

# Serotonin–mushroom body circuit modulating the formation of anesthesia-resistant memory in *Drosophila*

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**Pavlovian olfactory learning in *Drosophila* produces two genetically distinct forms of intermediate-term memories: anesthesia-sensitive memory, which requires the *amnesiac* gene, and anesthesia-resistant memory (ARM), which requires the *radish* gene. Here, we report that ARM is specifically enhanced or inhibited in flies with elevated or reduced serotonin (5HT) levels, respectively. The requirement for 5HT was additive with the memory defect of the *amnesiac* mutation but was occluded by the *radish* mutation. This result suggests that 5HT and Radish protein act on the same pathway for ARM formation. Three supporting lines of evidence indicate that ARM formation requires 5HT released from only two dorsal paired medial (DPM) neurons onto the mushroom bodies (MBs), the olfactory learning and memory center in *Drosophila*: (i) DPM neurons were 5HT-antibody immunopositive; (ii) temporal inhibition of 5HT synthesis or release from DPM neurons, but not from other serotonergic neurons, impaired ARM formation; (iii) knocking down the expression of d5HT1A serotonin receptors in  $\alpha/\beta$  MB neurons, which are innervated by DPM neurons, inhibited ARM formation. Thus, in addition to the Amnesiac peptide required for anesthesia-sensitive memory formation, the two DPM neurons also release 5HT acting on MB neurons for ARM formation.**

brain | olfaction | aversive conditioning | neurotransmitter

Pavlovian olfactory learning in *Drosophila* involves coincidence detection of a conditioned stimulus (CS), an odor, and an unconditioned stimulus (US), an electric shock (1). After one session of aversive olfactory conditioning, flies can form short- and intermediate-term memories but not long-term memory (LTM), which requires repetitive spaced training and is dependent on protein synthesis (2, 3). The intermediate-term memory has been dissected further into an anesthesia-sensitive form (ASM) that requires a gene called “*amnesiac*” and an anesthesia-resistant form (ARM) that requires the *radish* gene (4–6). Three hours after one session of training, ARM and ASM contribute equally to performance and can be distinguished by application of a short cold shock-induced anesthesia that impairs ASM but not ARM. Importantly, although ARM is consolidated, in the sense that it is resistant to cold shock, it is not thought to be a protein synthesis-dependent memory because it is resistant to cycloheximide (CXM) (2). One day after repetitive spaced training, both ARM and LTM are thought to contribute to memory performance. In contrast, repetitive massed training without rest intervals induces only *radish*-dependent ARM without detectable cAMP response element binding protein-dependent LTM measured 1 d after training (2, 3) (but see ref. 2 for an alternative model). Thus, more than one genetically distinct memory-storage system contributes to performance both at intermediate time points (e.g., 3 h after one training session) and at later time points (e.g., 24 h after repetitive training). Given the complexity of memory consolidation observed at the genetic level, much effort has been directed to-

ward attempts to integrate conceptually the genetically defined memory phases with biochemical signaling and neural circuits.

In adult *Drosophila*, each of the paired mushroom bodies (MBs) consists of ~2,500 intrinsic Kenyon cells whose segregated dendritic fields in the calyx receive olfactory information from the antennal lobes (8–10). Based on birth timing and axon configurations, Kenyon cells fall into three major classes: the early-born  $\gamma$  neurons forming a single medial lobe;  $\alpha'/\beta'$  neurons branching into a vertical  $\alpha'$  and a medial  $\beta'$  lobe; and the late-born  $\alpha/\beta$  neurons branching into a vertical  $\alpha$  and a medial  $\beta$  lobe (11, 12). A coherent picture has begun to emerge in which CS and US information converges on MBs, and coincidence detection within the MBs leads to the plasticity that underlies learning (13–15). Formation of aversive olfactory memory requires normal expression of many genes in the MBs (13), including the *rutabaga* gene encoding  $\text{Ca}^{2+}$ -responsive adenylyl cyclase (16). Importantly, two dorsal paired medial (DPM) neurons that innervate all MB lobes are believed to release the Amnesiac peptide required for modulation of the cAMP-signaling pathway in the MB neurons (17). In contrast, it remains unknown which presynaptic neurons and which neurotransmitter modulate ARM formation, although blocking MB  $\alpha/\beta$  and  $\gamma$  output after learning abolishes retrieval of the intermediate-term memory which includes both ASM and ARM (18–21).

We investigated the role of serotonin (5HT), a monoamine neurotransmitter synthesized from the amino acid L-tryptophan by a metabolic pathway, with two enzymes: tryptophan hydroxylase and amino acid decarboxylase (DDC) (22). In mammals, 5HT and its receptors play a key role in memory formation through modulation of enzymes including the cAMP-dependent protein kinase, the  $\text{Ca}^{2+}$  calmodulin-dependent protein kinase II, and MAPKs (23). 5HT also modulates glutamate, GABA, and acetylcholine which is involved in various aspects of cognitive functions including learning and memory (24). In *Aplysia*, when 5HT application is coupled with synaptic activity, PKC is activated, and a form of intermediate-term facilitation is induced that lasts for hours after stimulation (25). In *Drosophila*, four types of 5HT receptors have been reported: d5HT1A, d5HT1B, d5HT2,

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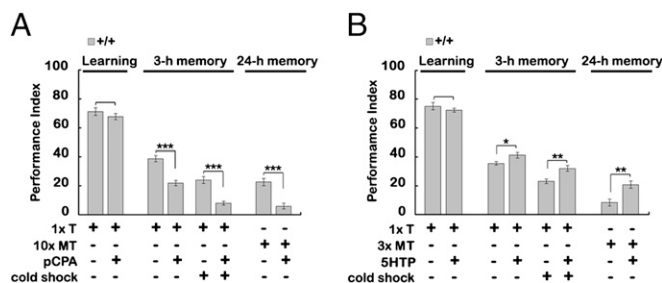
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and d5HT7 (26). d5HT1B, expressed in the eight clock neurons, is required for normal entrainment of circadian rhythm (27), and d5HT1A, expressed in the MB, is required for normal sleep (28). Because of the broad serotonergic innervations and differential expression of the distinct 5HT receptors, it is unclear how 5HT modulates specific behavioral functions. With genetic tools for spatiotemporal manipulation, we found that two DPM neurons synthesize and release 5HT acting via d5HT1A receptors on the  $\alpha/\beta$  neurons for ARM formation without affecting learning, ASM, or LTM.

## Results

**5HT Is Required for ARM Formation.** To address whether 5HT is required for aversive olfactory associative memory in *Drosophila*, we started by manipulating 5HT levels pharmacologically. Feeding wild-type flies with DL-p-chlorophenylalanine (pCPA), an inhibitor of 5HT synthesis (29), produced a significant suppression of intermediate-term memory, measured 3 h after training, without affecting immediate short-term memory, measured 2 min after training (Fig. 1A). This memory suppression predominantly impacted ARM, because cold-induced anesthesia that abolishes ASM but not ARM could suppress memory further in the pCPA-fed flies. The specificity of the 5HT effect on ARM was strengthened further by the result that pCPA impaired 24-h memory after massed training that produced only ARM. In contrast, feeding wild-type flies with L-5-hydroxytryptophan (5HTP), a 5HT precursor (30), enhanced intermediate-term memory without affecting learning after only one session of olfactory conditioning (Fig. 1B). Again, cold-induced anesthesia did not alter the enhancement of intermediate-term memory, suggesting that ARM rather than ASM was enhanced by 5HTP. The 5HT-dependent ARM enhancement was confirmed again using a shortened massed training protocol that induced lower ARM formation 1 d after learning. The effectiveness of pharmacological manipulation was verified by 5HT-antibody immunohistochemistry showing an elevated immunoreactive signal in the central brain after 5HTP feeding and a reduced immunoreactive signal after pCPA feeding (Fig. S1A–C). Also, 5HT immunolabeling showed that the elevated 5HT immunoreactive signal did not occur in tyrosine hydroxylase (TH) neurons (Fig. S2), excluding the possibility of ectopic serotonin synthesis in dopamine neurons caused by 5HTP feeding as reported in larval CNS (31). Together, through manipulation of 5HT levels, our results suggest that 5HT is required specifically for the formation of ARM and not for the acquisition of ASM.

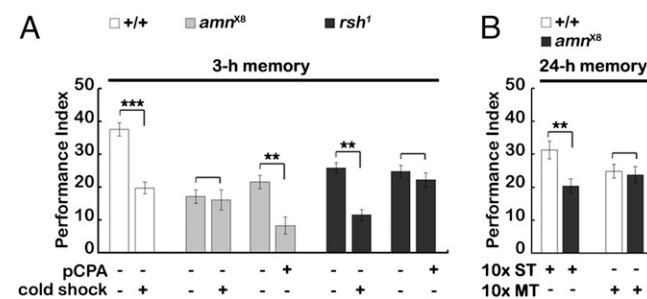


**Fig. 1.** Effects of pharmacological manipulation of 5HT level on aversive olfactory memory. (A) Learning and memories in flies fed with pCPA, a tryptophan hydroxylase inhibitor. (B) Learning and memories in flies fed with 5HTP, a 5HT precursor. Flies were subjected to 1 session of training (1x T), 3 sessions of massed training (3x MT), 3 sessions of spaced training (3x ST), or 10 sessions of massed training (10x MT). Memory was measured immediately (Learning), 3 h, or 24 h after training. Cold-induced anesthesia was performed 2 h after training. The mean  $\pm$  SEM was plotted for each treatment;  $n = 8$ –10 values for each group. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

**Reduced 5HT Synthesis Impairs ARM Similar to the Effect of *radish* Mutant.** Cold-induced anesthesia during memory consolidation blocks ASM formation and is dependent on *amnesiac* gene expression in the two DPM neurons. In contrast, Amnesiac protein is not required for ARM formation, which instead is dependent on the *radish* gene (6, 17, 32, 33), whose product is expressed in MB (33). To address whether 5HT and Radish protein are part of the same pathway for ARM formation, we performed the following experiments (Fig. 2). We first confirmed that 3-h memory impairment after cold-induced anesthesia was reduced further in the *radish* mutant (*rsh*<sup>1</sup>) but not in the *amnesiac* mutant (*amn*<sup>X8</sup>) (Fig. 2A). In contrast, reducing 5HT levels by pCPA feeding reduced 3-h memory in *amn*<sup>X8</sup> but had no effect in *rsh*<sup>1</sup>, suggesting that 5HT and Radish protein are in the same pathway for ARM formation (Fig. 2A). Furthermore, 24-h memory in *amn*<sup>X8</sup> was impaired after 10 sessions of spaced training but not after massed training, suggesting that ARM remains intact in *amn*<sup>X8</sup> (Fig. 2B).

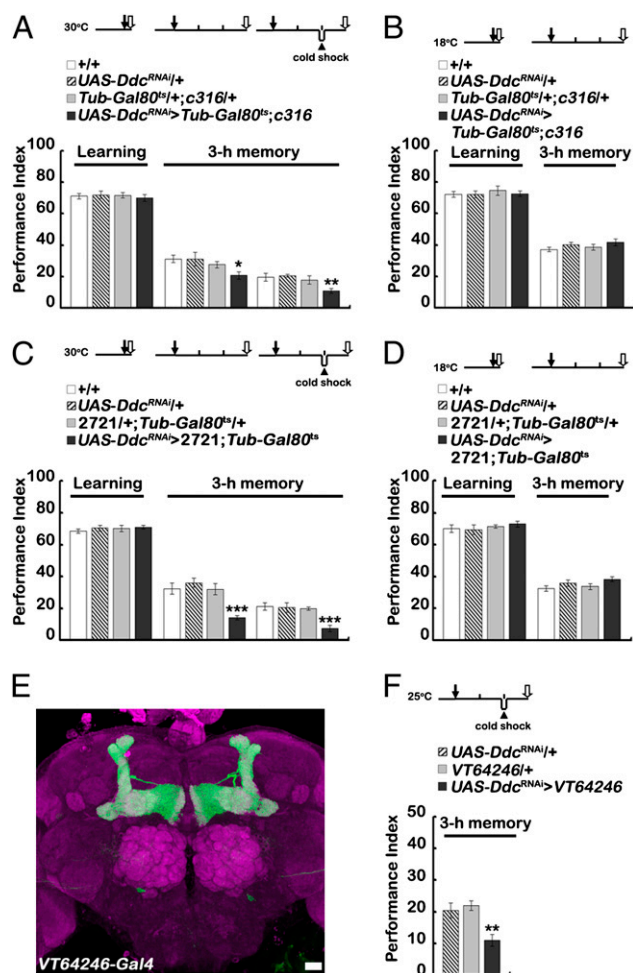
**5HT Acts on  $\alpha/\beta$  Neurons for ARM Formation.** Serotonergic neurons project to most brain regions in *Drosophila* (34). Both Radish protein (33) and the d5HT1A serotonin receptors are expressed in the MBs (28), prompting us to examine whether 5HT acts on MB neurons for ARM formation. We addressed this question by RNAi-mediated down-regulation of specific 5HT receptors in all or subsets of MB neurons (Fig. S3). Using *OK107-Gal4* to drive expression in the whole MB, we found that normal expression of the d5HT1A receptor but not the d5HT1B receptor in the MB neurons is necessary for the formation of 3-h memory (Fig. 3A). Next, by knocking down the expression of the d5HT1A receptor locally, we found that it was required only in the  $\alpha/\beta$  neurons (c739), but not in the  $\alpha'/\beta'$  neurons (c305a) or  $\gamma$  neurons (13109), for normal 3-h memory (Fig. 3B). To minimize any developmental contribution, we confirmed the necessity of d5HT1A in  $\alpha/\beta$  neurons by temporally regulated reduced expression in adults with *Gal80<sup>ts</sup>* (Fig. 3C) after showing that control flies kept at low temperature had normal formation of 3-h memory. Finally, cold-induced anesthesia further suppressed 3-h memory caused by down-regulation of the d5HT1A receptor in  $\alpha/\beta$  neurons (Fig. 3C), an effect similar to that seen in reducing 5HT levels (Fig. 1A) or in the *radish* mutant (Fig. 2). Thus, these data suggest that 5HT and its d5HT1A receptors acts via the Radish pathway for ARM formation in the  $\alpha/\beta$  neurons.

**Two DPM Neurons Are Serotonergic.** Next, we asked which neurons release 5HT to modulate ARM formation in the  $\alpha/\beta$  neurons. To manipulate specific subsets of serotonergic neurons, we used five different *Gal4* drivers with preferential expression in serotonergic neurons: *Trh247-Gal4*, *Trh493-Gal4*, *Trh819-Gal4*, *Trh996-Gal4*



**Fig. 2.** Effects of pCPA feeding in *amn*<sup>X8</sup> and *rsh*<sup>1</sup> mutants. (A) Effects on 3-h memory after a single session of training. Cold-induced anesthesia was performed 2 h after training. (B) Effects on 24-h memory after 10 sessions of spaced training (10x ST) or massed training (10x MT). The mean  $\pm$  SEM was plotted for each treatment;  $n = 8$  values for each group. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .





**Fig. 5.** The role of 5HT synthesis in DPM neurons in learning and memory. (A) Effects of temporal expression of *Ddc<sup>RNAi</sup>* in the *c316-Gal4* neurons. (B) Control *c316-Gal4* flies kept at 18 °C have normal learning and 3-h memory. (C) Effects of postdevelopmental temporal induction of *Ddc<sup>RNAi</sup>* in the *2721-Gal4* neurons. (D) Control *2721-Gal4* flies kept at 18 °C have normal learning and 3-h memory. (E) *VT64246-Gal4* expression pattern reported by *UAS-mCD8::GFP* (green). Brain structure was counterstained with Discs large-antibody immunostain (magenta). (Scale bar: 20  $\mu$ m.) (F) Impairment of 3-h memory by driving *Ddc<sup>RNAi</sup>* expression in *VT64246-Gal4* and cold shock. Black arrows indicate the start of training. Open arrows indicate testing time. The arrowhead indicates cold-shock time. The mean  $\pm$  SEM was plotted for each genotype;  $n = 8$ –15 values for each group. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

DDC also is required for dopamine synthesis, but several lines of evidence indicate that *Ddc<sup>RNAi</sup>* specifically down-regulates serotonin production in DPM neurons. First, the effectiveness of the *Ddc<sup>RNAi</sup>* after heat-shock induction was confirmed by Western blot showing a reduced amount of DDC (Fig. S7A). Second, serotonin production was reduced significantly in flies carrying *elav-Gal4*>*UAS-Ddc<sup>RNAi</sup>* transgenes (Fig. S7B). Third, *Th<sup>RNAi</sup>*-induced down-regulation of TH mRNA encoding another enzyme required for dopamine synthesis in the DPM neurons did not affect learning and 3-h memory (Fig. S7C). The effectiveness of *Th<sup>RNAi</sup>* was confirmed by its expression in *Th-Gal4* neurons, leading to impaired olfactory learning (Fig. S7D). Fourth, DPM neurons were immunonegative for antibody staining of TH (Fig. S7E–G). Fifth, spatial specificity of the requirement of DDC in the DPM neuron for normal ARM was confirmed by driving *Ddc<sup>RNAi</sup>* with *VT64246-Gal4*, a DPM-specific *Gal4* driver (Fig. 5 E and F). Finally, by temporal induction after development of both *Ddc<sup>RNAi</sup>* and *Trh<sup>RNAi</sup>*

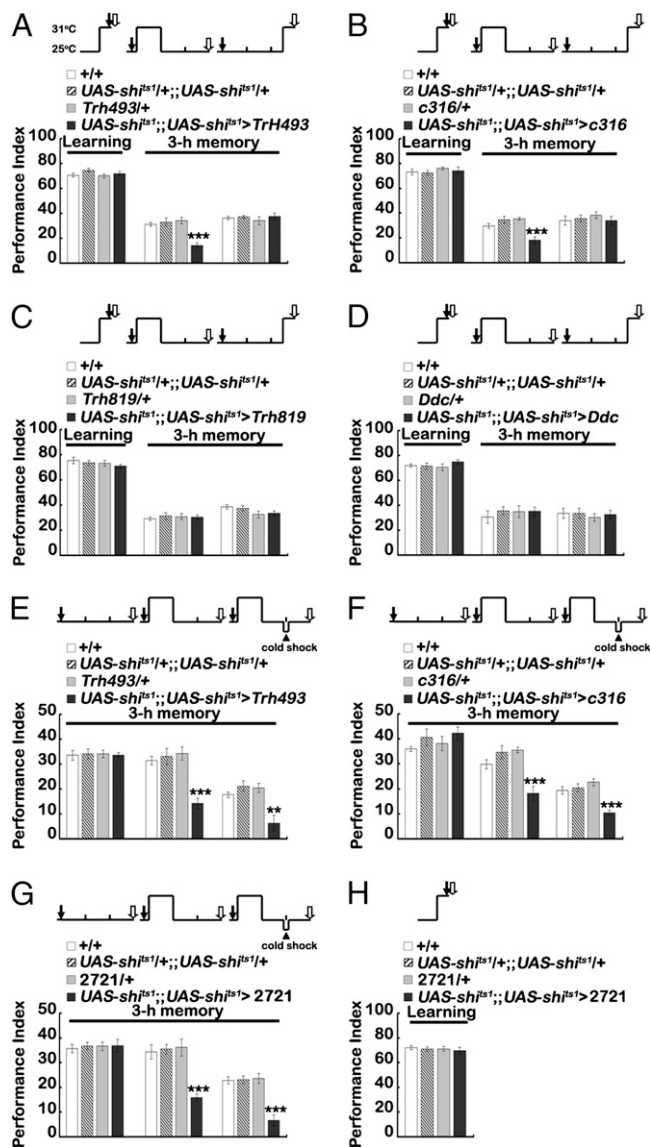
in the *c316-Gal4* neurons, we confirmed again that 5HT from DPM neurons is required for ARM formation (Fig. S7H).

**Other Serotonergic Neurons Are Not Involved in ARM Formation.** Multiple *Gal4* drivers expressed in different subsets of serotonergic neurons provide an opportunity to address whether other non-DPM serotonergic neurons also are required for aversive olfactory memory formation. Using *L0111-LexA* as a reference for labeling the two DPM neurons, we first confirmed that *c316-Gal4*, *L0111-LexA*, and *Trh493-Gal4* contained the same DPM neurons that were not labeled by *Trh819-Gal4* and *Ddc-Gal4* (Fig. S8). By blocking neurotransmission with *UAS-shi<sup>ts1</sup>* at the restrictive temperature (37), we found that outputs from *Trh493-Gal4* and *c316-Gal4* neurons were required during memory consolidation but were not required during learning or retrieval of 3-h memory (Fig. 6 A and B) (38–40). In contrast, blocking neurotransmission from *Trh819-Gal4* and *Ddc-Gal4* neurons did not affect learning, consolidation, or retrieval of 3-h memory (Fig. 6 C and D). These results suggest that only the two DPM neurons, and not any other serotonergic neurons expressed in *Trh819-Gal4* and *Ddc-Gal4*, are required for 3-h memory.

Next, we asked if blocking neurotransmission from DPM neurons specifically impairs ARM rather than ASM. We used three independent *Gal4* drivers with preferential DPM expression. In each case, we measured 3-h memory performance after blocking neurotransmission from DPM neurons with *shi<sup>ts1</sup>* immediately after training for 1 h. In each case, we observed the expected decrement in 3-h memory performance. Also, in each case, performance was reduced further by blocking ASM via application of cold-induced anesthesia 2 h after training (Fig. 6 E–G). The outputs from *2721-Gal4* were not required during the learning assay (Fig. 6H), consistent with the original reports of DPM cell temporal requirements (17). These results are consistent with the effects of manipulation of 5HT biosynthesis, d5HT1A receptor expression, and the *radish* gene (Figs. 1–3). Together, these convergent data indicate that the two DPM neurons are essential for both ASM and ARM, and are the only serotonergic neurons required for ARM formation.

## Discussion

**5HT in Memory Formation.** The key finding of our study is that 5HT from the two DPM neurons innervating all MB lobes is required specifically for ARM formation in aversive olfactory conditioning in *Drosophila*. This conclusion is supported by five independent lines of evidence. First, pharmacological inhibition of 5HT synthesis reduces memory formation that is additive to impaired ASM (i.e., with the *amnesiac* mutant or in flies subjected to cold-induced anesthesia) but is occluded in the *radish* mutant in which ARM already is abolished (Figs. 1A and 2). Second, pharmacological increase of 5HT enhances memory formation that is unaffected by cold-induced anesthesia (Fig. 1B). Third, the two DPM neurons are immunoreactive to 5HT (Fig. 4) but not to TH (Fig. S7E–G). Fourth, temporal reduction of 5HT synthesis in the DPM neurons specifically abolishes ARM (Fig. 5). Fifth, blocking neurotransmission from the DPM neurons but not other serotonergic neurons specifically abolishes ARM without affecting acquisition or retrieval (Fig. 6 and Fig. S8). Although it also has been suggested that DPM neurons are cholinergic, because *Cha<sup>3.3kb</sup>-Gal80* suppresses *c316-Gal4* expression (38), further validation by more reliable methods such as immunostaining before and after down-regulation by *Cha<sup>RNAi</sup>* is necessary. Our results show that 5HT from the DPM neurons is the primary neurotransmitter involved in ARM formation because *Ddc<sup>RNAi</sup>* inhibition of 5HT synthesis or blocking neurotransmission with the *UAS-shi<sup>ts1</sup>* transgene results in a similar degree of memory impairment that is damaged further by cold-induced anesthesia (Figs. 5 and 6 and Fig. S6). On the other hand, 5HT is necessary for *Drosophila* place memory, as evidenced by genetic manipulation of the 5HT level and neuro-



**Fig. 6.** Effects of DPM neuron outputs on ARM. (A) *Trh493-Gal4*, (B) *c316-Gal4*, (C) *Trh819-Gal4*, and (D) *Ddc-Gal4* were used as drivers to express *UAS-shi<sup>ts1</sup>* for temporal manipulation of serotonergic outputs. Blocking neurotransmission from *Trh493-Gal4* neurons or *c316-Gal4* neurons impairs 3-h memory during formation but not during learning or retrieval. The same manipulation on *Trh819-Gal4* neurons or *Ddc-Gal4* neurons has no effect. (E) *Trh493-Gal4*, (F) *c316-Gal4*, and (G) *2721-Gal4* were used as drivers to express *UAS-shi<sup>ts1</sup>* in DPM neurons. (H) Blocking neurotransmission from *2721-Gal4* neurons does not affect learning. Dark arrows indicate start training. Open arrows indicate testing time. The arrowhead indicates cold-shock time. The mean  $\pm$  SEM was plotted for each genotype;  $n = 8$  values for each group. \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

transmission from the *Ddc-Gal4*-positive neurons (41). Whether the two serotonergic DPM neurons are involved in place memory requires further examination, because *Ddc-Gal4* does not express in the DPM neurons (Figs. S4 and S8).

**ARM Circuit.** Three lines of evidence suggest that ARM forms within the MB  $\alpha/\beta$  neurons. First, ARM formation requires normal expression of d5HT1A only in the  $\alpha/\beta$  neurons (Fig. 3). Second, Radish protein required for ARM formation is expressed preferentially in the  $\alpha/\beta$  and  $\gamma$  neurons, but not in the  $\alpha'/\beta'$  neurons (33). Third, ARM retrieval requires neurotransmission from  $\alpha/\beta$

neurons (7). Although the molecular basis for ARM formation is largely unknown, ARM formation generally is believed not to require new protein synthesis and may involve posttranslational modifications of existing proteins (2, 3). Thus, far, two proteins have demonstrated roles in ARM formation in *Drosophila* aversive olfactory conditioning: Radish (6, 33) and a persistently active, truncated, atypical PKC, atypical protein kinase M (PKM), (42). Mutant *Radish* flies show normal learning but fail to form ARM. Atypical PKM appears to act downstream of Radish, because induction of atypical PKM can rescue the *radish* mutant phenotype (42). Persistent activation of PKCs during memory formation has been shown in other animals, including honey bee and *Aplysia* (43, 44). Together, these data indicate that, through activation of d5HT1A receptor, 5HT released from the DPM neurons sets Radish and atypical PKM in action to form ARM in the  $\alpha/\beta$  neurons.

**Spatiotemporal Control of Amnesiac and 5HT.** How does the DPM neuron regulate its release of the Amnesiac peptide and 5HT to modulate ASM and ARM formation? Previous studies have shown that the DPM neuron is responsive to both US and CS in aversive olfactory conditioning and exhibits a delayed memory trace dependent on the normal function of Amnesiac (36). Blocking DPM neuron output with the *UAS-shi<sup>ts1</sup>* transgene produces an effect similar to mutation of the *amnesiac* gene, one that abolishes half or more of the intermediate-term memory (38). In contrast, our findings combining *UAS-shi<sup>ts1</sup>* transgene expression and cold-induced anesthesia (Fig. 6) suggest that the temporal defect of the dominant negative *shi<sup>ts1</sup>* blocks 5HT release. However, rescue experiments clearly indicate a role for the *amnesiac* gene within the DPM cells. Amnesiac is a putative neuropeptide whose release probably would be impacted by *shi<sup>ts1</sup>* on a slower time scale. Indeed there is some evidence that longer-duration blocking of *shi<sup>ts1</sup>* in DPM neurons can ablate performance fully (39, 40), an effect that, by definition, includes both ASM and ARM.

It also is particularly interesting that formation of olfactory memory requires sequential involvement of different subsets of MB neurons (20). The early memory trace has been visualized only in the  $\alpha'/\beta'$  neurons in which neurotransmission outputs are required for memory acquisition and consolidation but not for retrieval (20, 45). Consistent with this notion, developmental disruption of DPM neurons, so that their fibers project mostly but not entirely to the  $\alpha'/\beta'$  lobes, produces significantly higher memory than the *amnesiac* mutant (39), suggesting that the Amnesiac peptide acts primarily on  $\alpha'/\beta'$  neurons. However, DPM neurons ramify not only in the  $\alpha'/\beta'$  lobes but throughout all MB lobes, and their memory traces show no evidence of MB lobe specificity (36). Together with our findings, these results suggest that DPM neurons modulate two memory mechanisms by release of Amnesiac and 5HT separately in both time and place: Amnesiac modulates an  $\alpha'/\beta'$  activity needed to support the plasticity in  $\gamma$  and  $\alpha/\beta$  neurons that underlies ASM, whereas 5HT acts on its receptor on  $\alpha/\beta$  neurons for ARM formation. Nevertheless, the presence of 5HT in all terminals of the DPM, as revealed by high-resolution 3D imaging (Fig. S5 Q–T), suggests that it plays additional functions in other MB lobes as well.

It has been suggested that in mammals 5HT and its receptors play key roles in psychosis, cognition, and mood via modulation of complex intracellular signaling pathways. During memory formation, activation of the 5HT1A receptor inhibits calcium channels, increases potassium conductance, inhibits adenylyl cyclase activity, reduces cAMP accumulation, and activates phospholipase C (24, 46). Interestingly, *radish* mutants with impaired ARM formation show attention-deficit and hyperactivity-like behavior that can be rescued by Ritalin, a drug used to treat human attention-deficit hyperactivity disorder that involves dysfunctions of the dopamine and 5HT systems (47, 48). Given the purported role of 5HT in depression and many other brain disorders that affect memory,

*Drosophila* ARM facilitated by only two serotonergic DPM neurons provides a simple reductionist model, with sophisticated genetic tools that will allow dissection of the interactions between different memory systems.

## Materials and Methods

**Fly Strains.** Fly stocks were raised on standard cornmeal-yeast-agar medium at 25 °C and 60–70% relative humidity under a 12/12-h light/dark cycle. *Ddc-Gal4*, *UAS-mCD8::GFP*, *Tub-Gal80<sup>ts</sup>*, *13109-Gal4* (BG00015), and *2721-Gal4* (GawB 5015) were obtained from Bloomington Stock Center. *Th-Gal4* was from *Drosophila* Genomics Resource Center. *UAS-d5HT1A<sup>RNAi</sup>*, *UAS-d5HT1B<sup>RNAi</sup>*, *UAS-Ddc<sup>RNAi</sup>*, *UAS-Trh<sup>RNAi</sup>*, and *UAS-Th<sup>RNAi</sup>* lines were from Vienna *Drosophila* RNAi Center. *UAS-Dicer* and *VT64246-Gal4* were from Barry Dickson. *LexAop-GFP* was from Tzumin Lee. *Trh247-Gal4* (III), *Trh493-Gal4* (III), *Trh819-Gal4* (III), and *Trh996-Gal4* (X) were generated by *Trh* and *Gal4* fusions, with the numbers indicating the remaining number of 5' flanking bases. *Trh-GAL4* deletions containing the indicated amount of 5' flanking DNA were constructed by primer mutagenesis starting with the *Trh-GAL4* construct. To achieve acute and temporal down-regulation of the expression of *Ddc*, *Trh*, *Th*, *d5HT1A*, or *d5HT1B* without causing significant developmental defect, flies carrying specific RNAi were raised at 18 °C until eclosion and then were transferred to 30 °C for 5 d before behavior assays. To block neurotransmission acutely, flies carrying the *UAS-shi<sup>ts1</sup>*; *UAS-shi<sup>ts1</sup>*

transgenes crossed with specific *Gal4* drivers were raised at 18 °C until eclosion and then were transferred to 25 °C for 3 d to allow sufficient Shibire protein expression before experiments were performed.

**Statistics.** Statistical analyses were performed using KaleidaGraph 4.1 (Synergy Software). Behavioral data were evaluated via one-way ANOVA followed by planned comparisons among the relevant groups with a Tukey Honestly Significant Difference test. All data are presented as mean ± SEM. \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001.

Details of cold shock and drug feeding, behavior assay, immunohistochemistry, quantitative measurement of serotonin, and Western blot are given in *SI Materials and Methods*.

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