Supporting Information

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SI Materials and Methods

Behavior. Training and testing were performed under dim red light at 25 °C and 70% relative humidity. Flies were exposed to 1 min of an odor paired with 12 pulses of electric shock at 90 V [conditioned stimulus (CS), i.e., CS+] followed by 1 min of a second odor without shock (CS−). For 3-min memory measurements, flies were immediately loaded into a testing maze and allowed to choose for 2 min between the CS+ and CS− odors. To assay memory retention at later time points, trained flies were transferred back into food vials for the appropriate interval and then tested the same way. Five spaced training cycles with a rest of 15 min between each cycle were performed for generating long-term memory, whereas 5× massed training was performed with no intertrial interval. For behavioral measurements, two groups of flies were trained simultaneously using two different odors as the CS+. The one-half performance index (PI) for each odor was calculated as follows:

microscope and imaged by using a 488-nm excitation laser line. The emitted light was collected from 520 ± 15 nm. Odors were diluted in mineral oil, put on a piece of filter paper, and delivered from a micropipette in an air stream at a rate of 200 mL/min. The delivery of odors was under the control of a solenoid-activated, three-way Teflon valve and a programmable controller, such that fresh air could be delivered to each animal for a predetermined period with an instantaneous switch to odor-laced air without altering the overall flow rate. The calcium response to the 3-octanol (Oct) was assayed first by imaging with a 3-s odor exposure. After a 3-min interval, the calcium response to benzaldehyde (Ben) or 4-methylcyclohexanol was assayed in an identical way.

Images were acquired at 10 frames per second at a resolution of 256 × 256 pixels. Quantification of the responses was made from the pixels representing innervation of the vertical lobe of the mushroom body (MB) neuropil or the dorsal tip of the α′ lobe in

$$PI = (\text{number of flies that chose CS} - \text{minus number that chose CS} +)/(\text{number of flies that chose CS} - \text{plus number that chose CS} +)$$

The overall PI was then calculated as the average of the two one-half PIs for each odor. For odor avoidance, untrained flies were loaded directly into a testing maze and allowed 2 min to choose between an odor and air. For shock avoidance, both arms of the testing maze were replaced with shock tubes, and 90-V electric shocks were applied to one of the two arms for 1 min while the flies distributed between the arms according to their preference.

Functional Cellular Imaging. After paired or unpaired training, flies were transferred into a new food vial. One fly was aspirated from the vial and mounted in a pipette tip. A small area of cuticle on the dorsal aspect of the fly head was removed, and the opening was covered with a small piece of plastic wrap. The flies were then mounted beneath the objective lens of a Leica TCS confocal

each image. The baseline fluorescence value was calculated for each pixel within the region of interest as the fluorescence before odor application as averaged over five successive frames. The change in fluorescence was calculated for each pixel as the difference between the highest intensity during the 3-s odor application and baseline fluorescence.

As Fig. 3C shows, after each individual received conditioning, the calcium response to the Oct was assayed by imaging and, after a 3-min interval, the calcium response to Ben or 4-methylcyclohexanol was assayed in the same individual sequentially. We calculated the ratio of the CS+ response to the CS− response for each individual and then averaged the individual ratios for flies within the group.
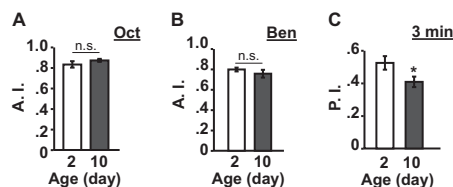

Fig. S1. Poor performance in 3-min memory for flies 10 d of age is a result of a defect in short-term memory rather than odor perception and avoidance. (A) Olfactory avoidance indices (A.I.) toward Oct using w(CS10) flies 2 d and 10 d of age at a high odor concentration (0.15%). Olfactory avoidance of Oct at 0.15% was not significantly different between 2- and 10-d-old flies (t test, n = 6 for each group). (B) Olfactory avoidance indices toward Ben using w(CS10) flies 2 d and 10 d of age at a high odor concentration (0.06%). Olfactory avoidance of Ben at 0.06% was not significantly different between 2- and 10-d-old flies (t test, n = 6 for each group). (C) The 3-min performance indices (P.I.) at the high odor concentration (Oct, 0.15%; Ben, 0.06%) for w(CS10) flies 2 d and 10 d of age. Under these conditions, the performance of 10-d-old flies was significantly lower than that of 2-d-old flies (t test, *P < 0.05; n = 6 for each group). Error bars indicate SEM.

Fig. S2. Artificial stimulation of MB α′/β′ neurons for short-term memory. (*A*) Activating MB α′/β′ neurons did not enhance 3-min memory in *c316>trpA1* flies at 10 d of age. Flies were shifted to 30 °C at 20 min before training and trained at 30 °C and tested immediately (black bar; +). Control flies were trained and tested for 3 min memory at 22 °C (open bar; −; *P < 0.05, Tukey post hoc following significant differences after one-way ANOVA; *n* ≥ 6 for all groups). (*B*) Activating MB α′/β′ neurons did not enhance 3-min memory in *c316>trpm8* flies at 10 d of age. Flies were shifted to 18 °C at 20 min before training and trained at 18 °C and tested immediately (black bar; +). Control flies were trained and tested for 3 min memory at 25 °C (open bar; −). Although cold treatment reduced 3-min memory, there was no genotype-specific significant difference in 3-min memory with cold treatment comparing *c316>trpm8*, *c316/+*, and *trpm8/+* (*P < 0.05, Tukey post hoc following significant differences after one-way ANOVA; *n* ≥ 6 for all groups).