Supporting Information

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SI Materials and Methods

Optical Imaging. Flies were dissected for optical imaging according to the protocol of Strutz et al. (69). Flies were briefly immobilized on ice and then mounted onto a custom-made stage. Protemp II composite (3M ESPE) was used to fix each head. We bent the anterior part of the fly's head with fine gold wire, and a small plastic plate having a round window was placed on top. We sealed the head with that plate using two-component silicone (Kwik Sil) and leaving the center part open to make a cut. The cuticle between the eyes and the ocelli was cut under saline (130 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 36 mM saccharose, 5 mM Hepes, 1 M NaOH, pH 7.3). The cuticle was either bent forward and fixed to the silicon or removed. After cleaning the fatty tissues and trachea, we were able to visualize the antennal lobes.

We used a Till Photonic imaging system with an upright Olympus microscope (BX51WI) and a 20× Olympus objective (XLUM Plan FL 20×/0.95 W), as described in ref. 19 for the functional imaging. Among the odorants, cVA (from Pherobank) was diluted in mineral oil (Carl Roth) to make concentrations of 10^{-1}, 10^{-2}, and 10^{-3}, and balsamic vinegar was diluted in double-distilled water to make concentrations of 10^{-1}, 10^{-2}, and 10^{-3}. Six microliters of these dilutions were pipetted on a filter paper (∼1 cm^2; Whatman), which was placed in Pasteur pipettes. For tests of odorant mixture, two filter papers, one containing cVA and one containing vinegar, were placed in the same pipette. We used filter papers with solvent alone as blanks. A stimulus controller (Stimulus Controller CS-55; Syntech) was used for odor application. Continuous airflow (1 L/min) and pulses of odor (0.1 L/min) were directed through an acrylic glass tube to the fly's antennae. Odor stimuli were injected into this airstream after 2 s for a duration of 2 s. The recording frequency during imaging was 4 Hz with 40 frames (i.e., 10 s) in total. Each odor was measured only once in each animal and the odor stimulation sequence was randomized for each experiment, while we always applied the odors with rising concentrations (i.e., from 10^{-3} over 10^{-2} to 10^{-1}). However, not all concentrations could always be measured in all animals. Therefore, the number of animals for each concentration might differ and is given in each plot. The interstimulus interval was at least 60 s to avoid any effects of adaptation or habituation. To test whether the odor responses, and in particular the mixture response, were reproducible from trial to trial, we measured repeated stimuli in single animals and observed that also three consecutive repetitions induced a significant synergistic mixture response.

Data Analysis. Further data were analyzed with custom-written IDL 6.4 software (ITT Visual Information Solutions). Manual movement correction and bleach correction were followed by the calculation of relative fluorescence changes ($\Delta F/F$) from the background. The glomeruli were identified according to ref. 70. The $\Delta F/F$ of all 40 frames was imported to an Excel file. The responses from frames 10–18 were averaged for the glomerulus of interest for all treatments. Wilcoxon matched paired test was used for all statistical analyses of the imaging data.

Photoactivation and Intensity Quantification. *UAS-C3PA* was driven under *Krasavietz-Gal4* in the background of *GH146-QF*, *QUAS mtd Tomato* for the photoactivation experiment. Four- to 5-d-old virgin females were dissected as described before. The photoactivation was performed on an MPCLSM (Zeiss LSM 710 NLO confocal microscope; Carl Zeiss) equipped with an infrared Chameleon Ultra diode-pumped laser (Coherent). An initial prephotoactivation scan of the whole antennal lobe was taken at 925 nm with 40× water immersion objective (W Plan-Apochromat 40×/1.0 DIC M27; Carl Zeiss). The DA1 glomerulus was identified based on a *GH146* projection pattern. A region of interest in the center of each DA1 glomerulus was photoactivated for ∼10 min (2-min photoactivation followed by 2-min rest) using 760 nm of laser. We allowed 10–15 min for photoactivated GFP to diffuse in more distal neural processes. The postphotoactivation scan was taken using the same set-up as that used for the prephotoactivation scan. The average fluorescence intensity was measured using Fiji software. The average intensity was divided by the area of selection to obtain intensity per square micrometer. The intensity was calculated in the photoactivated DA1 glomerulus and vinegar-responsive glomeruli and compared with the before and after photoactivated brains. A Wilcoxon matched paired test was used for all statistical analyses.

Single Sensillum Recording. Four- to 6-d-old virgin flies were immobilized by wedging each into a pipette tip while fixing the protruding head with wax. The antenna was stabilized on a coverslip with a glass pipette between the second and third antennal segments. Tungsten electrodes were electrolytically sharpened by immersing them in a KNO_2 solution. The reference electrode was inserted into the eye of the fly. To measure the olfactory response to cVA, the recording electrode was placed into long trichoid sensilla, which were identified based on morphology and their characteristic odor response profile. Each time, the complete odor set including all concentrations was tested at one sensillum per fly. Changes in extracellular potentials were measured with the computer software Auto Spike 32 (v3.7). Signals were amplified 10× (Syntech Universal AC/DC probe), sampled with 10,666 Hz, and filtered (300–3 kHz with 50/60 Hz suppression). The stimulus controller Syntech IDAC-4 controlled and defined the properties of the odor puff. The pulse duration of the odor stimulation was 500 ms. Neuronal activity was recorded 3 s before and 10 s after pulse stimulation. A main and a pulse flow of 0.5 L/min were maintained.

Serial dilutions of 10^{-3}, 10^{-2}, and 10^{-1} (vol/vol) were made. While cVA was diluted in mineral oil, double-distilled water was used for balsamic vinegar. Solvents were also used as control stimuli. Pasteur pipettes containing two filter papers were used for odor stimulations. Filter papers were loaded with 6 μL of the aliquots.

To analyze the action potential frequency (spikes per second) over the total recording interval, a bin width of 25 ms was set. We quantified the physiological response of the odor stimuli by subtracting the calculated maximum frequency of 1 s before from 1 s after stimulus onset. The Wilcoxon matched paired test was used for all statistical analyses.

Behavior. Males and females were collected after eclosion and raised individually and in groups, respectively, for 4–6 d. For each experiment, typically, 24 courtship assays were performed in a (1-cm diameter × 0.5-cm depth) chamber covered with a plastic slide. The base of the chamber had a small pore in which 2 μL of vinegar (10^{-3}) or water (solvent control) was placed to perfume the chamber. Plastic mesh was placed underneath the mating chamber to restrict the flies' contact with vinegar or water. Courtship behaviors were recorded for 20 min and analyzed. All mating experiments were performed under red light (660-nm wavelength) at 25 °C and 70% humidity. Each video was ana-

lyzed for copulation success, which was measured by the percentage of males that copulated successfully in the first 20 min, and copulation latency, which was measured as the time taken by each male until copulation. For courtship experiments, females were decapitated with a clean razor blade, to avoid any successful mating during the courtship process. The assay was performed 20 min after decapitation. Courtship index was measured by a researcher who was blinded to genotype. Courtship index was calculated as the portion of time a male fly was engaged in any step of the courtship (chasing, orienting, wing vibration, abdominal curling, and copulation) in the first 10 min of the assay. The χ^2 test with Yates correction was used to statistically analyze the copulation success and the Mann–Whitney test was used for the copulation latency and the courtship index.

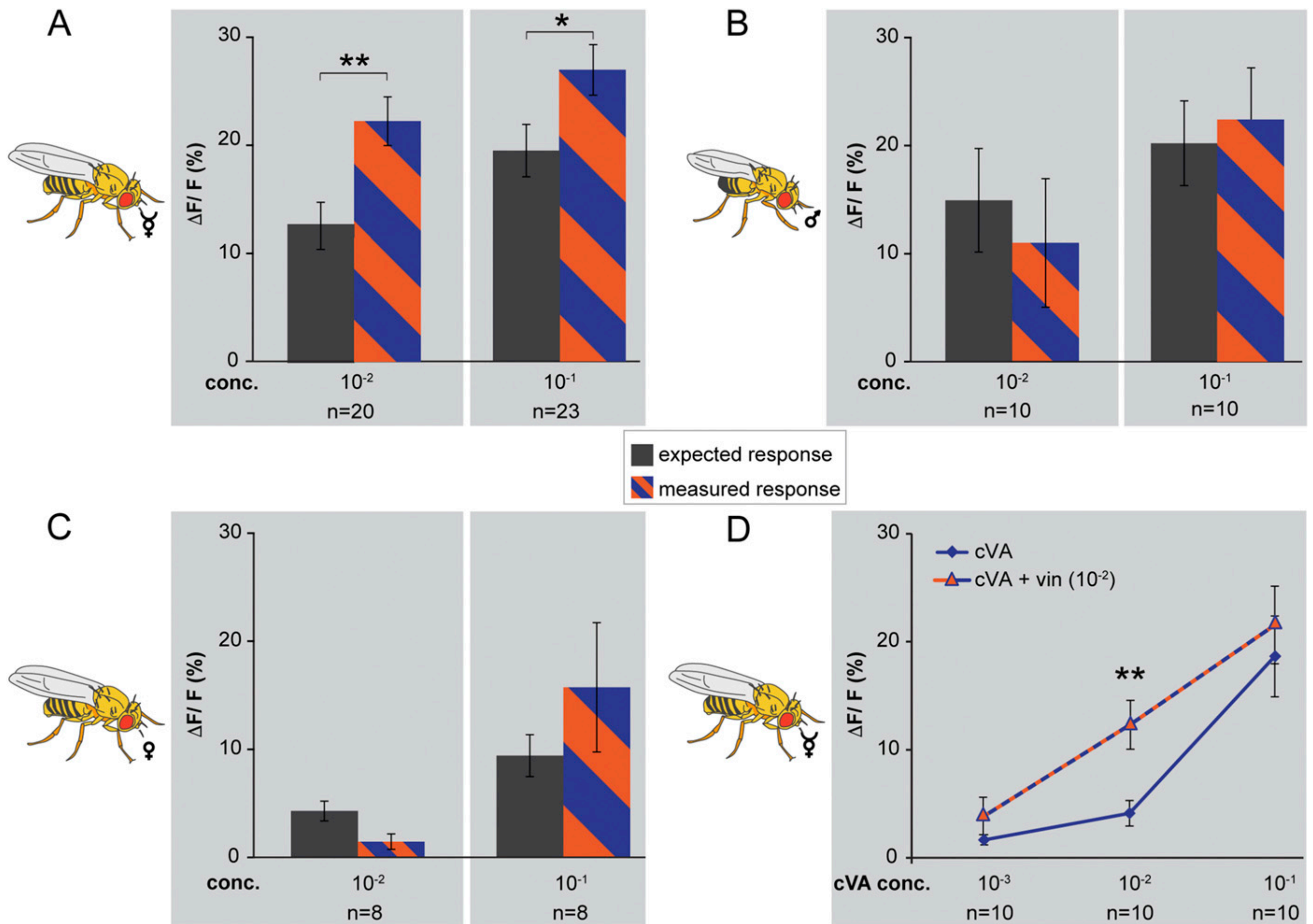

Fig. S1. PNs in glomerulus DA1 reveal synergism to the mixture of cVA and vinegar in virgin females, while males and mated females do not show any mixture interaction. (*A*–*C*) Comparison between expected (dark gray) and measured (striped) mixture response of PNs in glomerulus DA1 in virgin females (*A*), virgin males (*B*), and mated females (*C*) at 10^{-2} and 10^{-1} concentrations. The expected response was calculated by adding the individual responses of flies to vinegar and cVA. Only virgin females show a mixture synergism (**$P < 0.01$, *$P < 0.05$; Wilcoxon matched paired test). (*D*) Vinegar synergizes the cVA response in a ratio-dependent manner. Dose–response curve of cVA of PN responses ($\Delta F/F$) in glomerulus DA1 in virgin females with (striped line) or without vinegar (blue line) (10^{-2} concentration) in the background. A synergistic response is only visible at a 1:1 concentration of cVA and vinegar (**$P < 0.01$; Wilcoxon matched paired test).

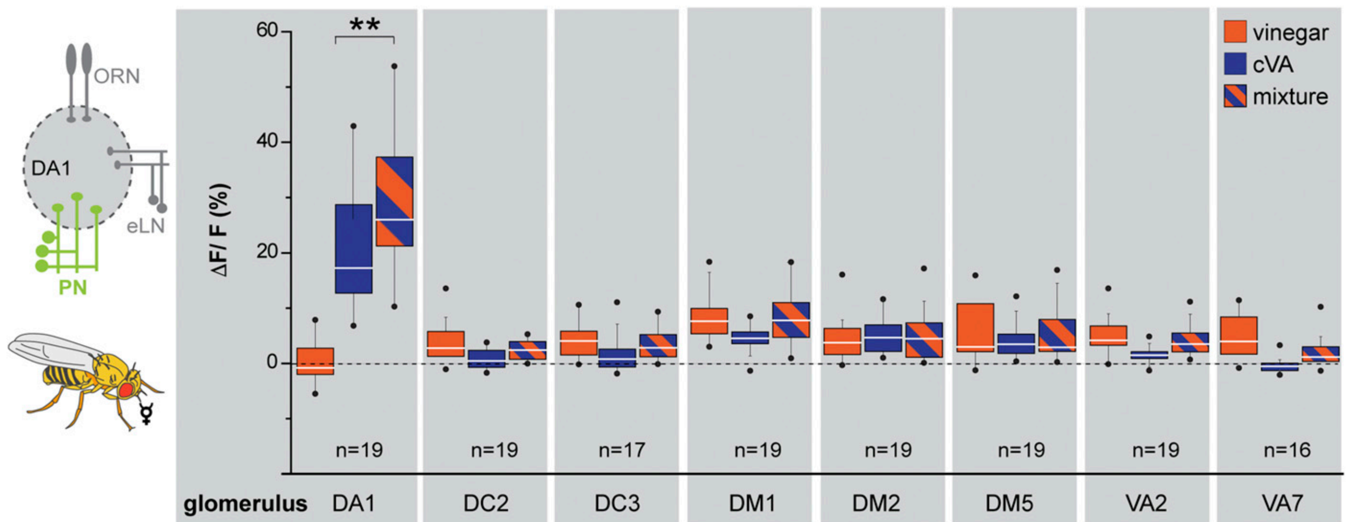

Fig. S2. Synergism in DA1 occurs in a glomerulus-selective manner. Box plots represent Δ*F/F* responses of PNs in different glomeruli in virgin females to vinegar (orange), cVA (blue), and their binary mixture (striped) (10^{-1} concentration). The white line in the box represents the median. Only glomerulus DA1 reveals a mixture synergism (**$P < 0.01$; Wilcoxon matched paired test).

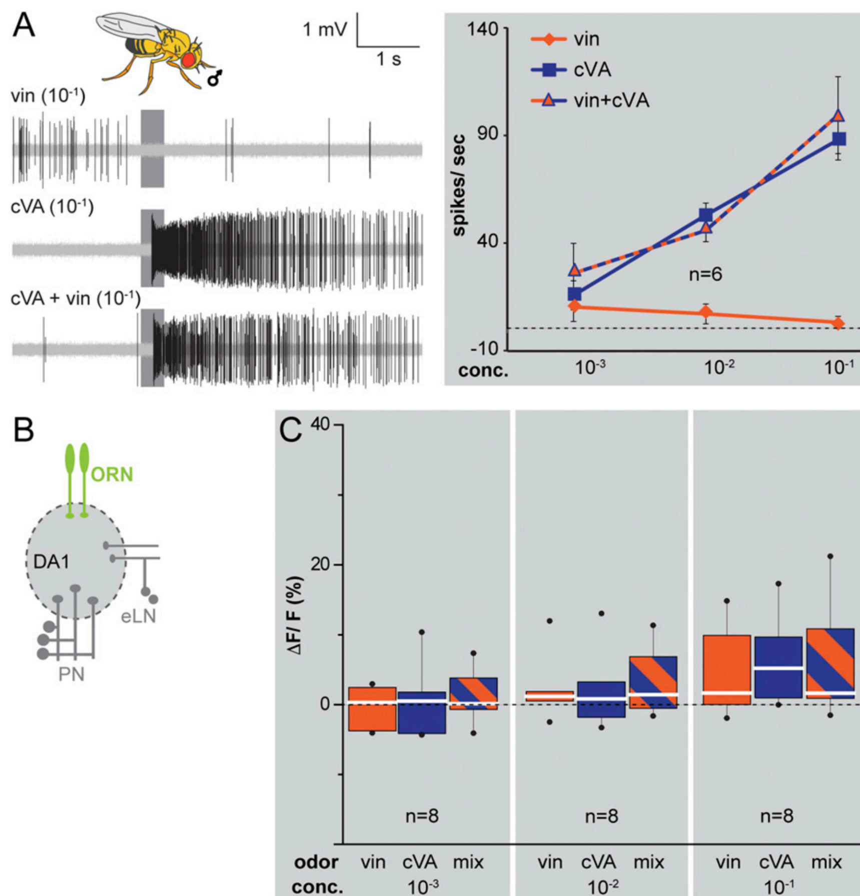

Fig. S3. Synergism does not occur at the sensory level in males. In vivo extracellular SSRs from the at1 sensillum expressing OR67d. (*A*, *Left*) Representative traces display the response of OR67d ORNs in virgin males to vinegar, cVA, and their binary mixture (10^{-1} concentration). (*Right*) Line curves represent the averaged responses (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 males. (*C*) Box plots represent Δ*F/F* responses of ORNs in glomerulus DA1 in males 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).

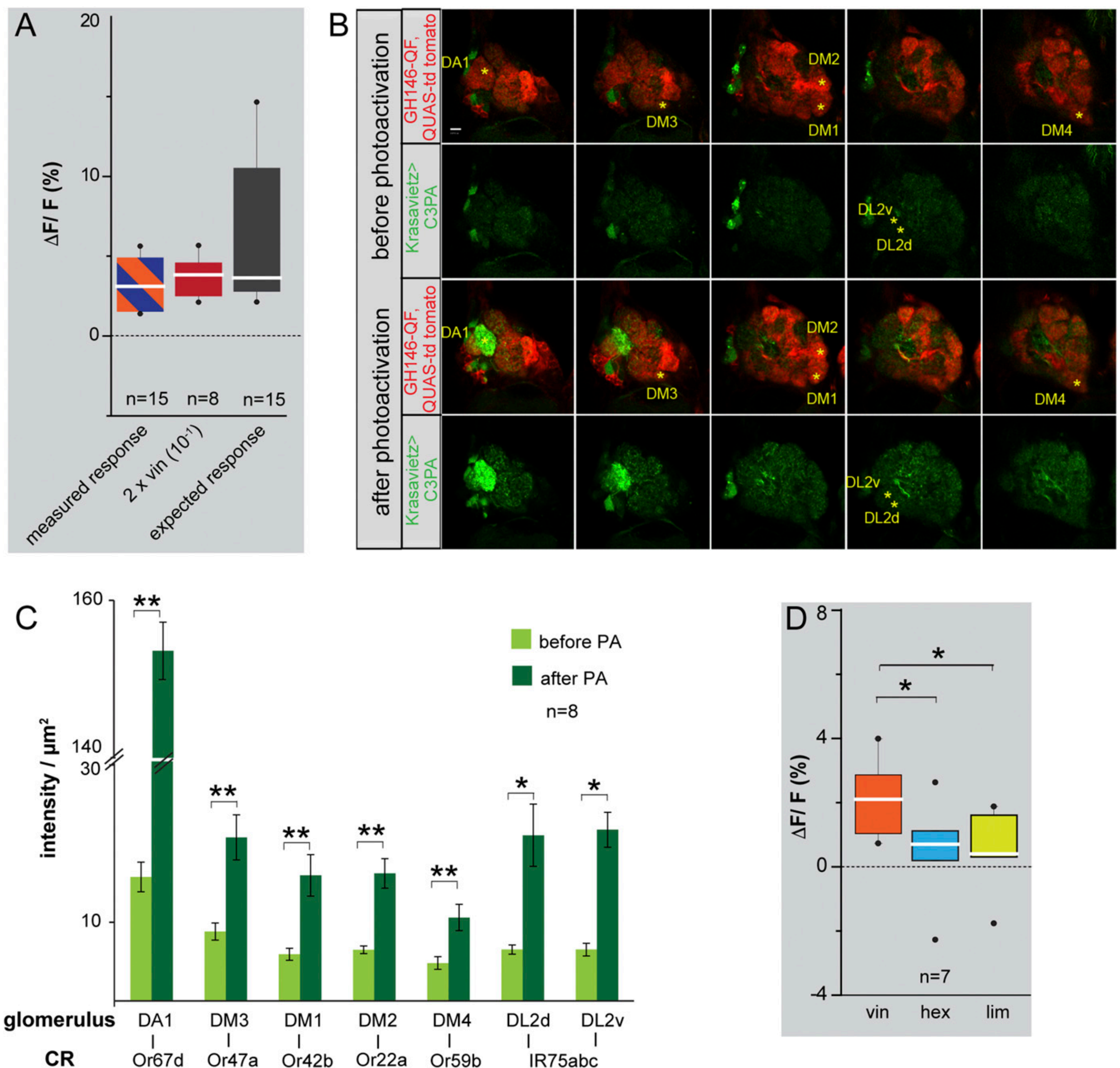

Fig. S4. Excitatory LNs do not show mixture synergism although they innervate both DA1 and vinegar-responsive glomeruli. (*A*) Comparison between expected (dark gray) and measured (striped) mixture response at 10^{-1} concentration and the response to double amount of vinegar at 10^{-1} of PNs in glomerulus DA1 in virgin females. The expected response was calculated by adding the individual responses of flies to vinegar and cVA. The measured mixture response is equal to the expected as well as the response to the double amount of the stronger component, (i.e., vinegar) ($P > 0.05$; Wilcoxon matched paired test). (*B*) Photoactivatable GFP *(UAS-C3PA)* expressed in eLNs using *Krasavietz-Gal4* (in green) and only the glomerulus DA1 was photoactivated. The different vinegar-responsive glomeruli (yellow asterisks) were identified based on their glomerular structure and visualized with *GH146 QF-QUAS td-tomato* in the background (in red). (*Upper*) The glomeruli at different focal planes before photoactivation. (*Lower*) The same glomeruli after photoactivation. (Scale bar, 10 μm.) (*C*) The fluorescence intensity per square micromete of GFP in different glomeruli (with their corresponding chemosensory receptor, CR) was quantified and compared before and after photoactivation. (**$P < 0.01$, *$P < 0.05$; Wilcoxon matched paired test). (*D*) Comparison of Krasavietz-positive eLNs response in DA1 to vinegar (vin), 1-hexanol (hex), and limonene (lim) at a concentration of 10^{-1}. The response to vinegar in DA1 is significantly higher than to the other two odors (*$P < 0.05$; Wilcoxon matched paired test).