Supplemental Figures

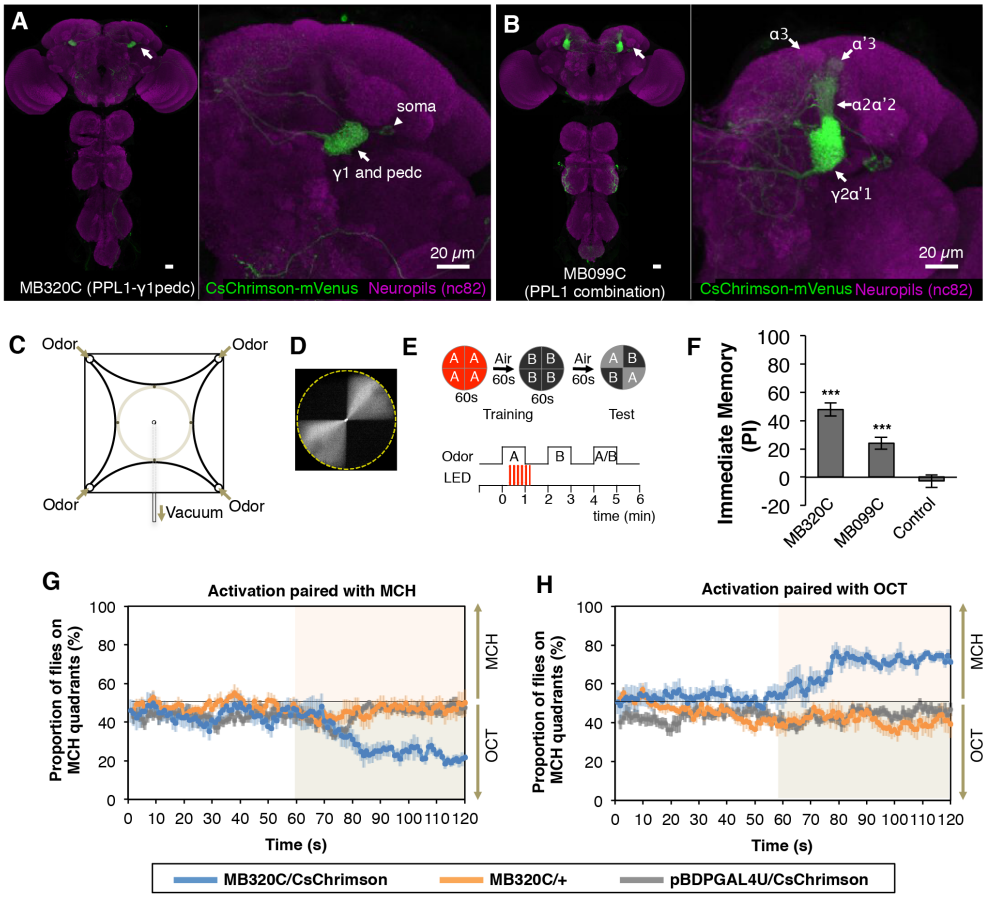

Figure S1. Aversive Olfactory Conditioning via Optogenetic Activation of DANs, Related to Figures 1 and 5

(A) Expression pattern of MB320C driving 20xUAS-CsChrimson-mVenus (in attP18) in brains and ventral nerve cord (left) and magnified view (right). We observed consistent expression in one PPL1-γ1pedc per brain hemisphere (arrowhead). Occasionally we also observed weak and stochastic expression in PPL1-α′2α2, PPL1-α′3 and other cells in optic lobes and ventral nerve cord; because this other expression was weak and variable, it could not account for the reproducible effects we observed in multiple animals. Arrow indicates terminals of PPL1-γ1pedc in γ1 and the core of the pedunculus (pedc) where α/β KCs send axon bundles.

(B) Expression pattern of MB099C driving 20xUAS-CsChrimson-mVenus (in attP18). MB099C reliably labels PPL1-γ2α′1 and PPL1-α′2α2. We also observed stochastic expression in PPL1-α′3 and PPL1-α3, but PPL1-γ1pedc was never labeled in this driver. Sparse off-targeted expression in VNC was also observed.

(C) Diagram of the four-field olfactometer. Odorized air was injected through each arm of the arena to the outlet at the center. Flies are confined in a 3-mm high and 10-cm diameter arena (gray circle).

(D) Separation of airflow in four quadrants of arena was verified by introducing smoke of ammonium chloride

to two opposing quadrants.
(E) Protocol for olfactory conditioning. Approximately 20 retinal-fed female flies were placed into the arena in the dark and exposed to odor for 60 s. 10 s after the onset of the first odor, CsChrimson-stimulation was delivered as 12 × 500ms pulses of red (627 nm) light were delivered from the bottom of arena through the diffuser, at 5-s intervals. After the last light pulse, we waited 60 s and then delivered the second odor for 60 s. After 60 s, memory was tested by delivering the two odors (CS+, odor paired with DAN activation and CS-, control odor not paired with DAN activation) to opposing quadrants. We monitored the location of flies for 60 s while they were allowed to walk freely.
(F) Memory acquisition following US-substitution with two different sets of DANs. Either OCT or MCH was used as CS+. Performance Index (PI) was calculated as [(# flies in CS- - # flies in CS+)/total # flies] during the last 30 s of the odor choice period (mean ± SEM, n = 6–8). Asterisks indicate significantly higher score of experimental genotype (split-GAL4/20xUAS-CsChrimson-mVenus) than an empty GAL4 control (pBDPGAL4U/20xUAS-CsChrimson-mVenus) ($p < 0.001$; one-way ANOVA followed by Dunnett's multiple comparison test).
(G, H) Time courses of the mean spatial distribution of flies for one of the reciprocal experiments shown in F. Shading represents SEM.

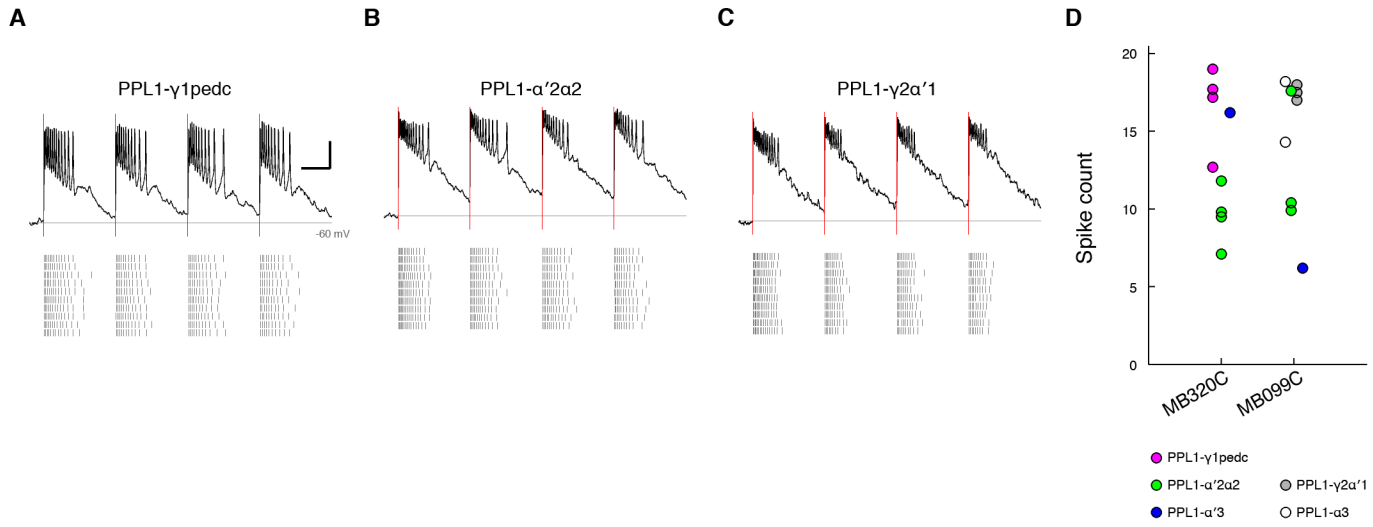

Figure S2. Optogenetic Activation of DANs, Related to Figures 1, 5 and 6

(A) A representative recording of light-evoked responses from PPL1-γ1pedc. Expression of CsChrimson was driven by MB320C. Red lines indicate the timing of the light pulses (1 ms in duration) delivered at intervals of 0.5 s, the same stimulation used for pairing experiments. Gray line, -60 mV. Scale bars, 10 mV and 200 ms. Raster plot of ten successive trials is shown below.

(B, C) A representative recording from PPL1-α′2α2 (B) and PPL1-γ2α′1 (C). Expression of CsChrimson was driven by MB099C.

(D) Summary of spike counts evoked by the first 1-ms light pulse. Recordings were made from cells that were positive for CsChrimson-mVenus signal (n = 9 cells per driver). Cells with lower expression level were also targeted for recording so that all the cell types labeled in each driver are recorded. Cell types were identified by *post hoc* immunohistochemistry with biocytin. With both drivers, CsChrimson-expressing neurons exhibited robust spikes in response to light.

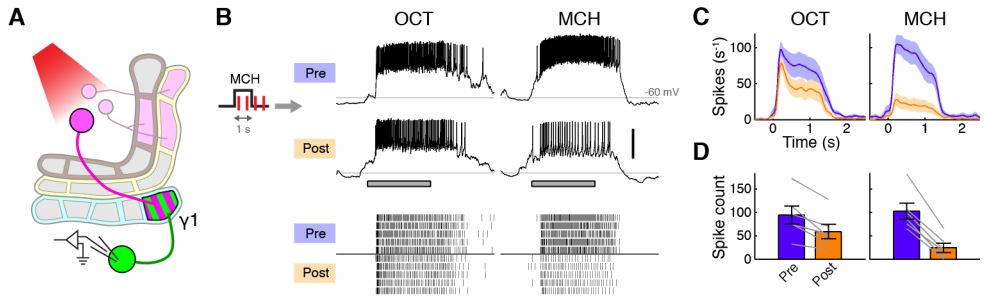

Figure S3. Induction of Plasticity to MCH Response, Related to Figure 1

(A) Expression of CsChrimson and GFP was driven by MB320C and R12G04-LexA, respectively. Recordings were made from MBON-γ1pedc.

(B) Representative single-cell data, showing odor responses before (Pre) and after (Post) pairing. The pairing protocol was the same as shown in Figure 1C except that CS+ is MCH. Gray bar, 1-s odor presentation. Scale bar, 20 mV. Raster plots (bottom) show spikes.

(C) Mean spike rates over several experiments displayed as peristimulus time histogram (PSTH; ± SEM, shaded area; $n = 6$).

(D) Mean odor-evoked spike count (± SEM). Gray lines indicate data from individual flies. Spike counts decreased in both CS+ (MCH, $p < 0.001$, Tukey's *post hoc* test) and CS- (OCT, $p < 0.01$), but the effect of pairing was significantly different between odors ($p < 0.005$, repeated measures two-way ANOVA).

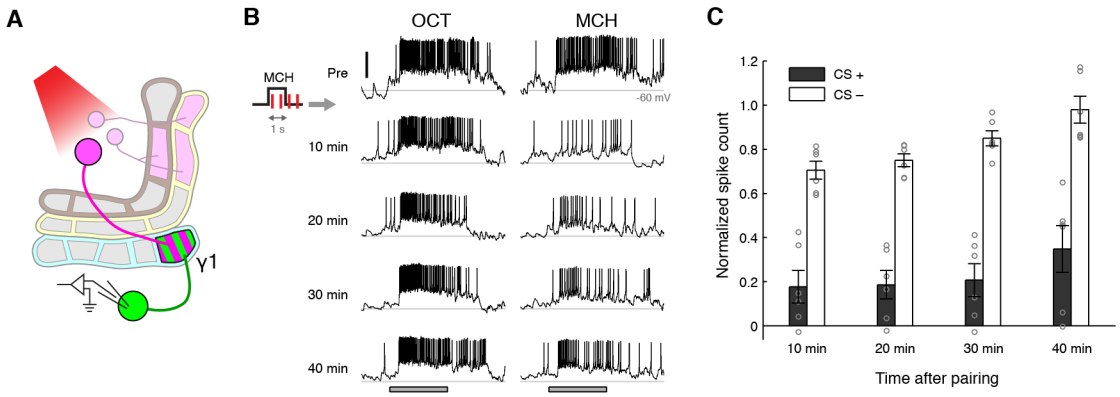

Figure S4. Pairing-induced Depression Lasts over 40 min, Related to Figure 1

(A) Expression of CsChrimson and GFP was driven by MB320C and R12G04-LexA, respectively. Recordings were made from MBON-γ1pedc.

(B) Representative single-cell data, showing odor responses before (Pre) and 10, 20, 30 and 40 min after 1-sec pairing. MCH was used as CS+. Gray bar, 1-s odor presentation. Scale bar, 20 mV.

(C) Mean odor-evoked spike count normalized to pre-pairing data (± SEM; $n = 6$). Gray circles indicate data from individual flies. Either OCT ($n = 3$) or MCH ($n = 3$) was used as CS+. CS+ responses remained depressed at all time points ($p < 0.05$, Tukey's *post hoc* test following repeated measures two-way ANOVA), while CS- responses were no longer significantly depressed after 30 min ($p > 0.1$).

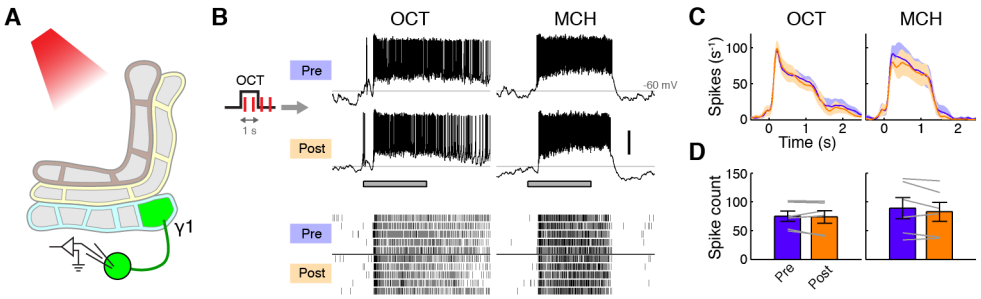

Figure S5. Induction of Plasticity Requires DAN Activation , Related to Figure 1

(A) Whole-cell recordings were made from MBON-γ1pedc. Odor-light pairing (1-s odor with 1-ms light pulses x 4) was performed in flies lacking the driver to express CsChrimson. The genotype of the flies is R12G04-LexA/13xLexAop-IVS-GFP-p10; 20xUAS-CsChrimson-mVenus/+.

(B) Representative single-cell data, showing odor responses before (Pre) and after (Post) pairing. Gray bar, 1-s odor presentation. Scale bar, 20 mV. Raster plots (bottom) show spikes.

(C) Mean PSTH (± SEM, shaded area; $n = 6$).

(D) Mean odor-evoked spike count (± SEM). Gray lines indicate data from individual flies. Pairing had no significant effect ($p > 0.3$, repeated measures two-way ANOVA).

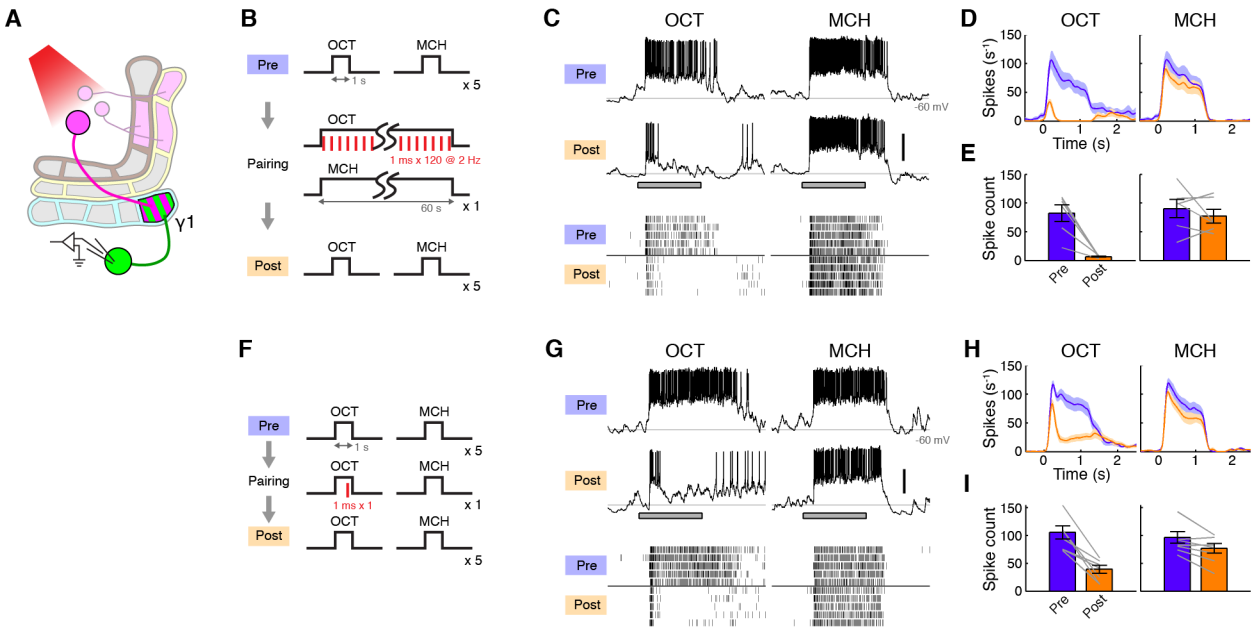

Figure S6. Induction of Plasticity with Long and Short Pairing Protocols, Related to Figures 1 and 7

(A) Expression of CsChrimson and GFP was driven by MB320C and R12G04-LexA, respectively. Recordings were made from MBON-γ1pedc.

(B) 1-min pairing protocol.

(C) Representative single-cell data, showing odor responses before (Pre) and after (Post) pairing. Gray bar, 1-s odor presentation. Scale bar, 20 mV. Raster plots (bottom) show spikes.

(D) Mean PSTH (± SEM, shaded area; $n = 6$).

(E) Mean odor-evoked spike count (± SEM). Gray lines indicate data from individual flies. Spike counts decreased in CS+ ($p < 0.005$, Tukey's *post hoc* test) but not in CS- ($p > 0.4$), and the effect of pairing was significantly different between the two odors ($p < 0.005$, repeated measures two-way ANOVA).

(F) Mild 1-sec pairing protocol. Single 1-ms light pulse was delivered 0.8 s after the onset of 1-s odor pulse.

(G) Representative single-cell data, showing odor responses before (Pre) and after (Post) pairing. Gray bar, 1-s odor presentation. Scale bar, 20 mV. Raster plots (bottom) show spikes.

(H) Mean PSTH (± SEM, shaded area; $n = 5$).

(I) Mean odor-evoked spike count (± SEM). Gray lines indicate data from individual flies. Spike counts decreased in both CS+ ($p < 0.001$, Tukey's *post hoc* test) and CS- ($p < 0.05$), but the effect of pairing was significantly different between the two odors ($p < 0.005$, repeated measures two-way ANOVA).

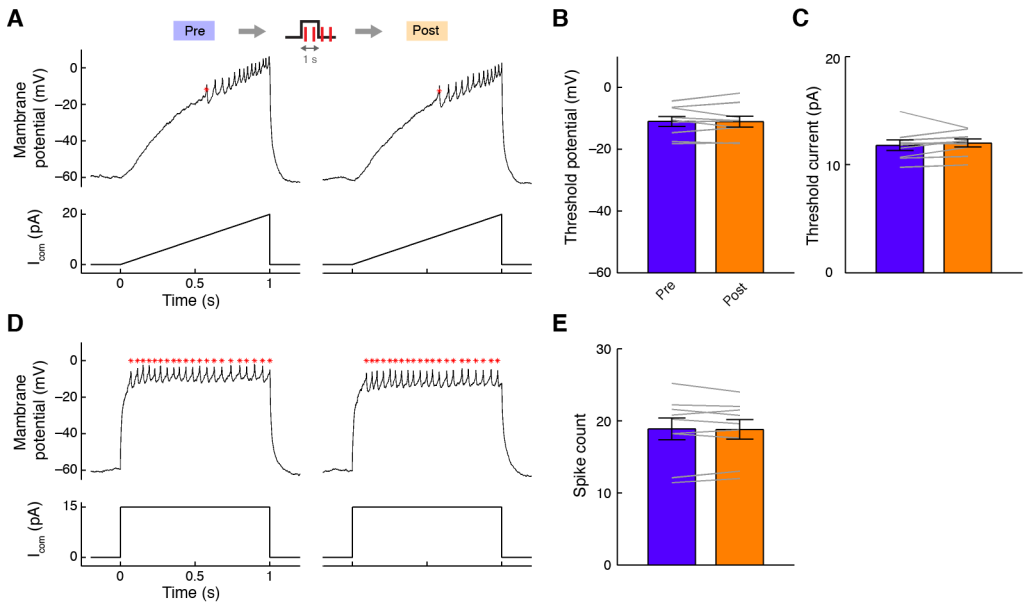

Figure S7. Excitability of γ KCs Does Not Change after Pairing Depolarization with DAN Activation, Related to Figure 2

(A) Measuring spike threshold in a γ KC before (Pre) and after (Post) pairing using a ramped current injection. Odor-DAN pairing was mimicked by pairing KC depolarization induced by current injection (15 pA, 1 s) and light activation of DANs (1 ms x 4 at 2 Hz). γ KC was targeted for recording by expressing GFP with R14H06-LexA. Expression of CsChrimson in PPL1-γ1pedc was driven by MB320C. Red asterisks show the detected shoulder of spikes.

(B) Pairing induced no significant change in spike threshold potential ($p > 0.9$; $n = 9$)

(C) Pairing induced no significant change in spike threshold current ($p > 0.4$)

(D) Measuring sustained spike rate in γ KC. Recordings from the same cell as (A) are shown. Red asterisks indicate the spikes.

(E) Pairing induced no significant change in sustained spike rate ($p > 0.7$).

Supplemental Experimental Procedures

Behavior assay

For olfactory conditioning with optogenetic activation of dopaminergic neurons, we modified a previously described (Aso et al., 2014b; Pettersson, 1970; Vet et al., 1983) four-field olfactometer and LED array. The four-field olfactometer consisted of star-shaped arena made of non-odor-binding ultra-high-molecular-weight polyethylene, a glass lid coated with Sigmacoat (Sigma–Aldrich) and a 2-mm high ring insert which confined flies to a 10-cm diameter and 3-mm high circular arena (Figure S1C). Diluted odors or solvent (8 mL) were placed in 40 mL tubes and 3-way solenoid valves and a custom built controller were used to direct airflow. Airflow to each arm of the olfactometer was maintained at 100 ml/min by a rotary vane pump (G 12/01 EB, Thomas) and a mass-flow controller (MCW-200SCCM, Alicat). Air suction through the outlet at the center was maintained by another rotary vane pump (400 mL/min). The bottom of arena was a light diffuser (optically clear cast acrylic sheet, 1/8-inch thick, 12 inch × 12 inch, McMaster 8560K239). Arrays of 627 nm LEDs (Red LUXEON Rebel LED—106 lm; Luxeon Star LEDs, Brantford, Ontario, Canada) and 800 nm infrared red LEDs were placed below the diffuser and above the heat sink, and were controlled by a microcontroller (Arduino) using custom software. Intensity of red light (627 nm) above diffuser was 20.4 mW/cm^2. The odors were diluted in paraffin oil (Sigma–Aldrich); 3-octanol (OCT; 1:1000; Merck) and 4-methylcyclohexanol (MCH; 1:1000; Sigma–Aldrich), 2-heptanone (HP: 1:10000; Sigma–Aldrich), pentyl acetate (PA: 1:10000; Sigma–Aldrich), butyl acetate (BA: 1:10000; Sigma–Aldrich) and ethyl lactate (EL: 1:10000; Sigma–Aldrich). Groups of approximately twenty 4–10d post-eclosion females were trained and tested at 25°C at 50% relative humidity in a dark chamber (Figure S1E).

Videography was performed with IR back light using a camera (ROHS 1.3 MP B&W Flea3 USB 3.0 Camera; POINT GREY, Richmond, BC, Canada) with an 800-nm long pass filter (B&W filter; Schneider Optics) at 30 frames per sec, 1024 × 1024 pixel resolution and analyzed using Fiji (SchindelinCardona2012).

Supplemental References

Pettersson, J. (1970). An Aphid Sex Attractant. Insect Systematics & Evolution *1*, 63–73.

Vet, L.E.M., Lenteren, J.C.V., Heymans, M., and Meelis, E. (1983). An airflow olfactometer for measuring olfactory responses of hymenopterous parasitoids and other small insects. Physiol Entomol *8*, 97–106.