Supporting Information

Lee et al. 10.1073/pnas.1019483108

SI Materials and Methods

Cold Shock and Drug Feeding. To induce anesthesia, flies were transferred into a vial in ice 2 h after olfactory conditioning for 2 min. The anesthetized flies then were allowed to recover in vials with fresh food for 1 h at 25 °C before the memory test. For drug feeding, flies 2–3 d after eclosion were kept in a bottle containing a 6 × 6 cm Chrome-Paper sheet (Sartorius) loaded with 2 mL drug/glucose solutions overnight [for L-5-hydroxytryptophan (5HTP) and cycloheximide (CXM)] or for 3 d [for DL-p-chlorophenylalanine (pCPA)]. 5HTP (2 mg/mL) (Alfa Aesar) and CXM (35 mM) (Sigma) were prepared in 5% glucose solution. pCPA (100 mg/mL) (Sigma) was dissolved in DMSO as stock and then was diluted to 10 mg/mL in 5% glucose. The control flies were kept in a bottle with vehicle alone (2 mL 5% glucose solution without drugs). Food was stained with colored dye, and ingestion of drugs was confirmed by the visualization of red color on the fly's abdomen. The drugs did not seem to affect feeding negatively, based on colored abdomens of flies exposed to the red dye solution. For the 24-h memory assay, flies were kept in the vial with drugs or vehicle until test.

Behavior. The genetic background for all flies used in the behavior assay except *VT64246-Gal4* flies was "Cantonized" by outcrossing with Canton-S w^{1118} (iso1CJ) wild-type flies for at least five generations. Aversive olfactory learning was performed by training 3- to 5-d-old adult flies in a T-maze apparatus with a Pavlovian conditioning procedure as described previously (1, 2). One training session consisted of ∼100 flies, each of which was electrically shocked in the presence of one of two odors (3-octanol and 4-methylcyclohexanol). Learning was measured immediately after one training session. Three-hour memory was tested 3 h after one training session. One-day memory was evaluated after 10× massed or spaced training. Massed training consisted of 10 cycles of one-session training, with one session immediately following the previous one. Spaced training consisted of 10 cycles of one-session training with a 15-min rest interval introduced between each session. In some cases (noted in figure legends), flies were subjected to only three cycles of massed or spaced training to generate lower levels of 1-d memory for higher sensitivity in the detection of the enhancement effect. In all cases, the shock-associated odors were alternated between 3-octanol and 4-methylcyclohexanol. Memory performance was calculated as the number of flies avoiding the conditioned odor minus the number of flies avoiding the unconditioned odor divided by the total number of flies. The Performance Index (PI) was calculated as the average score of the two memory performances.

Immunohistochemistry. Whole-mount immunolabeling of the adult brain was performed as previously described (3). Briefly, the dissected brains were fixed in 4% paraformaldehyde in PBS on ice for 2 h, transferred to 4% paraformaldehyde in PBS with 2% Triton X-100 in a room temperature, left in vacuum for 1 h and left overnight in the same solution at 4 °C. For immunohistochemical staining, the following primary antibodies were used: mouse anti-Discs large (anti-DLG) (1:50 dilution), rat anti-amino acid decarboxylase (anti-DDC) (1:500 dilution), and rabbit anti-serotonin (anti-5HT) (1:1,000 dilution). Secondary antibodies were used as follows: biotin-conjugated goat anti-mouse, biotin-conjugated goat anti-rat, and biotin-conjugated goat anti-rabbit IgGs, all diluted 1:200. Biotin-conjugated IgGs (Invitrogen) were probed by Alexa Fluor streptavidin 633 (1:500 dilution) (Invitrogen). Immunostained brains were cleared and mounted in FocusClear (Celexplorer) and imaged with a Zeiss LSM710 Confocal Microscope under a 40× C-Apochromat water immersion objective lens or 63× plan-Neofluar glycerol immersion objective lens. For 3D colocalization analysis of dorsal paired medial (DPM) fibers and anti-5HT immunostains, images were taken under a 63× Plan-Apochromat oil immersion objective lens. Images taken under 633-nm excitations were corrected for a red shift at the *z* axis (0.35 μm) before colocalization analysis with images taken under 488 nm excitations, based on the calibration using a standard fluorescence bead (Invitrogen).

Quantitative Measurement of 5HT. We used an Agilent 1200 Series of high-performance liquid chromatography coupled with a fluorescence detector system (HPLC-FLD) that detects 5HT fluorescence intensities on both the excitation (280-mm) and emission (320-mm) wavelengths. Sample preparation was similar to that reported previously (4). Briefly, 100 heads of 5-d-old adult flies were homogenized in 100 μL ice-cold solution containing 50 mM citrate/acetate (pH 4.5), 11 mM 1-decanesulfonic acid and 18% acetonitrile. After an equal volume of ice-cold methanol was added and the solution was run through a 0.22-μm Spin-X filter (Costar), 20 μL homogenate (equivalent to 10 heads) was allowed to flow through the sample to ensure equal mixing, and an internal standard of α-methylserotonin (800 pg/mL) was injected. Reverse-phase chromatography was performed using a ZORBAX Rx-C_{18} column (250 mm × 4.6 mm i.d.) with a 5-μm particle size and 80-Å pore size (Agilent). A Reliance cartridge guard column (12.5 mm × 4.6 mm i.d.) (Agilent) was placed between the injector and the analytical column. The mobile phase moving through the chromatography column was delivered at a flow rate of 0.6 mL/min to separate and detect 5HT. The 5HT was quantified and analyzed based on a standard curve using the HPCORE ChemStation software (Agilent).

Western Blot. Heat-shock induction with the same *hs-Gal4* driver and Western blotting was performed as described previously (3). In brief, flies in glass vials were immersed in a 37 °C water bath for 30 min two times with a 30-min interval at 25 °C for recovery between heat shocks. Flies were heat shocked once every day for 4 d and killed on the fifth day. Protein samples were extracted from homogenized head tissues in an SDS buffer (3% SDS, 10% glycerol, 65 mM Tris·HCl at pH 6.8, 5.25% 2-mercaptoethanol, 0.2 mM PMSF, 1 mg/mL aprotinin, 10 mg/mL chymostatin, 2 mg/mL leupeptin, 1 mg/mL pepstatin). Lysate proteins (20 mg per well) were electrophoresed on a 12% SDS/PAGE and then were electroblotted onto PVDF membranes. Immobilized proteins were probed with rat anti-DDC (1:20,000 dilution) or mouse anti–β-tubulin (1:20,000 dilution) (Sigma), incubated with HRP-conjugated goat anti-rat or goat anti-mouse IgG secondary antibody (1:10,000 dilution) (Invitrogen), and then visualized with a chemiluminescence kit (Pierce).

1. Tully T, Quinn WG (1985) Classical conditioning and retention in normal and mutant *Drosophila melanogaster*. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* 157: 263–277.
2. Tully T, Preat T, Boynton SC, Del Vecchio M (1994) Genetic dissection of consolidated memory in *Drosophila*. *Cell* 79:35–47.
3. Xia S, et al. (2005) NMDA receptors mediate olfactory learning and memory in *Drosophila*. *Curr Biol* 15:603–615.
4. Hardie SL, Hirsh J (2006) An improved method for the separation and detection of biogenic amines in adult *Drosophila* brain extracts by high performance liquid chromatography. *J Neurosci Methods* 153:243–249.

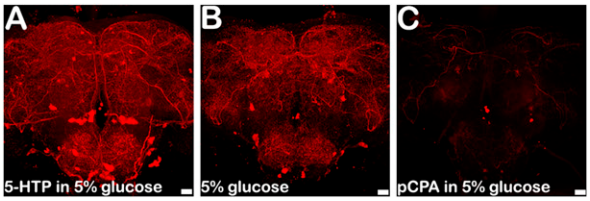


Fig. S1. Pharmacological effects on 5HT levels indicated by 5HT-antibody immunohistochemistry in the whole-mount representative brains. (*A*) Flies fed 2 mg/mL 5HTP in 5% glucose overnight. (*B*) Flies fed 5% glucose overnight. (*C*) Flies fed 10 mg/mL pCPA in 5% glucose for 3 d. Confocal images were taken under the detection sensitivity optimized for the control. Immunoreactive signals were elevated after 5HTP feeding and reduced after pCPA feeding as compared with controls with only glucose feeding. (Scale bars: 20 μm.)

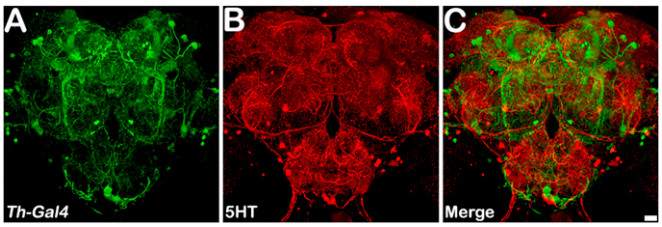


Fig. S2. Absence of 5HT in tyrosine hydroxylase (TH) neurons in flies fed with 5HTP. (*A*) The expression pattern of *Th-Gal4* in the adult brain. (*B*) 5HT-antibody immunolabeling. (*C*) Merged image showing that all TH neurons are 5HT-antibody immunonegative. Brain samples were derived from day-3 flies carrying *mCD8::GFP*; *Th-Gal4/mCD8::GFP* transgenes and fed with 2 mg/mL 5HTP overnight. (Scale bar: 20 μm.)

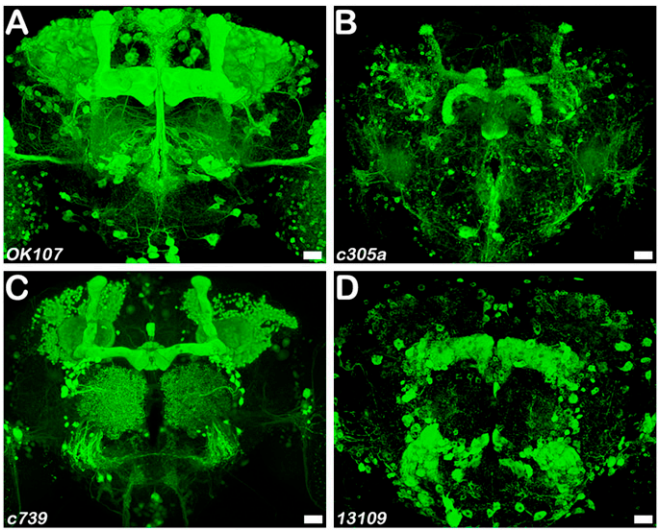


Fig. S3. GFP expression patterns of *Gal4* drivers preferentially expressed in subsets of mushroom body (MB) neurons. (*A*) *OK107-Gal4* for all MB neurons. (*B*) *c305a-Gal4* for α′/β′ neurons. (*C*) *c739-Gal4* for α/β neurons. (*D*) *13109-Gal4* for γ neurons. Note that each of these widely used *Gal4* drivers, although relatively specific for the manipulation of subsets of MB neurons, expresses in many other neurons outside MBs. All images are frontal views. (Scale bars: 20 μm.)

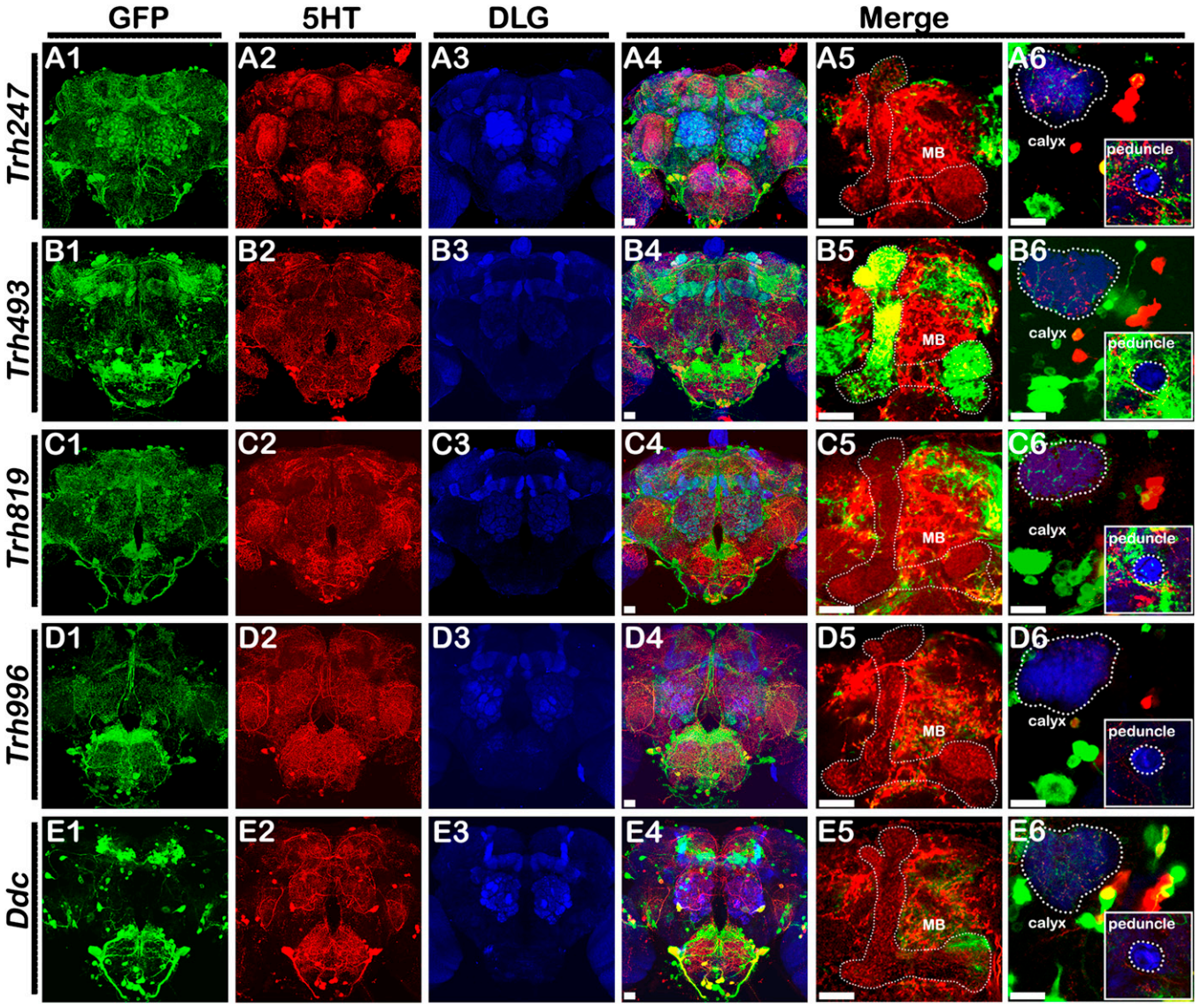


Fig. S4. The expression pattern of serotonergic *Gal4* drivers. (*A*) *Trh247-Gal4*. (*B*) *Trh493-Gal4*. (*C*) *Trh819-Gal4*. (*D*) *Trh996-Gal4*. (*E*) *Ddc-Gal4*. Most but not all Gal4 neurons (green in *A1*–*E1*) are 5HT-antibody immunopositive (red in *A2*–*E2*) as indicated by double labeling (yellow in *A4*–*E4*). Brain structures are counterstained with DLG-antibody immunostain (blue in *A3*–*E3*). Close-up views (*A5*–*E6*) show that only *Trh493-Gal4* (*B5*) contains GFP^+ (green) and 5HT-immunoreactive (red) fibers in the MB lobes (dashed outlines). Each close-up image is a projection of several neighboring optical slices through MB lobes (*A5*–*E5*), calyx, or peduncle (*A6*–*E6*). *Ddc-Gal4* (*E5*) also has GFP^+ fibers in the distal part of the β' lobes (green), but these fibers are 5HT immunonegative. Some 5HT-immunoreactive fibers appear in the calyx and are labeled by GFP expressed in *Trh247-Gal4* (*A6*), *Trh819-Gal4* (C6), and *Ddc-Gal4* (*E6*) but not in *Trh493-Gal4* (*B6*). *Trh996-Gal4* does not express in the calyx (*D6*). These 5HT-immunoreactive fibers in the calyx are derived from the contralaterally projecting 5HT-immunoreactive deutocerebral interneurons (1). *Trh819-Gal4* also contains some GFP^+ fibers in the peduncle. (Scale bars: 20 μm.)

1. Roy B, et al. (2007) Metamorphosis of an identified serotonergic neuron in the *Drosophila* olfactory system. *Neural Dev* 2:20.

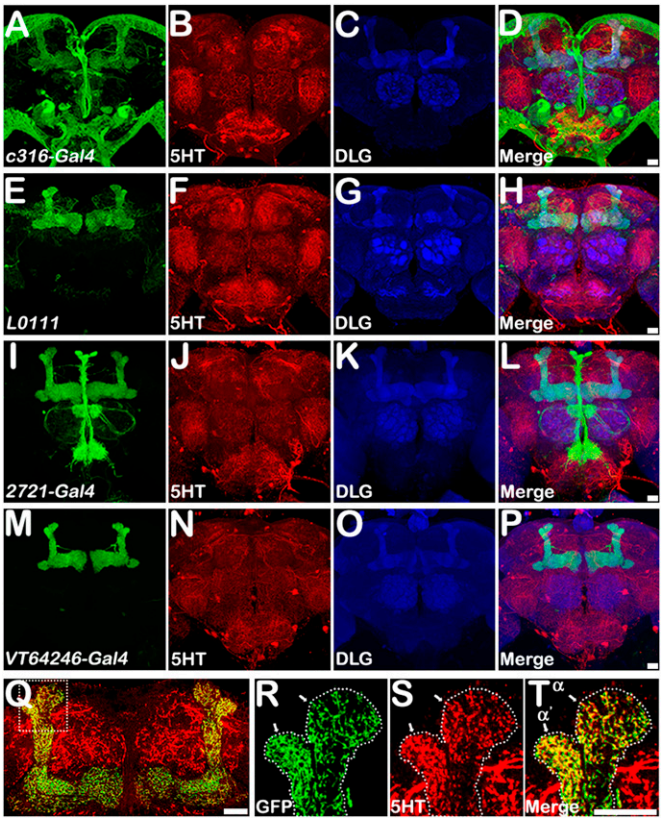


Fig. S5. Spatial distribution of 5HT-antibody immunoreactive signals in the MB: (*A–D*) *c316-Gal4*. (*E–H*) *L0111-LexA*, (*I–L*) *2721-Gal4*, (*M–P*) *VT64246-Gal4*. Expression patterns were reported by *mCD8::GFP*. *VT64246-Gal4* shows no detectable expression elsewhere in the brain (*M*). Brains are immunostained with anti-5HT and anti-DLG antibodies. (*Q*) High-resolution imaging of optical sections through MB lobes of *VT64246-Gal4*. (*R–T*) Close-up views of the projection of four neighboring slices at the tips of α/α′ lobes (arrows) demonstrate that 5HT-antibody immunoreactive spots (*S*) are all localized on the DPM fibers (*R* and *T*). Green, GFP; red, 5HT-antibody immunoreactive signals; yellow, double labeling. (Scale bars, 20 μm.)

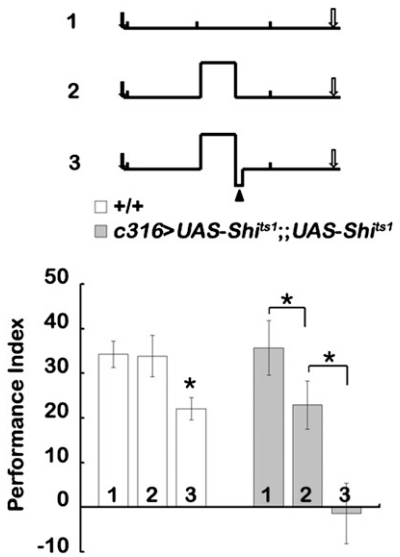


Fig. S6. An additive effect of neurotransmission from DPM neurons and cold-induced anesthesia for 3-h memory. *c316-Gal4* was used as a driver to test the roles of serotonergic neurons on ARM formation after neurotransmission output was blocked at 31 °C. (*Upper*) Cold-induced anesthesia was performed for discrimination of ASM and ARM. In temperature-shift schemes 1–3, dark arrows indicate start training, open arrows indicate testing time, and the arrowhead indicates cold-shock time. (*Lower*) The mean ± SEM was plotted for each treatment. $n = 8$ values for each group. $^*P < 0.05$.

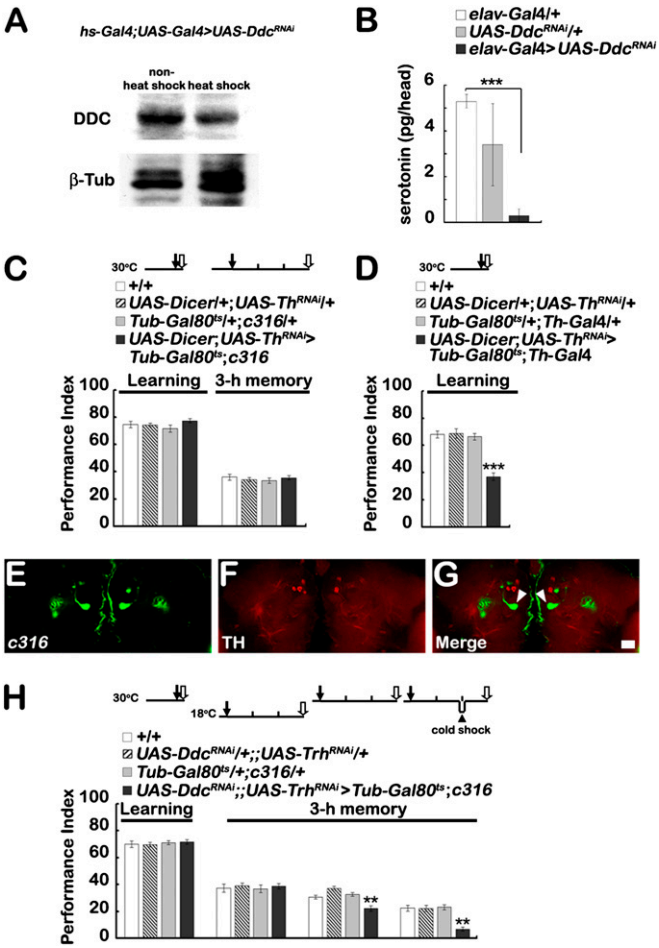


Fig. S7. The effectiveness of Ddc^{RNAi} for down-regulation of DDC and 5HT production. (*A*) Western blot showing reduced amount of DDC protein in the fly head after heat-shock induction of Ddc^{RNAi} in flies carrying *UAS-*Ddc^{RNAi}*/+;hs-Gal4/+;UAS-Gal4/+* transgenes. DDC and β-tubulin were detected by DDC-antibody and β-tubulin–antibody immunostaining, respectively. β-Tubulin was used as an internal control. (*B*) Reduced 5HT level by RNAi mediated knockdown of DDC. The amount of 5HT in head extracts was measured by the *HPLC-FLD* method. The 5HT level was reduced significantly in flies carrying *elav-Gal4>UAS-*Ddc^{RNAi} transgenes as compared with control flies carrying *elav-Gal4/+* or *UAS-*Ddc^{RNAi}*/+* transgenes. ***$P < 0.001$ (student's *t* test). $n = 7$ for each test. (*C*) Normal learning and 3-h memory after temporal down-regulation of TH with Th^{RNAi} in the *c316-Gal4* neurons. (*D*) Impaired learning after temporal expression of Th^{RNAi} in the *Th-Gal4* neurons. (*E–G*) DPM neurons are TH-antibody immunonegative. (*E*) The expression pattern of *UAS-mCD8::GFP;c316-Gal4*. (*F*) TH-antibody immunostaining. (*G*) The projection shows cell bodies of DPM neurons (arrowheads) and several other neighboring TH-immunoreactive neurons (red) in flies carrying *UAS-mCD8::GFP;c316-Gal4* transgenes. (Scale bar: 20 μm.) (*H*) Impaired 3-h memory after temporal expressions of Ddc^{RNAi} and Trh^{RNAi} in the *c316-Gal4* neurons. $Gal80^{ts}$ inhibition was removed in flies kept at 30 °C for 5 d before training. Control flies always were kept at 18 °C. Cold shock (arrowhead) was performed 2 h after training. Black arrows indicate start of training. Open arrows indicate testing time. The mean ± SEM was plotted for each genotype; $n = 8$ values for each group. **$P < 0.01$, ***$P < 0.001$.

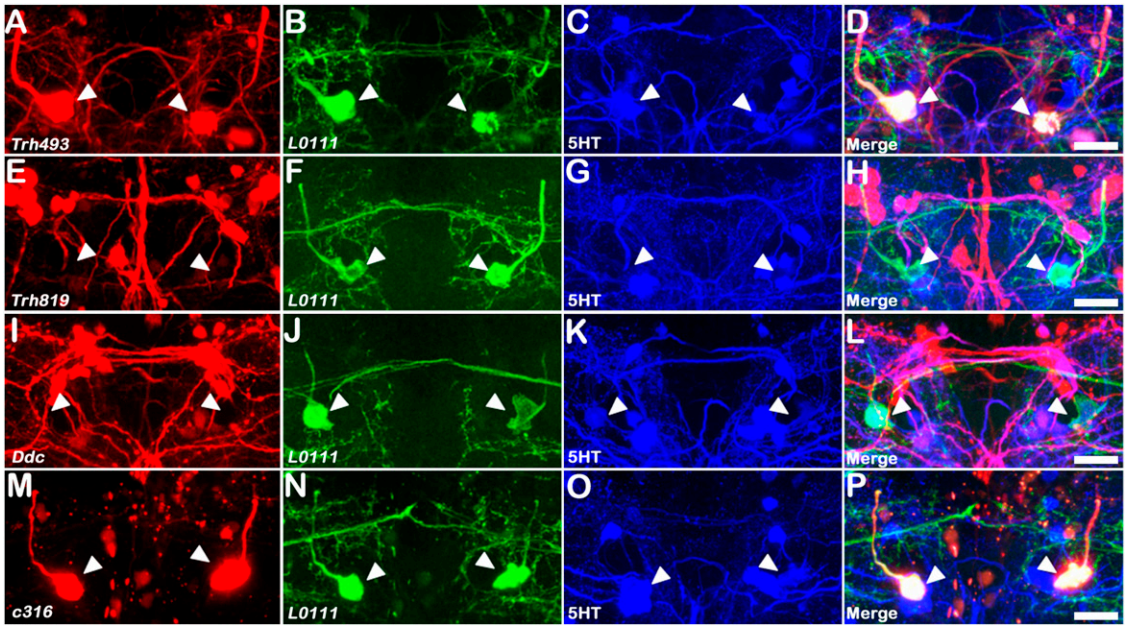
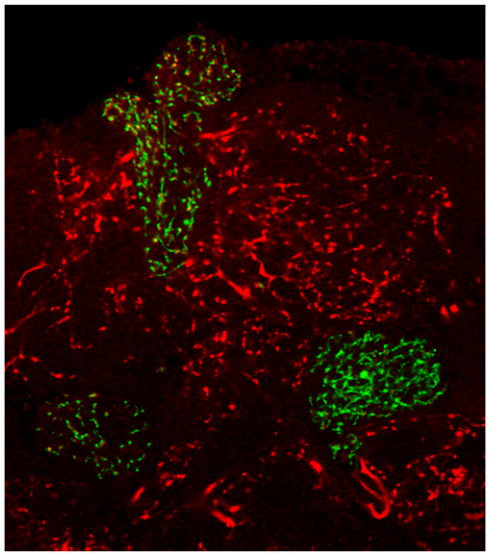


Fig. S8. *Trh-Gal4s* may or may not express in the two serotonergic DPM neurons. *L0111-LexA* (green) was used as a reference to examine if the same DPM neurons also expressed in the following *Gal4* drivers: (*A*) *Trh493-Gal4*; (*E*) *Trh819-Gal4*; (*I*) *Ddc-Gal4*; and (*M*) *c316-Gal4*. 5HT-antibody immunostaining (*C*, *G*, *K*, *O*) confirmed that *Trh493-Gal4* and *c316-Gal4* contained the serotonergic DPM neurons that were not present in *Trh819-Gal4* and *Ddc-Gal4*. Each image is a projection of several neighboring optical slices through the soma of the two DPM neurons. Arrowheads indicate the cell body of the DPM neuron. The expression patterns were reported by *UAS-mKO* (*A* and *M*), *LexAop-GFP (B, F, J, N)*, or *UAS-DsRed* (*E* and *I*). (Scale bars: 20 μm.)

Table S1. Number of serotonergic neurons in protocerebrum and suboesophageal ganglion

Gal4 driver	GFP neurons	5HT neurons	GFP and 5HT neurons
Trh247	260.0 ± 24.3	41.7 ± 1.5	27.3 ± 1.5
*Trh493**	280.0 ± 14.6	59.7 ± 2.3	25.3 ± 1.8
Trh819	317.3 ± 56.0	55.7 ± 4.7	34.7 ± 3.3
Trh996	177.0 ± 1.5	50.3 ± 2.4	30.0 ± 1.7
Ddc	277.3 ± 16.9	54.3 ± 4.1	34.0 ± 1.5

Gal4 expression patterns were reported by *UAS-mKO,UAS-mKO;UAS-nls::GFP*. The numbers of GFP^+ nuclei and 5HT-antibody immunoreactive neurons were counted automatically in Amira software after a landmark sphere was placed manually on each nucleus. The mean ± SEM was determined for each genotype; $n = 3$ values for each group.
*The expression contains the two DPM neurons.



Movie S1. Spatial relationship between the distributions of 5HT-antibody immunoreactive signals and DPM fibers in the MB. Series of optical sections are presented from posterior (DPM cell body) to anterior (DPM fibers) in the right MB. In the MB lobes, all 5HT-antibody immunoreactive signals are localized on the DPM neurons. The brain sample is the same as shown in Fig. S5 *Q–T*. Green, GFP; red, 5HT-antibody immunoreactive signal; yellow, double labeling. Genotyping: UAS-mCD8::GFP/+; VT64246-Gal4/ UAS-mCD8::GFP.

Movie S1