in the DA1 response when presented along with cVA, compared with when presented alone (Fig. 1 *J* and *K*). Interestingly, acetic acid, the main volatile component of vinegar, did not evoke any synergism in combination with cVA (Fig. 1 *J* and *K*), although it elicits behavioral attraction as a single compound (34). However, as Becher et al. (34) also observed, acetic acid alone does not nearly evoke the same grade of attraction as vinegar. It is therefore likely that the complete vinegar blend is necessary to elicit mixture synergism in combination with cVA and not just a single compound. Taking these data together, we find that the synergistic response of the glomerulus DA1 can be said to occur only in virgin females, in an odorant-specific and glomerulus-selective manner.

Synergism Between Pheromone and Vinegar Does Not Occur at the Sensory Level. We next wondered whether the synergism evolves at and derives from the peripheral level, and therefore performed extracellular single sensillum recordings (SSRs). As synergism was observed only in the cVA-responsive DA1 glomerulus, we limited our recordings to the at1 sensillum, which houses OR67d expressing ORNs. We examined the responses in virgin females to cVA, vinegar, and the binary mixture of both, again at three different concentrations. As expected, the OR67d-expressing ORNs responded specifically to cVA in a dose-dependent manner, but these ORNs did not show any response to vinegar alone (Fig. 2*A*). However, unlike the PNs, OR67d-expressing ORNs in virgin females did not show any enhanced response to the blend of cVA and vinegar (Fig. 2*A*). SSR data from the male at1 sensillum exhibited similar properties, wherein the response to the binary mixture revealed the same spike frequency as the response to cVA alone (Fig. S3*A*). We further performed functional imaging of ORNs in the AL by expressing GCaMP3 under the control of the *Orco* promoter (Fig. 2*B*). Because Orco expression is very heterogeneous in the different sensilla classes, and in particular low in trichoid sensilla (35), GCaMP expression in the AL varies accordingly. Hence, calcium signals in glomerulus DA1 are less sensitive compared with SSR and showed a clear calcium response to cVA only at a concentration of 10^{-1}. In accordance with the SSR data, neither the calcium responses in the female nor those in the male AL revealed any synergistic effect in the glomerulus DA1 to the mixture of cVA and vinegar (Fig. 2 *C* and *D* and Fig. S3 *B* and *C*). In addition, we performed optical imaging from IR8a-expressing ORNs in different vinegar-responsive glomeruli in virgin females, as vinegar activates strongly some glomeruli (e.g., DP1m, DP1l, DL2d/v, and DC4) innervated by ionotropic receptors (IRs) (36) (Fig. 2 *E* and *F*). Still we did not observe any synergistic responses to the mixture in those glomeruli. The mixture response was always equal to the response to the stronger component, which was vinegar in this case (Fig. 2*G*). Taken together, these results demonstrate that the observed synergism does not occur at the sensory level and therefore likely emerges within the neuronal network of the AL.

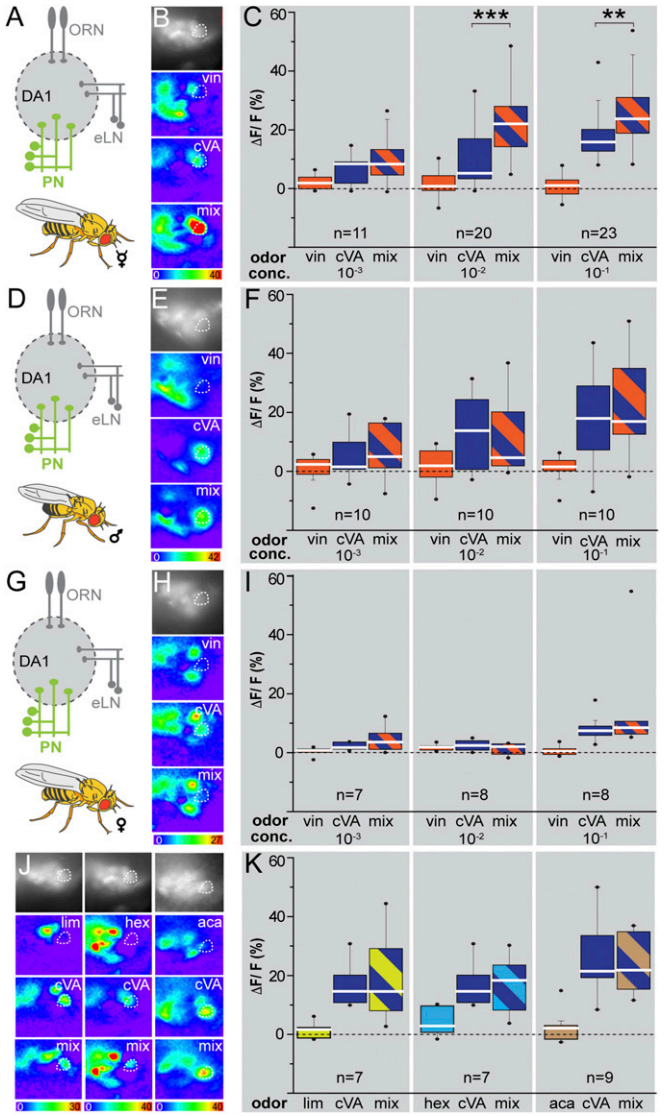

Fig. 1. PNs in the glomerulus DA1 reveal synergistic responses to the mixture of cVA and vinegar specifically in virgin females. (*A*) Schematic of the experimental approach: *UAS-GCaMP3* was expressed in PNs (green) using *GH146-Gal4* in virgin female flies. (*B*) Representative odor-evoked calcium responses of PNs in the AL of a virgin female to cVA, vinegar, and their binary mixture (10^{-1} concentration). (*C*) Box plots display $\Delta F/F$ responses in glomerulus DA1 in virgin females to vinegar (orange), cVA (blue), and their binary mixture (striped) at three different concentrations. The white line in the box represents the median. The mixture evokes a significantly enhanced response (***$P < 0.001$; **$P < 0.01$; Wilcoxon matched paired test). (*D*) Schematic of the experimental approach: *UAS-GCaMP3* was expressed in PNs (green) using *GH146-Gal4* in virgin male flies. (*E*) Representative odor-evoked calcium responses of PNs in the AL of a virgin male to cVA, vinegar, and their binary mixture (10^{-1} concentration). (*F*) Box plots display $\Delta F/F$ in DA1 in virgin males to vinegar (orange), cVA (blue), and their mixture (striped) at three different concentrations. The mixture evokes a similar response as cVA ($P > 0.05$; Wilcoxon matched paired test). (*G*) Schematic of the experimental approach: *UAS-GCaMP3* was expressed in PNs (green) using *GH146-Gal4* in mated female flies. (*H*) Representative odor-evoked calcium responses of PNs in the AL of a mated female to cVA, vinegar, and their mixture (10^{-1} concentration). (*I*) Box plots display $\Delta F/F$ in DA1 in mated females to vinegar (orange), cVA (blue), and their mixture (striped) at three different concentrations. The mixture evokes a similar response as cVA ($P > 0.05$; Wilcoxon matched paired test). (*J*) Representative odor-evoked calcium responses of PNs in the AL of a virgin female to limonene (lim), 1-hexanol (hex), acetic acid (aca), and their individual binary mixtures with cVA (10^{-1} concentration). (*K*) Box plots represent $\Delta F/F$ responses of PNs in DA1 to limonene (lim, yellow), 1-hexanol (hex, indigo), acetic acid (aca, brown), and cVA (blue), and the mixtures of cVA with the individual odors (striped boxes) at 10^{-1} concentration. None of the mixtures evokes a synergistic response ($P > 0.05$; Wilcoxon matched paired test). (Magnification in *B*, *E*, *H*, and *J*, 200×.)

Glomerulus DA1 Receives Input from Vinegar-Responsive ORNs Through Excitatory Local Interneurons. To pinpoint the origin of the synergistic effect, we proceeded to the next processing level along the olfactory pathways and examined the response to the mixture in local interneurons (LNs). As vinegar and the pheromone together induce a positive synergistic effect, we focused our interest on the population of excitatory LNs (eLNs). For this purpose, we expressed GCaMP3 using the enhancer trap line *Krasavietz-Gal4* and performed functional imaging of the AL (Fig. 3*A*). The majority of local interneurons, labeled by *Krasavietz-Gal4*, are excitatory in nature and coupled to other neurons through electrical synapses (37–39); they possess reciprocal synapses with PNs, inhibitory LNs (iLNs), and other eLNs, and transmit both depolarization and hyperpolarization, while chemical neurotransmission does not occur (38, 40). We analyzed the calcium responses of Krasavietz-positive eLNs in the glomerulus DA1 to cVA and to vinegar, and to their binary mixture at three different concentrations (Fig. 3*B*). We observed that, whereas these eLNs responded only minimally to all three odorants at the two lower concentrations (10^{-3} and 10^{-2}), they responded clearly and strongly to odorants at the highest concentration (i.e., 10^{-1}). Because LNs are multiglomerular in nature, eLNs in the DA1 glomerulus responded to both vinegar and to cVA. Interestingly, the binary mixture induced a significant stronger response compared with the response to the major component (i.e., here, vinegar) (Fig. 3*B*). However, as the measured response to the mixture was not significantly different compared with the predicted additive response to vinegar and cVA, this effect cannot be termed as synergism (Fig. S4*A*). In addition, we also measured the double concentration of vinegar, since the expected response to an odor mixture in the absence of interactions should not exceed the response to the double concentration of the stronger odor component (41). However, the response in glomerulus DA1 to the double vinegar concentration was equal to the measured mixture response (Fig. S4*A*), confirming that no synergistic response can be observed in eLNs.

Although no interaction takes place at the eLN level, it is still conceivable that these neurons are involved in initiating synergism to the mixture by conveying the input from ORNs responsive to food odors to the DA1 glomerulus, where the interaction takes place. Since Krasavietz-positive eLNs have been described as multiglomerular neurons (37, 42), these neurons should connect the pheromone glomerulus with the vinegar-responsive glomeruli and hence facilitate cross-talk at the AL level. To verify such a connection, we expressed photoactivatable GFP (*UAS-C3PA*) under control of the *Krasavietz-Gal4* driver line and photoactivated glomerulus DA1 to monitor the eLN processes from DA1 to other glomeruli throughout the whole AL (Fig. S4*B*). After the photoactivatable GFP diffused to other glomeruli, we quantified the intensity of those glomeruli that are responsive to vinegar before and after photoactivation (Fig. S4*C*). The observed significant increase in intensity in those glomeruli confirms that the Krasavietz-positive eLNs are connecting the glomerulus DA1 to other vinegar-specific glomeruli. Hence, it is conceivable that eLNs spread and transmit the olfactory input from vinegar-specific glomeruli to the cVA-specific glomerulus DA1, leading to a subsequent synergistic response in the downstream neurons (i.e., in PNs).

Next, we wondered why the mixture synergism could only be observed for the vinegar-cVA mixture but not for other odors blended with cVA, because eLNs are innervating the majority of glomeruli (37, 42). We therefore performed imaging from

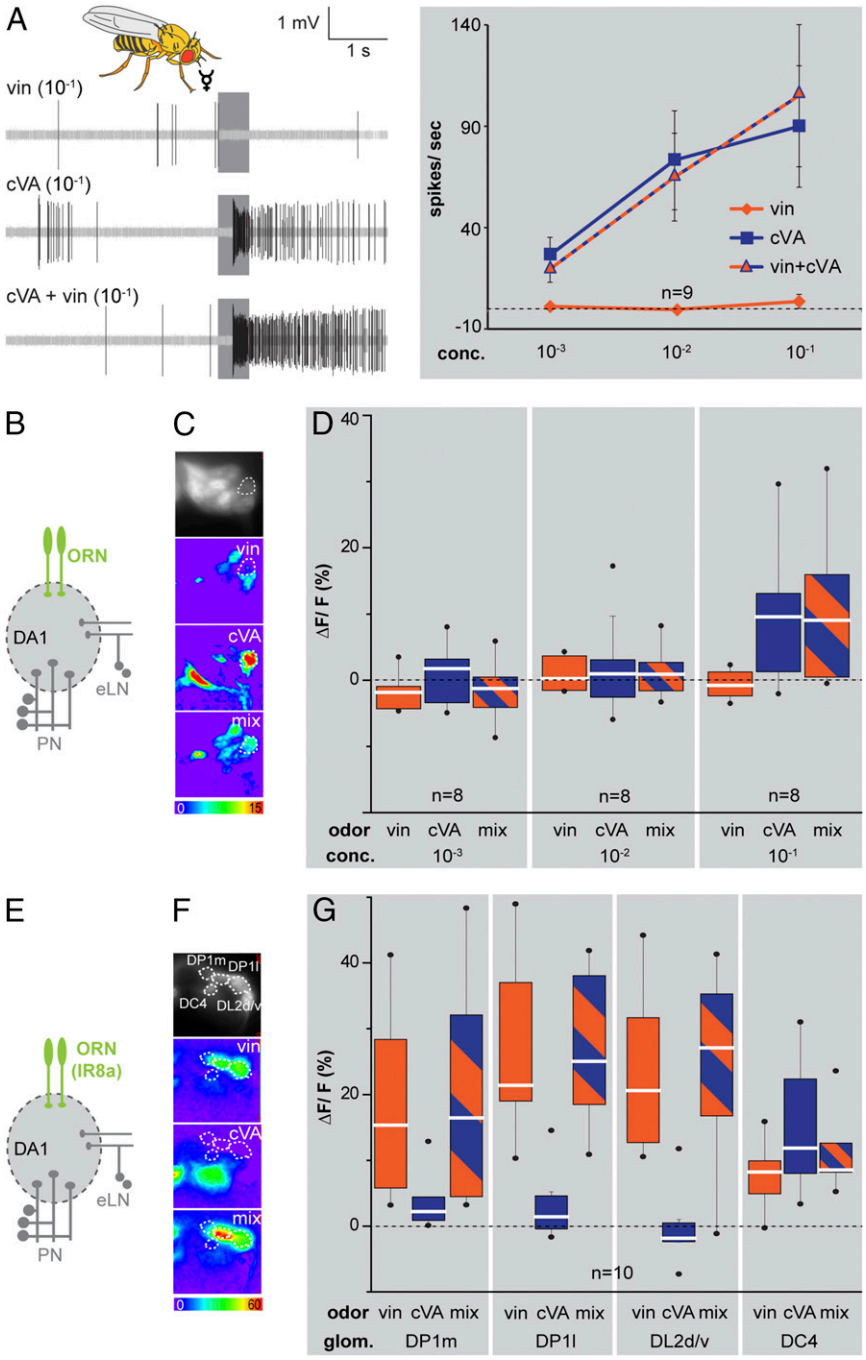

Fig. 2. Mixture synergism does not occur at the sensory level. In vivo extracellular SSRs from the at1 sensillum expressing OR67d. (*A*, *Left*) Representative traces display the response of OR67d ORNs in virgin females to vinegar, cVA and their binary mixture (10^{-1} concentration). (*Right*) Line curves represent the averaged neuronal activity (spikes per second) to vinegar (orange), cVA (blue), and their binary mixture (striped) at three different concentrations ($P > 0.05$; Wilcoxon matched paired test). (*B*) Schematic of the experimental approach: *UAS-GCaMP3* was expressed in the majority of ORNs (green) using *Orco-Gal4* in virgin females. (*C*) Representative odor-evoked calcium responses of ORNs in the AL of a virgin female to cVA, vinegar, and their binary mixture (10^{-1} concentration). (*D*) Box plots represent $\Delta F/F$ responses of ORNs in the glomerulus DA1 in virgin females to vinegar (orange), cVA (blue), and their binary mixture (striped boxes). The white line in the box represents the median. The ORN response to the mixture is equal to the response to the stronger component (i.e., cVA) ($P > 0.05$; Wilcoxon matched paired test). (*E*) Schematic of the experimental approach: *UAS-GCaMP3* was expressed in ORNs expressing IRs (green) using *IR8a-Gal4* in virgin females. (*F*) Representative odor-evoked calcium responses of IR8a-expressing ORNs in the AL of a virgin female to cVA, vinegar, and their binary mixture (10^{-1} concentration). (*G*) Box plots represent $\Delta F/F$ responses of IR8a-expressing ORNs in different vinegar-responsive glomeruli in virgin females to vinegar (orange), cVA (blue), and their binary mixture (striped boxes) at 10^{-1} concentration. The ORN response to the mixture is equal to the response to the stronger component (i.e., vinegar) ($P > 0.05$; Wilcoxon matched paired test). (Magnification in *C* and *F*, 200×.)

Krasavietz-positive eLNs to vinegar and two other previously used odors, 1-hexanol and limonene, to investigate whether they differentially activate glomerulus DA1. Notably, these two odors did not induce any synergistic mixture response when combined with the pheromone cVA (Fig. 1*K*). Indeed, the quantification of the eLN response reveals that vinegar induced

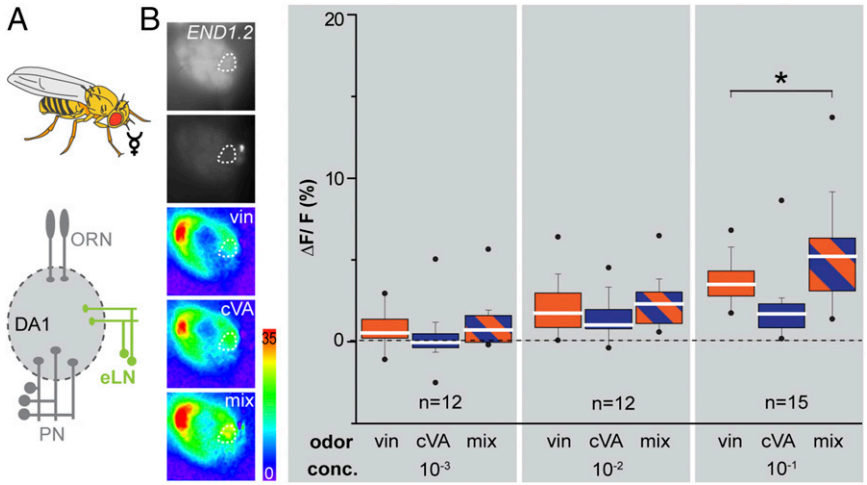

Fig. 3. Excitatory local interneurons do not reveal a synergistic mixture response. (*A*) Schematic of the experimental approach: *UAS-GCaMP3* was expressed in eLNs (green) using *Krasavietz-Gal4* in virgin female flies. (*B*, *Left*) Representative odor-evoked calcium responses of eLNs in the AL in the background of *END1-2* (elav-n-synaptobrevin:DsRed) of a virgin female to cVA, vinegar, and their binary mixture (10^{-1} concentration). (*Right*) Box plots display Δ*F*/*F* responses in the glomerulus DA1 in virgin females to vinegar (orange), cVA (blue), and their binary mixture (striped) at three different concentrations. The white line in the box represents the median. The eLN response to the mixture is significantly higher than the response to the stronger component (i.e., vinegar) at 10^{-1} concentration. ($P < 0.05$; Wilcoxon matched paired test). *$P = 0.03$. (Magnification in *B*, 200×.)

a significantly stronger activity in glomerulus DA1 than the other two odors (Fig. S4*D*). This result is in line with previously published electrophysiological recordings of Krasavietz-positive eLNs, demonstrating that they exhibit distinct odor response patterns (38). Hence, irrespective of the multiglomerular morphology of eLNs, their selective odor responses might drive the vinegar-specific synergism in glomerulus DA1.

Electrical Synapses Between eLNs and PNs Mediate Synergism. As mentioned above, eLNs are largely connected to PNs through gap junctions (38, 40). To investigate whether the synaptic connections between eLNs and PNs actually mediate the interaction between the two odors, vinegar and cVA, we analyzed whether the mixture-induced synergism in PNs is evident in flies with mutated gap junctions. In invertebrates, gap junctions are composed of intercellular channels formed by innexin proteins. Among eight types of innexins in *Drosophila*, *shakB* (inx8) is expressed in scattered neurons, the giant fiber neural pathway, and the AL (40, 43, 44). The *shakB*^{2} mutant exhibits disrupted electrical connections in the optic lobe and in the giant fiber escape pathway (45, 46). In the *Drosophila* AL, four kinds of synapses possess gap junctions and are therefore affected by the *shakB*^{2} mutation: eLNs-to-PNs, PNs-to-PNs, eLNs-to-iLNs, and eLNs-to-eLNs (38, 40). Hence, the olfactory input to the AL should function normally in the *shakB*^{2} mutant fly, while the synaptic transmission of eLNs should be disrupted. Notably, functional imaging from PNs in the *shakB*^{2} mutant background did not reveal any synergism in the glomerulus DA1 (Fig. 4 *A* and *B*), indicating that gap junctions are necessary to drive the synergism in the pheromone glomerulus that is induced by the exposure to both cVA and vinegar. As the *shakB*^{2} mutation causes a global loss of electrical synapses, which is not limited to the AL, we next used an RNAi construct against inx8 (i.e., RNAi of *shakB*) (47) to block gap junctions in olfactory PNs. To achieve this, we expressed *inx8-RNAi* in PNs of *GH146-Gal4* and monitored their response to the mixture as well as to the individual odors via functional imaging at two concentrations, 10^{-2} and 10^{-1} (Fig. 4*C*). In line with our previous observation, these flies failed to show any mixture-induced synergistic response in PNs of the glomerulus DA1. It is important to note here that the enhancer trap line *GH146-Gal4* does not label solely PNs, but also a few additional higher-order neurons, such as a subset of Kenyon cells in the mushroom body, a small group of descending interneurons ventral to the lateral protocerebrum (48), and a GABAergic anterior paired lateral neuron innervating the mushroom body (49). We therefore cannot rule out the possibility that those neurons were also affected by silencing gap junctions, although the importance of electrical coupling for odor processing has so far been proven solely for the AL (40).

As gap junctions are bidirectional and require the ShakB protein at both the presynaptic and the postsynaptic sites to function properly (45), we next rescued the wild-type ShakB protein in both eLNs and PNs. For this purpose, we employed a transgenic fly as a control strain in which Krasavietz-positive eLNs and GH146-positive PNs expressed GCaMP6s (Fig. 4*D*). We first verified that the synergistic response to the mixture was visible when we recorded eLNs along with PNs, and performed imaging from both sets of neurons to vinegar, cVA, and their binary mixture. Indeed, these control animals also showed a significantly increased response to the binary mixture compared with their response to the individual odors at two concentrations, 10^{-2} and 10^{-1} (Fig. 4*E*). Confirming our previous results, the *shakB*^{2} mutation abolished the synergistic response in PNs and eLNs. By expressing and rescuing wild-type *shakB.neural* in eLNs and PNs in the background of the *shakB*^{2} mutation, we were able to restore the synergism to the mixtures (Fig. 4*E*).

Altogether, our observations suggest that gap junctions between eLNs and PNs, and within PNs, are necessary and sufficient to drive synergism in the glomerulus DA1 and therefore to enhance the response to cVA in the presence of the complex food odor vinegar.

Exposure to Vinegar Modulates Female Receptivity, Which Requires Gap Junctions in PNs. Our functional imaging results indicate that vinegar modulates the olfactory system of virgin females in a way that enhances their sensitivity to cVA. However, what does that mean for a female fly in nature? In female flies cVA governs both aggregation and mating. A previous study has shown that the mixture of vinegar and cVA becomes behaviorally more attractive to virgin females than vinegar alone (19), meaning that the aggregation-promoting response of flies to cVA is increased by vinegar. However, do food odors also influence mating

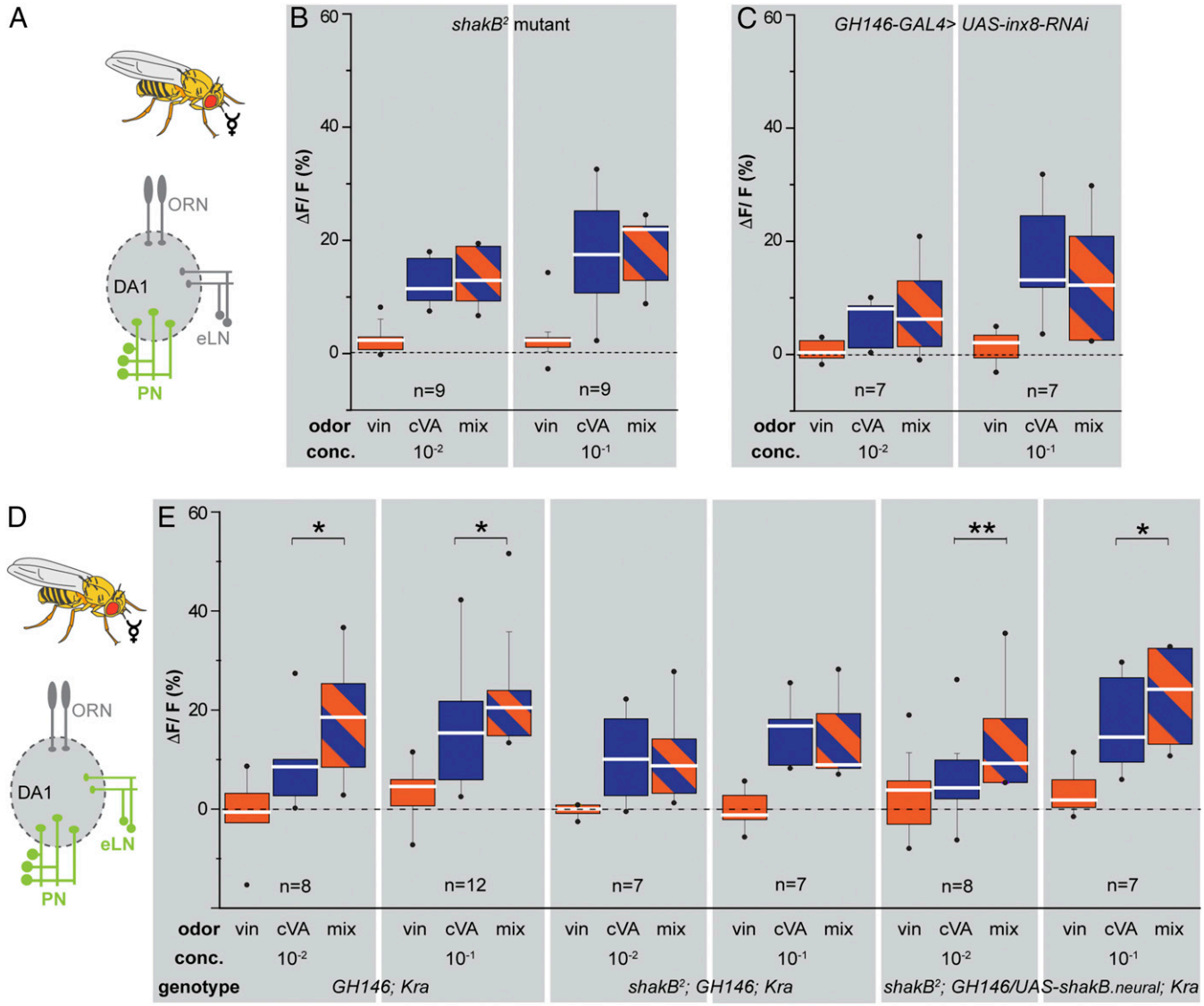

Fig. 4. Gap junctions between PNs and eLNs are necessary and sufficient to induce mixture synergism. (*A*) Schematic of the experimental approach: *UAS-GCaMP3* was expressed in PNs (green) using *GH146-Gal4* in virgin females. (*B*) Box plots display Δ*F*/*F* responses in the glomerulus DA1 in virgin females, in the background of the *shakB*^{2} mutant to vinegar (orange), cVA (blue), and their binary mixture (striped) at two different concentrations (10^{-2} and 10^{-1}). The white line in the box represents the median. The mixture does not evoke a synergistic response ($P > 0.05$; Wilcoxon matched paired test). (*C*) Box plots display Δ*F*/*F* responses in the glomerulus DA1 in virgin females to vinegar (orange), cVA (blue), and their binary mixture (striped) at two different concentrations (10^{-2} and 10^{-1}). Gap junctions have been blocked in PNs using RNAi against inx8. The mixture does not evoke a synergistic response ($P > 0.05$; Wilcoxon matched paired test). (*D*) Schematic of the experimental approach: *UAS-GCaMP6s* was expressed in PNs and eLNs (green) using *GH146-Gal4* and *Krasavietz-Gal4* in virgin females. (*E*) Box plots display Δ*F*/*F* responses in the glomerulus DA1 in virgin females to vinegar (orange), cVA (blue), and their binary mixture (striped) at 10^{-2} and 10^{-1} concentration. Genotypes are as follows: control line, *GH146-Gal4*; *Krasavietz-Gal4*; mutant line, *shakB*^{2}; *GH146-Gal4*; *Krasavietz-Gal4*; rescue line, *UAS- shakB.neural/GH146-Gal4*; *Krasavietz-Gal4* in the *shakB*^{2} mutant background. The control and rescue lines show a synergistic mixture response at both concentrations (*$P < 0.05$, **$P < 0.01$; Wilcoxon matched paired test).

behavior in flies? The presence of the food odors phenyl acetic acid and phenyl acetaldehyde increases courtship behavior in males via the IR84a-dependent pathway (29), although in females, mating behavior remains unaltered. Due to our findings of a synergism of cVA and vinegar, we asked whether the latter influences the courtship behavior of female flies. We therefore monitored the courtship behavior of a wild-type virgin male and female in a closed arena in the presence of either water or vinegar. Because the behavioral assay was performed in a closed small chamber for an extended period of time (20 min), all behavioral experiments were carried out with exposure to a low concentration of vinegar (i.e., 10^{-3}). Interestingly, while the copulation success of flies was not significantly affected by vinegar (Fig. 5*A*), flies mated significantly earlier in the presence of this food odor (Fig. 5*A*′). To analyze whether the female's receptivity or the male's perception was modulated by vinegar, we quantified the courtship index. However, the presence of vinegar does not affect the courtship index and therefore does not influence the male's courting behavior at all (Fig. 5*A*″), implying that only the female's receptivity is affected.

To verify whether vinegar modulates the female's receptivity only through the cVA pathway, we paired a *OR67d* mutant virgin female, which cannot detect cVA, with a wild-type virgin male in the courtship assay. As expected, only 21% of flies copulated in this experiment; this low percentage was shown previously to be due to the lack of pheromone perception (10) (Fig. 5*B*).

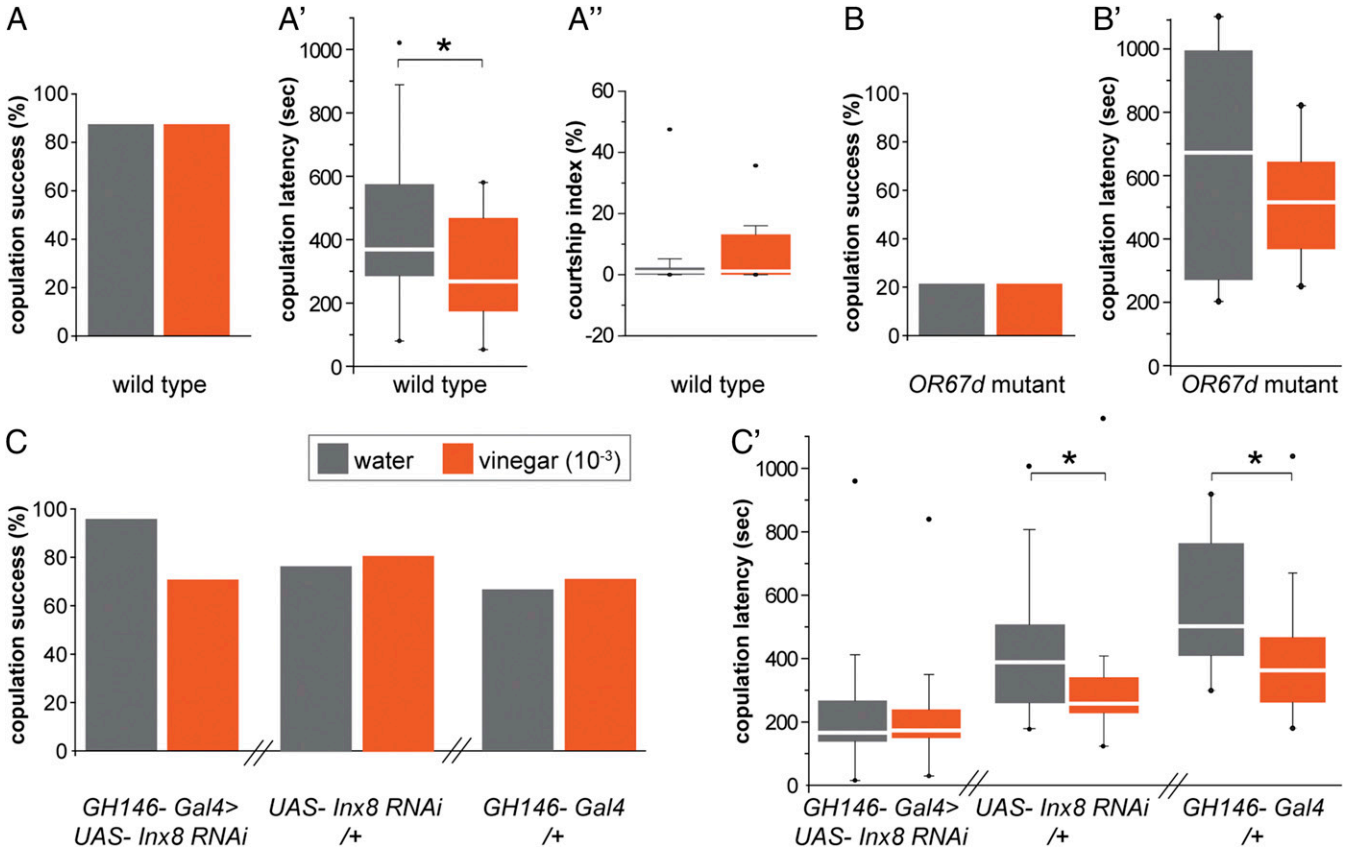

Fig. 5. Vinegar modulates copulation latency in females, which requires gap junctions in PNs. Courtship behavior assays performed with wild-type and different mutant flies in the presence of water (gray) or vinegar (10^{-3}, orange). (*A* and *A′*) Histograms represent copulation success and the box plots show the copulation latency of wild-type pairs of *D. melanogaster*. The presence of vinegar significantly reduces copulation latency, while copulation success is unaffected (*$P < 0.05$; Mann–Whitney test; $n = 24$). (*A″*) Box plots reveal courtship index of wild-type pairs. The presence of vinegar does not significantly affect the courtship index ($n = 12$). (*B* and *B′*) Histograms represent copulation success and the box plots show the copulation latency of wild-type males (*Canton-s*) and *OR67d* mutant females. Neither copulation success nor latency are influenced by the presence of vinegar ($P > 0.05$; Mann–Whitney test; $n = 24$). (*C* and *C′*) Histograms represent copulation success and the box plots show the copulation latency of wild-type males (*Canton-s*) and mutant females in which gap junctions have been blocked in PNs (*GH146-Gal4* > *UAS-inx8-RNAi*), as well as the parental control lines (*UAS-inx8-RNAi/+* and *GH146-Gal4/+*). Only the parental lines show a reduced copulation latency in the presence of vinegar (*$P < 0.05$; Mann–Whitney test; n = 24). χ^2 Test with Yates correction was used for copulation success and Mann–Whitney test was used for copulation latency.

However, the copulation latency of female flies in this experiment did not differ significantly between flies exposed to water or to vinegar (Fig. 5*B′*), indicating that vinegar acts exclusively through the OR67d pathway.

Next, we were curious to know whether the change in receptivity mediated by vinegar depends on the gap junction at the eLN-PN level, as implied by our functional imaging experiments. For this purpose, we paired a wild-type virgin male with a mutant virgin female whose gap junctions in PNs had been blocked by expressing *inx8-RNAi* under control of the *GH146-Gal4* driver line. Notably, we did not observe any significant difference regarding either copulation success or copulation latency in flies exposed to water or vinegar (Fig. 5 *C* and *C′*), indicating that the vinegar-induced reduction in receptivity depends on electrical synapses in PNs. As predicted, the parental controls (i.e., a *UAS-inx8-RNAi*/+ or *GH146-Gal4*/+ female paired with a wild-type male, respectively) became receptive more rapidly in the presence of vinegar while the level of copulation success remained similar to that observed in wild-type flies (Fig. 5 *C* and *C′*). Taken together, our results demonstrate that vinegar modulates and increases the female flies' sensitivity to cVA, by being mediated through electrical synapses at the eLN-PN level within the DA1 glomerulus (Fig. 6). Both odors, cVA and vinegar, activate glomerulus DA1 through two different pathways: while cVA directly activates glomerulus DA1 through OR67d-expressing ORNs, vinegar indirectly enhances the DA1 activation via lateral excitation by eLNs. At a later stage, the two different pathways converge at the output level of the AL and lead to a subsequent synergistic mixture response in glomerulus DA1 at the PN level. As a behavioral consequence, this modulation causes the virgin female to become receptive more rapidly to courting males.

Discussion

Interaction Between Food Odors and Sex Pheromone. In nature, odors always occur as blends, and each odor component may affect the perception of another odor. In the context of *Drosophila*, as flies always feed, mate, and oviposit on fermenting food, food odors are part of an ever-present, unavoidable background of every odor that flies encounter, such as aggregation cues, male-emitted sex pheromones, parasitoid odors (50), or oviposition cues (33). It is evident that a specific class of ionotropic receptors in *Drosophila*, namely IR84a, is activated not by fly-derived chemicals (the volatile sex pheromones) but by the compounds present in food which promote courtship behavior in males (29). Food odors are also reported to enhance the attraction of female *Drosophila* to the male-emitted cVA

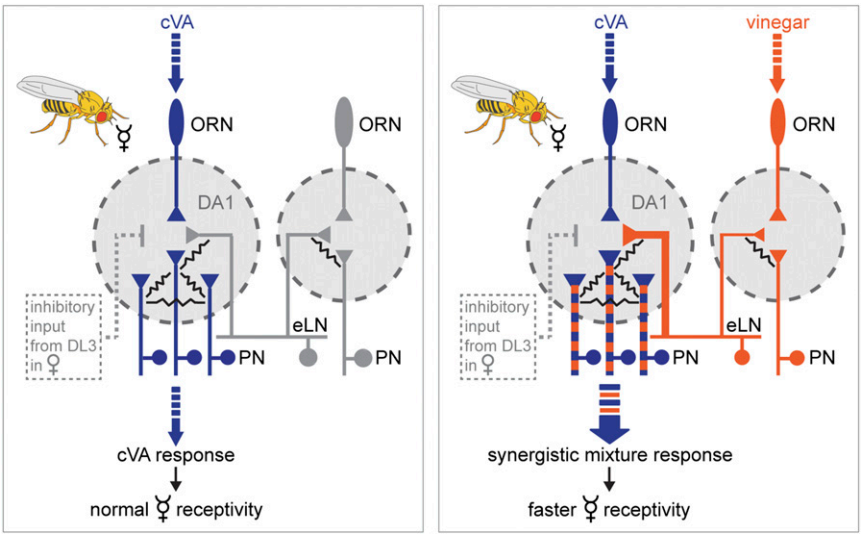

Fig. 6. Circuit model for mixture synergism. Proposed mechanism underlying the observed synergism in virgin females to the mixture of the sex pheromone cVA and the complex food odor vinegar. (*Left*) The sole cVA stimulation, which is detected by ORNs expressing OR67d that target glomerulus DA1 in the AL. As a result, PNs in glomerulus DA1 are activated, which transfer the cVA response to higher brain centers promoting courtship and virgin female receptivity. (*Right*) Illustration of how the simultaneous stimulation with vinegar and cVA enhances the activity of DA1 in a synergistic manner. Vinegar activates specific vinegar-responsive glomeruli which convey this input through eLNs to the DA1 and other glomeruli via electrical synapses. Since DA1 receives a stronger lateral excitation by vinegar (thick line) than other glomeruli (thin line), the PNs of DA1 are stronger activated. As glomerulus DA1 possesses a large number of electrically coupled sister PNs, the signal gets further amplified and leads to the observed synergistic mixture response. The resultant synergistic activity of DA1 is reflected behaviorally by a faster receptivity of virgin females to courting males in the presence of vinegar. As previously shown, in the mated female glomerulus DL3 suppresses the cVA response in glomerulus DA1 via inhibitory LNs; as a result, the synergism cannot occur in this scenario (32).

depending on their nutritional state (19), further supporting the fact that food odors interact with pheromone perception.

In our study, we have identified and characterized the neuronal mechanism underlying the interaction of exposure to the complex food odor vinegar and to the male-specific sex pheromone cVA at the primary olfactory circuit level. We demonstrated that exposure to vinegar synergistically enhanced the flies' response to cVA in PNs in a glomerulus-specific and odorant-selective manner. Moreover, we were able to show that this synergistic response is mediated through electrical synapses between eLNs and PNs in the fly AL. The food odor in this case enhanced the virgin female's sensitivity to cVA. As mentioned above, a similar influence of other food odors (phenyl acetic acid and phenyl acetaldehyde) on male courtship through IR84a has been reported (29). In their study, the food odor affected the behavior of males only. In our study, a different food odor modulated the response of virgin females to cVA without having an effect on males, indicating that environmental cues affect males and females differentially through separate neuronal mechanisms. Although there is evidence that odorant interactions take place at the level of ORNs (51–53), we did not observe any synergistic effect at the peripheral site. Vinegar is a complex blend of odors, where individual components activate different sets of ORs and IRs. Acetic acid alone, in combination with cVA, fails to evoke any synergism, suggesting that the complete vinegar blend is necessary to mediate a synergistic mixture response in PNs. It is conceivable that eLNs need to be activated in an optimum or strong level to achieve this synergism. Hence, the presence of all components of vinegar and consequently the activation of a specific OR/IR combination might be crucial, and need to be elucidated in further studies.

Different Aspects of Synergism. Can we term our observed mixture effect synergism, although vinegar does not directly activate glomerulus DA1? To induce a synergistic response, both stimulations do not necessarily need to share the same input pathways as, for example, demonstrated for synergistic interactions between different sensory modalities (2). Nociceptive and mechanosensory stimulations have been shown to lead to a synergistic behavioral output mediated by two different neuronal pathways that converge at a late stage of the sensory processing hierarchy. In our case, the direct cVA-mediated activation of glomerulus DA1 converges with an indirect lateral excitation induced by vinegar resulting in a synergistic glomerular activation at the AL output level and an enhanced behavioral output.

As already mentioned earlier, we wondered why the synergism via electrical synapses is confined only to vinegar and does not occur with other odor mixtures, since the Krasavietz-positive eLNs are multiglomerular and should therefore be activated also by other odors. We think the synergistic effect evoked by vinegar can be explained by the functional properties of these eLNs. The Krasavietz-positive eLNs have been shown to respond selectively to odor stimuli pronounced by their distinct firing patterns to different odors, while each odor elicited distinct responses in different Krasavietz-positive eLNs (38). Notably, this property is in contrast to the similar responses of inhibitory LNs to distinct odors (54, 55). According to our observation, vinegar activates the eLNs in glomerulus DA1 stronger than other odors, which in turn leads to a stronger activation of PNs in DA1 mediated by the eLNs-PNs gap junctions. This selective odor-response property of eLNs provides the basis for driving synergistic interaction in a glomerulus- and odor-specific manner.

Notably, we observed the synergistic effect of exposure to vinegar on courtship latency only in virgin females, while mated females failed to show this effect (Fig. S1*C*). This feature brings the plastic nature of the synergistic effect as the change in physiological state of the animal modulates the observed phenotype. The difference might be due to the chronic exposure to a high amount of cVA during mating, which activates the olfactory receptor OR65a targeting the DL3 glomerulus (32). OR65a ORNs decrease the activity of the DA1 glomerulus, most likely via inhibitory LNs. Decreased activity in DA1 results in an inhibition of cVA attraction behaviorally (32). Due to this inhibition onto glomerulus DA1, it is likely

that vinegar fails to enhance the activity of this glomerulus in combination with cVA, resulting in the absence of synergism.

Interestingly, virgin males also did not show any enhanced attraction to the odorant mixture in behavioral assays (19), which is well correlated to our observations derived from the functional imaging of AL PNs in males (Fig. 1). Although the branching patterns of cVA-specific PNs originating from the glomerulus DA1 differ in a gender-specific manner in their target area (i.e., the lateral horn) (25, 27), so far there is no evidence for any sex-specific innervation pattern at the level of the AL. However, although there seems to be no anatomical difference at the PN level of the glomerulus DA1, a sexually dimorphic response pattern has been reported: in males, PNs innervating DA1 responded preferably and more strongly to an ipsilateral cVA stimulus, whereas in females, PNs responded equally to both an ipsilateral and a contralateral stimulus (56). Whether this difference in the response pattern seen in glomerulus DA1 between males and females is in any way related to our observed sex-specific synergism needs to be addressed in further studies. In addition, it is conceivable that the innervation patterns of LNs is gender-specific in the pheromone-responsive glomeruli and could therefore lead to differential lateral processing between males and females. This assumption needs to be tested in future studies.

Functional Significance of Gap Junctions for Odor Tuning. We demonstrate here that exposure to vinegar enhances the fly's response to the sex pheromone cVA at the PN level. Although the population of eLNs does not show any synergistic response to the mixture, those neurons are necessary to initiate and mediate the synergism. It has been shown that eLNs significantly mediate lateral excitation in the AL (37, 39), and therefore they most likely convey the excitatory input from vinegar-responsive glomeruli to the DA1 glomerulus. eLNs labeled by the *Krasavitz-Gal4* line are connected to GH146-positive PNs only through reciprocal gap junctions (40), and the eLN-to-PN connection has a stronger impact than vice versa (40). The *Krasavietz-Gal4* line could be classified into two different LN subpopulations, namely type I and type II, based on their physiological properties and glomerular innervation patterns (38, 42). Among them only type I is coupled to other AL neurons via gap junctions. In addition, according to Huang et al. (38), type II Krasavietz neurons are probably inhibitory LNs. Since rescuing wild-type *shakB.neural* in Krasavietz-positive eLNs and -PNs rescued the mixture synergism, it is most likely that the gap junctions between type I eLNs and PNs are necessary to mediate the synergistic effect.

As eLNs are electrically coupled to GH146-positive PNs in multiple glomeruli, the question arises: How is the observed synergism limited to the DA1 glomerulus and not found in other glomeruli? The strength of the connectivity of eLNs to PNs is largely variable across glomeruli, and eLNs have been shown to respond selectively to odor stimuli (38). In addition, the glomerulus DA1 possesses an unusually large number of sister PNs (seven to eight PNs) compared with other glomeruli in the AL (35, 40, 57). As a result, the probability that dense electrical coupling will evolve between eLNs and PNs is higher in the glomerulus DA1 than in more broadly tuned glomeruli, such as the vinegar-responsive ones. These factors may explain why synergism is restricted to the cVA-responsive DA1 glomerulus. However, other narrowly tuned glomeruli with high PN innervations (35) might be the site of additional synergistic interactions and should be studied in the future.

PNs in the glomerulus DA1 detect cVA through OR67d-expressing ORNs located on the antennae, whereas they receive the vinegar-evoked signal most likely through electrically coupled eLNs. In the DA1 glomerulus, PNs possess two kinds of electrical synapses: eLNs-to-PNs and PNs-to-PNs connections (40). Gap junctions represent sophisticated synapses because of their high transmission speed, bidirectionality, and analogical nature, meaning that they transmit graded (i.e., also subthreshold) excitations and inhibitions (40, 58). Hence, neurons that are electrically coupled detect and transmit coincident subthreshold depolarization, which in turn increases neuronal excitability and promotes the temporal synchronization of firing (59–61). In sensory systems, electrical synapses have been shown to mediate lateral excitation and thereby improve sensory sensitivity (62–64). Applied to our results, the synchronous firing of electrically coupled eLNs-to-PNs and PNs-to-PNs, deriving from cVA- and vinegar-responsive glomeruli, leads to an enhancement of *Drosophila's* sensitivity to cVA in the presence of vinegar, such as food. Because cVA acts as a mating cue for the female, the presence of food during courtship increases the sexual receptivity of the virgin female without affecting male courtship. From an ecological point of view, this mechanism sounds logical, since reproductive behavior depends highly on the nutritional state of the female fly (19). Hence, the herein described circuit promotes mating when food is present: that is, when the nutritional supply of the female and its offspring is guaranteed. Future studies will elucidate how this synergism involving food and pheromone is relayed to higher processing centers, and will investigate whether this neuronal mechanism applies to other concurrent sensory inputs.

Materials and Methods

Flies were raised on autoclaved cornmeal-yeast-sucrose-agar food in a 12-h light/dark cycle at 25 °C incubator. Newly emerged flies were anesthetized with CO_2, and virgin males and females were collected, kept in separate vials, and fed fresh food for 4–7 d. Following lines have been used for functional imaging, *Orco-Gal4*, *GH146-Gal4* (II) (48), *Krasavietz-Gal4* (III) (37, 39), *IR8a-Gal4* (II) (65), *GH146-QF*, *QUAS mtd Tomato*, *UAS-GCaMP3* (66), and *UAS-GCaMP6s* (67). The above-mentioned stocks were obtained from Bloomington *Drosophila* Stock Center. For gap junction mutation, *shakB*^{2} (X) (40, 68) and *UAS-shakB.neural* (II) (40, 45) were obtained from Mani Ramaswami's laboratory (Department of Genetics, Trinity College Dublin, Dublin). For photoactivation experiments, *UAS-C3PA* (27) was used. *UAS-inx8 RNAi* was obtained from the Vienna *Drosophila* RNAi Center (VDRC); Canton-S, an OR67d knock-in mutant (10), obtained from the Research Institute of Molecular Pathology, was used for the behavioral experiments.

Details on optical imaging, data analysis, the photoactivation procedure, SSRs, and the behavioral assays are provided in *SI Materials and Methods*.

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