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Increased dopaminergic signaling impairs aversive olfactory memory retention in *Drosophila*

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ABSTRACT

Dopamine is necessary for the aversive olfactory associative memory formation in *Drosophila*, but its effect on other stages of memory is not known. Herein, we studied the effect of enhanced dopaminergic signaling on aversive olfactory memory retention in flies. We used L-3,4-dihydroxyphenylalanine (L-DOPA) to elevate dopamine levels: L-DOPA-treated flies exhibited a normal learning performance, but a decrease in 1-h memory. Dopamine transporter (DAT) mutant flies or flies treated with the DAT inhibitor desipramine exhibited poor memory retention. Flies subjected to heat stress after training exhibited a decrease in memory. Memory was restored by blocking dopaminergic neuronal output during heat stress, suggesting that dopamine is involved in heat stress-induced memory impairment in flies. Taken together, our findings suggest that increased dopaminergic signaling impairs aversive olfactory memory retention in flies.

In addition to its role in memory formation, dopamine is also involved in the modulation of memory in vertebrates [1]. In *Drosophila*, dopamine plays a critical role in the aversive olfactory associative memory formation [2]. However, the effect of dopamine on other memory stages in flies has not been reported. Herein, we investigate the effects of increasing dopaminergic signaling on aversive olfactory memory retention in flies.

In *Drosophila*, mushroom bodies (MBs) are the essential neural structures for olfactory memory formation, retrieval and consolidation [3,4]. MBs receive punitive unconditioned stimuli signals from dopaminergic neurons. Dopaminergic projections to the MB lobes can be activated strongly by electric shocks [5]. A previous study demonstrated that dopaminergic neuronal output is necessary for memory formation in flies [2]. Consistently, mutant flies with abnormal D1 dopamine receptors expressed in mushroom bodies are impaired in aversive olfactory learning [6]. A study of *Drosophila* larvae also showed that light-induced activation of dopaminergic neurons paired with an odor is sufficient to induce aversive memory formation [7].

Accumulating evidence suggests that dopamine may contribute to aversive olfactory memory processes in addition to memory formation. For example, the activities of dopaminergic neurons projecting to MBs are mildly increased by odor stimuli, but after conditioning, the conditioned odor-induced activities of dopaminergic neurons are prolonged [5]. The cyclic adenosine monophosphate (cAMP)-associated signaling cascade is necessary for normal olfactory memory [3]. Dopamine can increase the activity of adenylyl cyclase (AC) in flies [8], so increased dopaminergic signaling may disrupt other stages of olfactory memory which requires the normal cAMP signaling.

In the present study, we found that flies with increased dopaminergic signaling induced by treatment with L-3,4-dihydroxyphenylalanine (L-DOPA) or by inhibiting the function of the dopamine transporter (DAT) showed a decrease in aversive olfactory memory retention. Dopaminergic neurons were also involved in the memory impairment induced by heat stress which can lead to elevated dopamine levels in flies [9,10]. Collectively, these findings suggest that increasing dopaminergic signaling leads to aversive olfactory memory retention impairment in flies.

Materials and methods

Fly strains. All flies were raised on standard cornmeal food at 25 °C with 60% relative humidity on a 12 h:12 h light:dark cycle. The wild-type flies used in this study include Canton S (CS) and *w2202*. The *TH-Gal4* line was kindly provided by Prof. J. Hirsh. The *UAS-shi^{ts1}* line was kindly provided by Prof. T. Tully. The *fmn* and *w2202* strains were kindly provided by Prof. F.R. Jackson. The *UAS-dDAT/TM3* line was kindly provided by Prof. Y. Rao. For rescue of the *fmn* memory defect, TH-Gal4 driver and UAS-dDAT were crossed into flies with the *w*; *fmn* genetic background. All the flies used in the experiments were 2–5 days old. Prior to behavioral training, flies were transferred to fresh food vials for up to 12 h.

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Memory assay. A classical olfactory conditioning procedure was performed in a T-maze as described previously in Tully et al. [11] with slight modification. Training was conducted with 0.1% OCT and 0.14% MCH diluted in mineral oil. Groups of 80–100 flies were exposed to the first odor (CS^+) for 1 min in the presence of an unconditioned stimulus consisting of twelve 1.25 s pulses of electric shocks at 60 V with 3.75 s intervals between pulses, followed by exposure to a second odor (CS^-) without shock for 1 min. Flies were tested with the two odors presented for 2 min. A one-half performance index ($PI_{1/2}$) was determined as the percentage of flies that chose the CS^- minus the percentage of flies that chose the CS^+. A second set of flies was trained to associate the other odor with shock, and a separate $PI_{1/2}$ value was determined. A single performance index (PI) was calculated by averaging the two $PI_{1/2}$ values to rule out odor bias. The PI determined immediately after training was used as the learning index. For the memory retention test, flies were transferred to fresh food vials after training. Memory was tested by loading the flies to the chosen point at the indicated time. Except when indicated, the training and testing were performed under dim red light at 24 ± 1 °C with 75 ± 5% humidity.

Shock avoidance and olfactory acuity assay. Electric shock avoidance was performed in a T-maze in which each arm was equipped with an electric shock grid [11]. Groups of 80–100 flies were given 2 min to choose between the electrified and the non-electrified tube. The avoidance index was calculated as the percentage of flies choosing the non-electrified tube minus the percentage of flies choosing the electrified tube. Olfactory acuity was also tested in the T-maze. Flies were given 2 min to choose between the test odor used in conditioning and air bubbled through mineral oil. The avoidance index was calculated as the percentage of flies choosing the air tube minus the percentage of flies choosing the odor tube.

Heat stress. Heat stress was induced by exposing the flies to 38 °C for 19 min and then transferring them to their normal temperature environment.

Pharmacological treatments. Flies were treated with 3-iodotyrosine (3-IY) (Sigma) and L-DOPA (Sigma) as described in Bainton et al. [12] with slight modification. Briefly, L-DOPA (1 mg/ml) and/or 3-IY (10 mg/ml) were dissolved in an aqueous 5% sucrose, 2% yeast solution. Groups of 80–100 flies were placed in a vial containing one Kimwipe paper that was soaked in a total of 2 ml of solution for 48–56 h. Flies were fed 3 mg/ml desipramine (Sigma) in 4% glucose for 24–28 h before training. Groups of 80–100 flies were placed in feeding tubes (Falcon 2017) containing one 1.0 × 2.5 cm 3MM filter paper strip that was soaked with a total of 150 μl of solution. One hour before training, the flies were transferred to standard food vials to clean themselves.

Statistical analysis. In order to determine statistically significant differences between two groups, we used two-tailed, unpaired Student's *t*-tests. Analyses among multi-group data were conducted using one-way analysis of variance (ANOVA), followed by Scheffe's post hoc test. Differences among groups were considered significant if the probability of error was less than 0.05.

Results

The effect of elevated dopamine levels on memory retention in flies

We used 3-IY and L-DOPA to modulate dopamine levels in flies. L-DOPA is the dopamine precursor, which can increase dopamine levels in flies, whereas 3-IY is a competitive antagonist of tyrosine hydroxylase, which leads to a decrease in dopamine levels in flies [12].

There was no significant difference between L-DOPA-treated flies relative to vehicle-treated flies in learning performance (Fig. 1A; $P = 0.79$). Flies treated with 3-IY showed a significant reduction in learning performance compared to control flies ($P < 0.01$), but their odor or shock acuity was not affected by 3-IY (Table 1). We ascribed the reduction to a decrease in dopamine levels, because L-DOPA treatment restored the normal learning performance of 3-IY-treated flies. The 1-h memory of the flies treated with L-DOPA decreased significantly compared to control flies ($P = 0.037$); this memory defect could be rescued by treating the flies with L-DOPA and 3-IY together. Because the immediate memory of the L-DOPA-treated flies was not different from that of control flies, suggesting that the formation and retrieval of aversive olfactory memory was normal, these data provide evidence that elevated dopamine levels impair the retention of olfactory aversive memory in flies.

The effect of the DAT on memory retention in flies

We also determined the effect of enhanced dopaminergic signaling on memory retention by inhibiting the function of DAT. DAT deficits lead to elevated levels of extracellular dopamine [13]. A previous study showed that the *Drosophila* dopamine transporter (dDAT) mediated the uptake of dopamine in cell-based assays and responded to dopamine when expressed in *Xenopus laevis* oocytes [14]. Defective dDAT leads to altered dopaminergic signaling in *Drosophila* [15].

Fmn is a dDAT mutant fly that exhibits increased levels of locomotor activity [15]. Compared to *w2202* wild-type control flies, *fmn* flies showed a moderate level of learning (Fig. 2A; $P < 0.01$). Shock reactivity and olfactory acuity tests showed that the sensory acuity of *fmn* flies was indistinguishable from that of *w2202* control flies (Table 1). We next tested the memory of *fmn* flies at 15, 30 and 60 min, respectively, after training (Fig. 2A). The retention curve showed that the memory of *fmn* flies decreased nearly four times faster than that of wild-type control flies during the first half hour. In order to exclude the possible effect of initial learning performance on memory decay, we changed electric voltage to 25 V and at this voltage the learning performance of the *w2202* flies was indistinguishable from that of the *fmn* files trained with 60 V electric shock (Fig. 2B). However, after 30 min, the memory was significantly better in *w2202* flies than in *fmn* flies ($P < 0.01$).

Expression of dDAT in dopaminergic neurons with TH-Gal4 rescued the learning deficit phenotype of *fmn* flies (Fig. 2C, left). The immediate memory of *fmn; TH-Gal4/UAS-dDAT* flies was indistin-

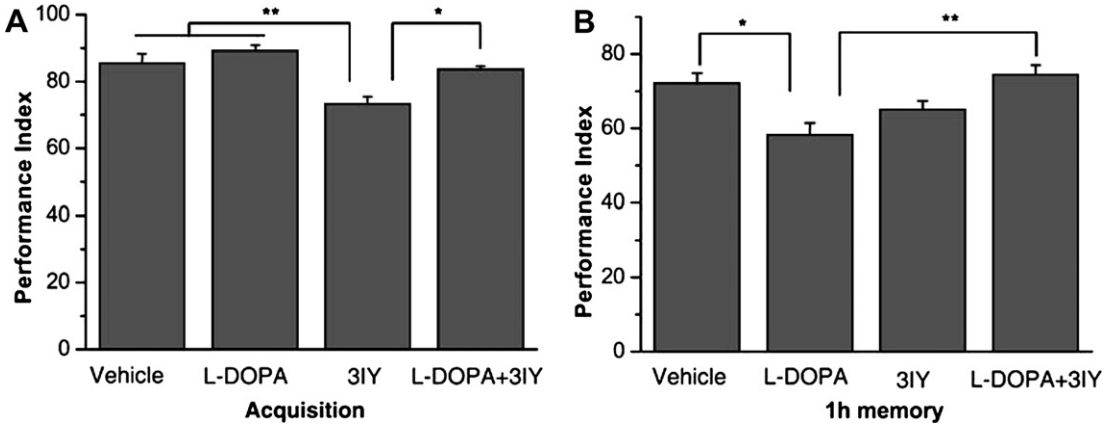

Fig. 1. Pharmacological elevation of dopamine impaired olfactory aversive memory retention in flies. (A) The learning performance (acquisition) of CS flies treated with 3-IY, L-DOPA, both or neither. (B) The 1-h memory of CS flies fed with 3-IY, L-DOPA, both or neither. Performance indices (PIs) are shown for each group ($n = 6–12$). Error bars indicate SEM (standard error of mean). Statistical significance ($^*P < 0.05$; $^{**}P < 0.01$) is indicated.

Table 1
Olfactory acuity and shock avoidance test

Genotype	Olfactory acuity		Shock avoidance
	MCH	OCT	
CS + vehicle	55 ± 5	55 ± 3	87 ± 5
CS + 3-IY	54 ± 3	55 ± 5	87 ± 4
w2202	41 ± 5	56 ± 5	81 ± 3
fmn	38 ± 5	61 ± 5	75 ± 3
w2202	49 ± 3	58 ± 4	77 ± 5
fmn; TH-Gal4/+	51 ± 4	56 ± 3	78 ± 2
fmn; UAS-dDAT/+	51 ± 2	52 ± 7	76 ± 4
fmn; TH-Gal4/UAS-dDAT	49 ± 4	65 ± 5	80 ± 3

Olfactory acuity and shock avoidance of experimental and control flies (n = 8–10 for each group). Data represent means ± SEM. There were no significant differences detected between the experimental and control groups.

guishable from that of *w2202* control flies (P = 0.997), but was better than that of *fmn; TH-Gal4/+* or *fmn; UAS-dDAT/+* flies (P < 0.01). The *fmn; TH-Gal4/UAS-dDAT* flies showed a partial rescue of the memory retention deficit: the 30-min memory of the *fmn; TH-Gal4/UAS-dDAT* was lower than that of *w2202* control flies (P = 0.015), but was better than that of *fmn; TH-Gal4/+* or *fmn; UAS-dDAT/+* flies (Fig. 2C, right; P < 0.01). After 30 min, the memory of *fmn; TH-Gal4/UAS-dDAT* was reduced by 25%, which was slower than that of *fmn; TH-Gal4/+* flies (71%) and *fmn; UAS-dDAT/+* flies (65%), but was faster than that of wild-type flies (12%).

We also used desipramine, which is a dDAT inhibitor with high affinity for dDAT [14], to determine the effect of dDAT on memory retention in flies (Fig. 2D). After 12–18 h of desipramine treatment, the CS flies showed a slight, but not significant, decrease in learning performance (P = 0.47). However, the 30-min memory was significantly lower in desipramine-treated flies than in vehicle-treated flies (P = 0.026).

Dopaminergic neurons were involved in the memory impairment induced by heat stress

Heat stress can increase the dopamine levels in flies [9,10]. Here we subject the flies to heat stress and observe the effect of elevated dopamine level after training on the memory retention. Heat stress was induced by transferring the flies to 38 °C for 1 min after conditioning for 19 min (Fig. 3A). After the heat stress, the flies were transferred back to 24 °C and were tested later. Results of these experiments showed that CS flies exposed to heat shock exhibited a significant decrease in olfactory memory at 45, 90 and 180 min, respectively, after training compared to the unstressed groups (P < 0.05 in all groups). The heat stress-induced memory impairment was not due to failed memory retrieval, because flies that were trained 25 min after heat stress and were tested afterwards did not show a difference in memory performance compared to the unstressed group (Fig. 3B; P = 0.77). Therefore, the memory impairment induced by heat stress is likely attributable to their impact on the memory retention.

In order to determine the role of dopaminergic neurons in heat stress-induced memory loss, we blocked synaptic output from dopaminergic neurons by expressing the UAS-shi^{ts1} allele using the transgenic *TH-Gal4* line. Shi^{ts1} is a temperature-sensitive allele: a shift from the permissive to the restrictive temperature leads to rapid and reversible effects on synaptic transmission of shi^{ts1}-

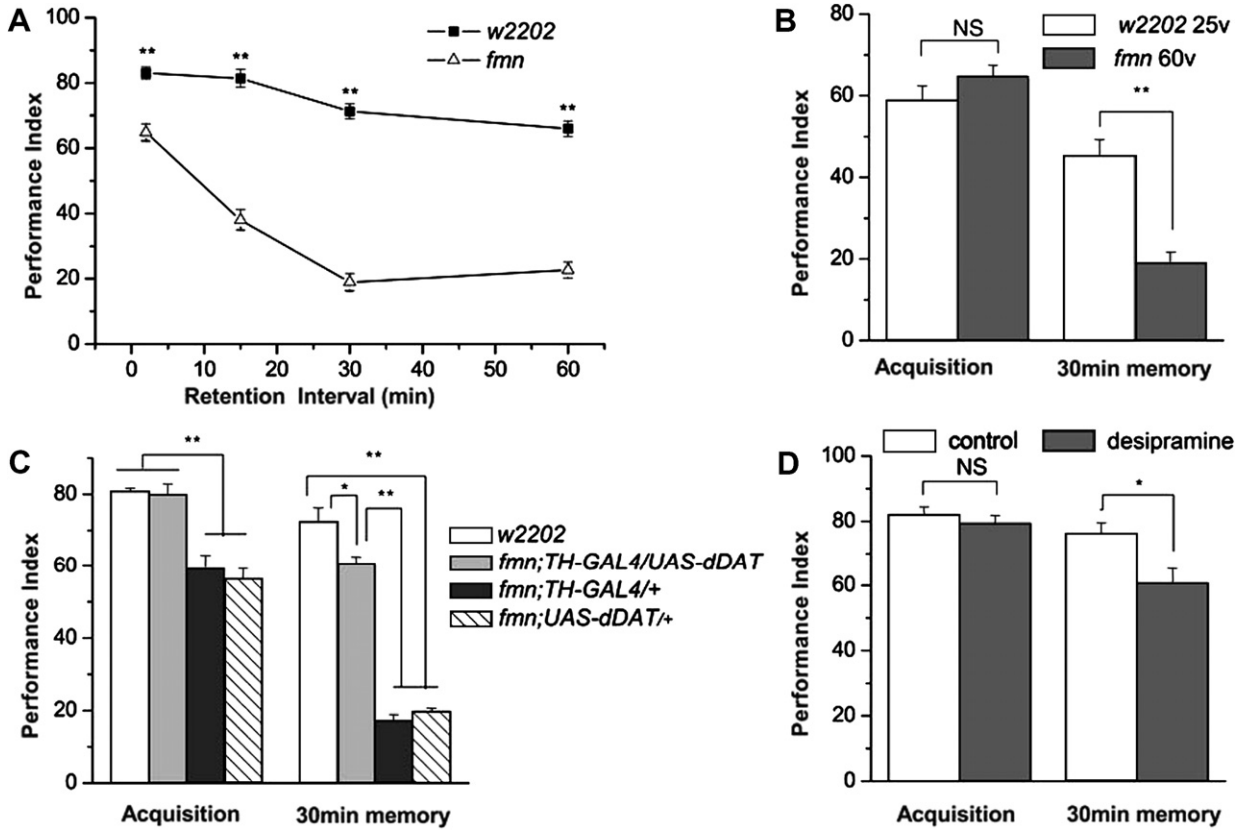

Fig. 2. Defective DAT led to a disruption of memory retention in flies. (A) The memory of *fmn* and wild-type control flies (*w2202*) was determined at 2, 15, 30 and 60 min after training. (B) The learning performance (acquisition) and 30-min memory of *w2202* with 25 V electric shocks as unconditioned stimulus (US) and the *fmn* flies with 60 V electric shocks as US. (C) Expressing dDAT in dopaminergic neurons produced a complete rescue of the *fmn*-associated learning defect and a partial rescue of the *fmn* 30-min memory defect. (D) The learning performance and 30-min memory of desipramine-treated flies. PIs ± SEM are shown for each group (n = 5–6). Statistical significance ($^{*}P$ < 0.05; $^{**}P$ < 0.01) or not significant (NS) is indicated.

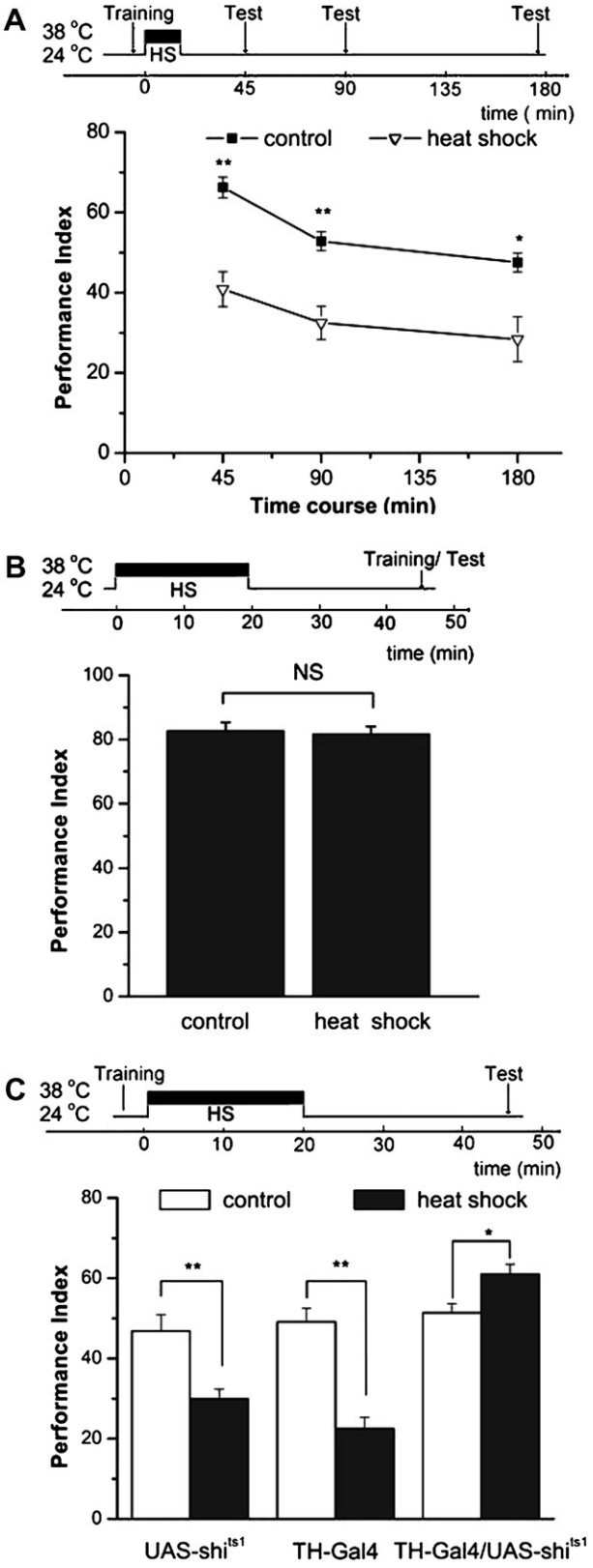

Fig. 3. Dopaminergic neurons were involved in the memory impairment induced by heat stress. (A) CS flies were exposed to heat stress (HS) 1 min after training and memory was tested at 45, 90 and 180 min ($n = 5–6$). (B) Training and immediate memory tests were performed 25 min after heat stress. (C) The 45-min memory of *TH-Gal4*, *UAS-shi^{ts1}* and *TH-Gal4/UAS-shi^{ts1}* flies exposed to heat stress. Except when indicated, each group was $n = 6$. Error bars indicate SEM. Statistical significance ($^{*}P < 0.05$; $^{**}P < 0.01$) or not significant (NS) is indicated.

expressing neurons [16]. Because the temperature used to induce heat stress was higher than the restrictive temperature for the shi^{ts1} allele, synaptic transmission of dopaminergic neurons was disrupted in the *TH-Gal4/UAS-shi^{ts1}* flies exposed to heat stress. As same as the CS flies, *TH-Gal4* and *UAS-shi^{ts1}* flies subjected to the heat shock showed a significant decrease in olfactory memory compared to unstressed flies of the same genotype (Fig. 3C; $P < 0.01$); however, the memory of *TH-Gal4/UAS-shi^{ts1}* flies exposed to heat shock was better than that of the unstressed flies ($P = 0.017$).

Discussion

In flies, dopamine is involved in many cognitive behaviors, including attention-like behavior [17], decision making [18] and aversive olfactory associative memory formation [2]. In the current studies, our results showed that the altered dopaminergic transmission also affected the memory retention in flies.

Dopamine biosynthesis involves the hydroxylation of L-tyrosine to L-DOPA via tyrosine hydroxylase, followed by decarboxylation of L-DOPA by DOPA decarboxylase [19]. Therefore, 3-IY and L-DOPA treatment can modulate global dopamine levels in the flies via modulation of this biosynthetic pathway. Because flies do not express norepinephrine or epinephrine [19], these treatments will specifically target dopamine biosynthesis. Our results showed that elevated dopamine levels did not impact memory acquisition but led to impaired memory retention.

We also altered dopamine neurotransmission by inhibiting DAT in flies. DAT is the primary mechanism used to remove dopamine from the synaptic cleft. Studies using a mouse model show that loss of DAT function leads to a persistent elevation of extracellular dopamine levels and a decrease in intracellular dopamine levels [13]. Our results showed that the dDAT mutant exhibited moderate learning performance and had impaired memory retention. Flies treated with desipramine also exhibited impaired memory performance, but showed normal learning performance. We attribute this difference to a lower inhibitory effect of desipramine on dDAT compared to that of *fmn* flies. Taken together with our findings that changes in dopamine levels can lead to altered memory acquisition and retention, these data suggest that elevated extracellular dopamine levels may be responsible for impaired memory retention.

Memory retention of *fmn* flies was only partially rescued by expression of dDAT under the control of TH-Gal4. A similar phenomenon was described in experiments by Kume et al. [15] showing that the hyperactivity phenotype of *fmn* flies could be rescued partially by expression of the dDAT driven by Elav-Gal4, whereas dDAT expression driven by TH-Gal4 led to a weaker rescue; this result was attributed to potential compensatory postsynaptic changes in response to strong TH-Gal4-induced expression of dDAT. The possibility of postsynaptic compensatory changes is supported by a study showing that postsynaptic receptor sensitivity increased in flies with decreased dopamine and 5-HT synthesis and release [20]. This compensatory effect may require a long duration of dopamine decrease in the synaptic cleft, because short-term depletion of dopamine leads to reduced cocaine sensitivity in flies [12]. Our findings also showed that transitory inhibition of dopaminergic neuronal output led to an increase in memory, rather than a decrease. We observed a similar discrepancy in a previous study showing that long-term and short-term dopamine blockades led to different effects on attention-like behavior in flies [17].

Previous studies have demonstrated that heat stress can increase the dopaminergic signaling [9,10]. In our studies, flies subjected to the heat stress showed a decrease in memory. We employed flies expressing *TH-Gal4/UAS-shi^{ts1}* to block the output

of dopaminergic neurons, and found that disruption of dopaminergic neural transmission restored heat stress-induced memory deficits. Furthermore, inhibition of dopamine release in heat stressed flies not only eliminated the heat shock-induced amnesia, but also led to an increase in 45-min memory. In *TH-GAL4*|*UAS-shi*^{ts1} flies, the output of dopaminergic neurons blocked at the restrictive temperature includes both heat stress-induced dopamine release and the spontaneous dopamine release. Altered memory in these flies may be due to a combination of both processes. Collectively, our findings suggest that the output of dopaminergic neurons may be involved in heat stress-induced memory impairment in flies.

In conclusion, our studies showed that an increase in dopaminergic signaling had detrimental effects on aversive olfactory memory retention in flies. Previous studies have established that dopamine plays a critical role in aversive learning [2,6], and our study extends these findings by demonstrating that aversive memory retention is also affected by dopaminergic signaling. However, the underlying molecular mechanisms linking increased dopaminergic signaling to modulation of memory remain elusive. The stimulatory effect of dopamine on adenylyl cyclase [8] and the critical role of cAMP signaling in aversive memory make it tempting to speculate that altered cAMP signaling is involved. Further studies will be required to elucidate it.

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