Behavioral/Systems/Cognitive

D_1 Dopamine Receptor dDA1 Is Required in the Mushroom Body Neurons for Aversive and Appetitive Learning in *Drosophila*

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***Drosophila* has robust behavioral plasticity to avoid or prefer the odor that predicts punishment or food reward, respectively. Both types of plasticity are mediated by the mushroom body (MB) neurons in the brain, in which various signaling molecules play crucial roles. However, important yet unresolved molecules are the receptors that initiate aversive or appetitive learning cascades in the MB. We have shown previously that D_1 dopamine receptor dDA1 is highly enriched in the MB neuropil. Here, we demonstrate that dDA1 is a key receptor that mediates both aversive and appetitive learning in pavlovian olfactory conditioning. We identified two mutants, *dumb*^{1} and *dumb*^{2}, with abnormal dDA1 expression. When trained with the same conditioned stimuli, both *dumb* alleles showed negligible learning in electric shock-mediated conditioning while they exhibited moderately impaired learning in sugar-mediated conditioning. These phenotypes were not attributable to anomalous sensory modalities of *dumb* mutants because their olfactory acuity, shock reactivity, and sugar preference were comparable to those of control lines. Remarkably, the *dumb* mutant's impaired performance in both paradigms was fully rescued by reinstating dDA1 expression in the same subset of MB neurons, indicating the critical roles of the MB dDA1 in aversive as well as appetitive learning. Previous studies using dopamine receptor antagonists implicate the involvement of D_1/D_5 receptors in various pavlovian conditioning tasks in mammals; however, these have not been supported by the studies of D_1- or D_5-deficient animals. The findings described here unambiguously clarify the critical roles of D_1 dopamine receptor in aversive and appetitive pavlovian conditioning.**

Key words: **dopamine receptor; pavlovian conditioning; punishment; reward; mushroom body neurons; learning memory**

Introduction

Pavlovian (classical) olfactory conditioning tests the animal's ability to learn and remember the odor [conditioned stimulus (CS)] associated with diverse unconditioned stimuli (US) in *Drosophila* and is instrumental in investigating the neural and cellular mechanisms underlying distinct learning and memory processes. When subjected to concurrent odor (CS+) and electric shock (aversive US) presentation, flies learn to avoid the CS+ odor in the absence of shock. Conversely, flies learn to prefer the CS+ odor after concurrent odor (CS+) and sugar (appetitive US) exposure. Thus, the same CS+ triggers either avoidance or preference behavior depending on previous experience of the flies. Several key questions arise regarding the underlying mechanisms. Specifically, are common or separate neural systems required for aversive versus appetitive learning and memory? What are the critical molecular and cellular events that distinguish reward versus punishment information?

Two key components are essential for both aversive and appetitive conditioning. One component is the cAMP signaling pathway. Flies defective in cAMP metabolism, such as *dunce* (cAMP-specific phosphodiesterase) and *rutabaga* [*rut*; calcium/calmodulin (CaM)-dependent adenylyl cyclase (AC)], or flies with altered activities of cAMP effectors protein kinase A and dCREB2 are impaired in learning and/or memory in aversive conditioning (for review, see Davis, 2005). Likewise, appetitive conditioning requires cAMP because *rut* mutants display poor learning (Schwaerzel et al., 2003). The other component is the mushroom body (MB) brain structure. Flies with ablated MB structures or functions are completely defective in aversive learning (de Belle and Heisenberg, 1994; Connolly et al., 1996). Moreover, synaptic output of different MB lobes is involved in memory formation or retrieval in aversive and appetitive conditioning (Dubnau et al., 2001; McGuire et al., 2003; Schwaerzel et al., 2003; Isabel et al., 2004; Krashes et al., 2007). These indicate the MB as a central neural substrate for olfactory learning and memory. This poses a fundamental question regarding the neuromodulators and their receptors that initiate the cAMP cascade in the MB for aversive and appetitive learning and their memories.

The neuromodulators that are crucial for olfactory condition-

Received March 15, 2007; revised June 1, 2007; accepted June 8, 2007.

This work was supported by grants from the National Institute of Child Health and Human Development and the National Science Foundation to K.-A.H. We greatly appreciate Drs. Scott Waddell, Martin Heisenberg, and Ronald Davis and the Bloomington stock center and Harvard Exelixis stock collection for providing fly lines.

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DOI:10.1523/JNEUROSCI.1167-07.2007

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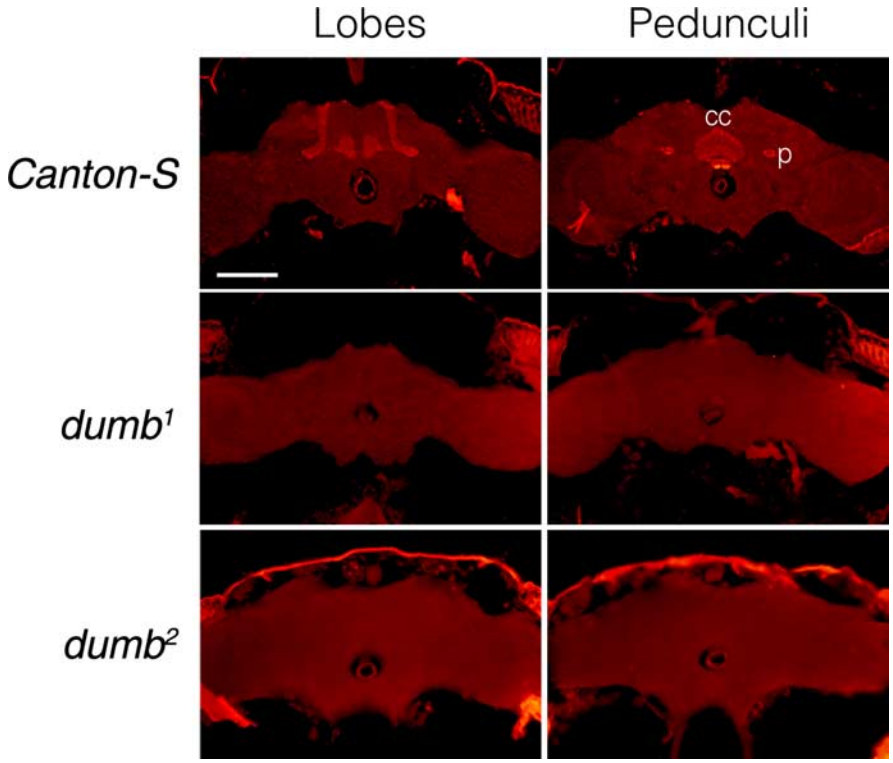


Figure 1. dDA1 IR in the adult head sections of *Canton-S*, *dumb*^{1}, and *dumb*^{2} flies. The frontal sections at the levels of the MB lobes (top) and pedunculi along with the central complex (bottom) are shown. dDA1 IR is visualized by red florescence. *Canton-S* has prominent dDA1 IR in the MB lobes and pedunculi as well as the central complex; however, no dDA1 IR is visible in those structures of *dumb*^{1} and *dumb*^{2}. p, Pedunculus; cc, central complex. All images are at the same magnification. Scale bar, 100 μm.

ing and activate cAMP increases are dopamine and octopamine. Previous studies of *Drosophila* larvae and adults show that dopaminergic neuronal activities are essential for aversive, but not for appetitive, learning, whereas octopamine or octopaminergic neuronal activities are necessary only for appetitive learning (Schwaerzel et al., 2003; Schroll et al., 2006). Consistently, the activities of dopaminergic neurons projecting to the MB are mildly increased by odor stimuli and strongly by electric shock (Riemensperger et al., 2005). Moreover, duration of their activities is prolonged when the CS+ odor is presented, suggesting the role of dopamine neurons in US prediction. However, it is yet unknown whether dopamine directly activates the MB for aversive learning. To uncover the signal(s) activating the learning and memory cascade, we previously identified three receptors that are highly enriched in the MB and increase cAMP levels, and they are two dopamine receptors, dDA1 and DAMB, and an octopamine receptor, OAMB (Han et al., 1996, 1998; Kim et al., 2003). Here, we show that dDA1 is required in the MB for aversive and appetitive learning.

Materials and Methods

Drosophila *stocks and culture.* Wild-type *Canton-S* and isogenic w^{1118} were used as controls. The control, deficiency, and inversion lines used in this study were obtained from the Bloomington stock center and *f02676* from the Harvard Exelixis stock collection. *MB247-GAL4*, *Elav-GAL4*, and *GAL80*^{ts} lines were kindly provided by Drs. S. Waddell, M. Heisenberg, and R. Davis, respectively. The X and second chromosomes of the inversion line *In(3LR)234* were replaced with those of *Canton-S*. *f02676* was backcrossed with w^{1118} for at least five generations. Flies were reared on standard cornmeal/agar medium at 25°C and ~50% relative humidity on a 12 h light/dark cycle. The 4- to 7-d-old flies of mixed genders were used for behavioral tests. In appetitive conditioning, flies were starved for 22 h in *Drosophila* vials containing water-soaked Kimwipes before training (Kim et al., 2007).

Immunohistochemistry and molecular analyses. Immunostaining was performed as described previously using mouse anti-dDA1 antibody (1:200 for sections; 1:1000 for whole mounts) and Alexa 555-conjugated anti-mouse IgG (1:1000; Invitrogen, Eugene, OR) (Han et al., 1996; Kim et al., 2003). Images were taken by a DMR epifluorescent (Leica, Heidelberg, Germany) or a FluoView confocal (Olympus, Melville, NY) microscope. RNA preparation and reverse transcription (RT)-PCR were performed using Qiagen (Chatsworth, CA) kits according to the manufacturer's instruction.

Behavioral tests. The protocol described by Beck et al. (2000) was adopted for aversive conditioning with minor modifications. Appetitive conditioning was performed as described previously (Kim et al., 2007). Briefly, 50–60 flies were exposed to a first odor (CS+) in the presence of pulses of 90 V electric shock or 2 M sucrose for 1 min, followed by 30 s air. After exposure to a second odor (CS−) without shock or sucrose for 1 min, flies were tested in a T-maze with two odors presented for 2 min. In sucrose-mediated conditioning, flies received another cycle of training with a 30 s intertraining interval. A second set of flies was simultaneously trained with the odors presented in a reversed order to counterbalance any possible odor bias in conditioning. The test was performed immediately after or at 1 h after training. The performance index (PI) was calculated by subtracting the percentage of flies that chose CS+ (incorrect choice) from the percentage of flies that chose CS− (correct choice). An average PI of two sets of flies conditioned with counterbalanced odors was used as one data point. Odorants used for conditioning were 1% 3-octanol (OCT), 0.5% benzaldehyde (BA), 2% ethyl acetate (EA), and 2% isoamyl acetate (IAA). Odorants were diluted in mineral oil and sucrose in deionized water. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

For olfactory acuity tests, flies were placed in a T-maze, with one of the arms carrying air and the other carrying an odor, into which flies dispersed from a central choice point, and allowed to choose air versus the odor for 2 min. The tests were performed with the concentrations of odorants used for conditioning and with the five-times lower concentrations. Electric shock avoidance was tested in a T-maze with two tubes lined with copper grids, in one of which flies received pulses of 90 or 30 V electric shock. Sugar preference was also tested in a T-maze with both tubes covered with filter papers, one of which had 2 or 0.2 M sucrose. Avoidance and preference scores were calculated similar to PI.

All data are reported as mean ± SEM. Statistical analyses were performed using Minitab 14 (Minitab, State College, PA). ANOVA with *post hoc* Tukey–Kramer or Student's *t* test was used for normally distributed data. If data were not normally distributed, the Kruskal–Wallis test was used.

Results

Identification of dDA1 mutants

To identify dDA1 mutants, we surveyed multiple fly lines with lesions that are known to map at the chromosomal location 88A where the dDA1 gene resides. Two lines showed abnormal dDA1 immunoreactivities (IRs) in the brain. One of them is the inversion line *In(3LR)234*, which has the break points at 67D and 88A-88B (Craymer, 1984). The other is *f02676* containing the transposable element piggyBac inserted at the first intron in the

Table 1. Sensory modalities including olfactory acuity and shock reactivity [$n = 6$ for all groups: *Df(3R)*, *Df(3R)su(Hw)7*;*Df(3L)*, *Df(3L)AC1*]

		CS	*Df(3R)*/+	*dumb*^{1}/*Df(3L)*	*dumb*^{1}/+	*dumb*^{1}/*Df(3R)*	*dumb*^{1}	*p* values	*w*	*dumb*^{2}/+	*dumb*^{2}	*dumb*^{1}/*dumb*^{2}	*p* values
Odor													
avoidance	1% OCT	56.5 ± 4.0	52.6 ± 2.5	52.6 ± 6.6	63.1 ± 4.7	56.3 ± 1.8	47.8 ± 2.9	0.188	52.4 ± 4.4	49.4 ± 1.4	49.4 ± 4.0	52.8 ± 4.3	0.869
	0.2% OCT	16.1 ± 5.3	18.7 ± 4.6	17.8 ± 4.6	13.3 ± 5.0	13.6 ± 4.6	5.1 ± 5.8	0.437	17.2 ± 4.3	15.8 ± 1.5	17.9 ± 2.2	17.1 ± 2.1	0.959
	0.5% BA	70.9 ± 4.3	76.0 ± 2.2	64.5 ± 3.1	76.9 ± 3.9	65.2 ± 3.7	65.6 ± 2.0	0.032	63.0 ± 3.0	65.5 ± 1.4	62.1 ± 3.3	67.8 ± 4.6	0.604
	0.1% BA	10.8 ± 3.0	11.2 ± 2.9	9.7 ± 3.4	14.6 ± 1.5	12.7 ± 3.6	7.2 ± 3.0	0.608	16.8 ± 3.8	15.3 ± 1.8	17.0 ± 1.3	17.4 ± 3.9	0.962
Shock													
avoidance	90 V	69.9 ± 3.5	66.1 ± 4.6	68.8 ± 6.7	63.6 ± 4.4	66.7 ± 4.5	69.2 ± 2.5	0.927	57.8 ± 4.4	62.4 ± 4.4	57.5 ± 3.9	54.7 ± 4.0	0.635
	30 V	39.3 ± 3.3	28.5 ± 2.9	26.6 ± 3.2	29.7 ± 6.3	31.5 ± 3.4	27.0 ± 2.5	0.212	25.8 ± 4.0	27.1 ± 8.1	27.5 ± 4.3	24.8 ± 4.2	0.984

dDA1 locus (Thibault et al., 2004). Previously, we have shown that dDA1 is highly enriched in the MB lobes, the central complex, a few scattered cells in the brain, and the Apterous-positive cells in the thoracico-abdominal ganglion (Kim et al., 2003; Park et al., 2004). Both *In(3LR)234* and *f02676* have negligible dDA1 IRs in the MB and the central complex (Fig. 1) but intact IRs in the scattered and Apterous-positive cells (data not shown). Consistently, full-length dDA1 transcripts were detected in both lines by RT-PCR (data not shown). Thus, *In(3LR)234* and *f02676* appear to have lesions in the regulatory sequence for tissue-specific dDA1 expression, representing hypomorphic dDA1 alleles, and are designated as *dumb*^{1} [D1(uno) in mushroom bodies] and *dumb*^{2}, respectively.

Table 2. Sensory modalities including olfactory acuity and shock reactivity ($n = 6$ for all groups)

		247/+;*dumb*^{1}	*247*/+;*dumb*^{2}	*247*/+; *dumb*^{1}/*dumb*^{2}	*247*/+; *dumb*^{1}/+	*p* values
Odor avoidance	1% OCT	50.9 ± 4.1	53.0 ± 3.6	53.9 ± 3.3	52.0 ± 6.0	0.965
	0.5% BA	62.1 ± 3.2	56.0 ± 4.6	59.3 ± 2.7	57.6 ± 2.2	0.609
Shock avoidance	90 V	60.5 ± 2.8	57.3 ± 2.7	59.5 ± 3.3	56.9 ± 3.6	0.820
	30 V	31.3 ± 3.9	28.8 ± 3.5	26.5 ± 3.4	30.3 ± 3.6	0.798

Table 3. Sensory modalities including olfactory acuity and taste perception ($n = 6$ for all groups)

		CS	*dumb*^{1}	*dumb*^{2}	*247*/+; *dumb*^{2}	*p* values
Sugar preference	2 M sucrose	68.2 ± 2.9	70.0 ± 3.5	68.6 ± 2.2	70.4 ± 2.9	0.942
	0.2 M sucrose	38.1 ± 3.1	37.0 ± 2.9	39.9 ± 3.9	36.4 ± 3.0	0.881
Odor avoidance	2% IAA	64.4 ± 2.7	62.4 ± 2.8	60.3 ± 3.1	61.8 ± 3.1	0.798
	2% EA	64.4 ± 2.3	61.6 ± 3.0	64.3 ± 3.4	61.9 ± 2.9	0.841

Impaired learning of *dumb* mutants in aversive conditioning

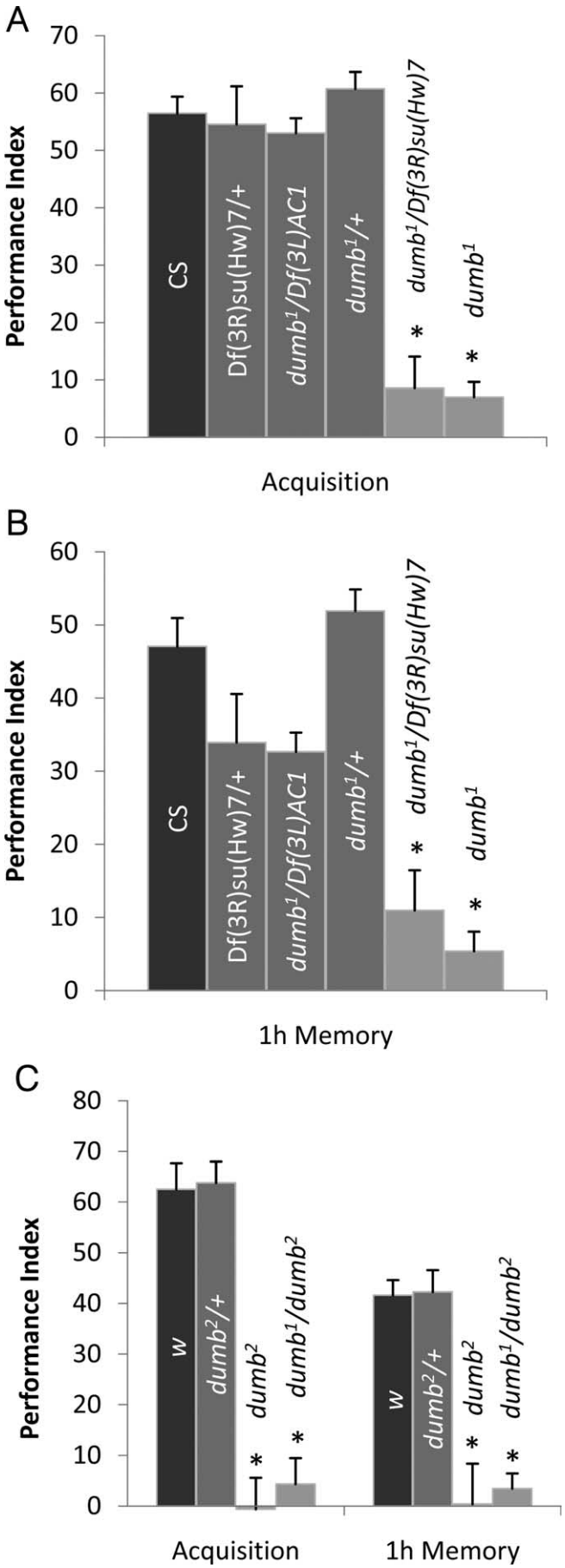
The observations that dDA1 is concentrated in the MB neuropil and can activate the cAMP pathway (Sugamori et al., 1995; Kim et al., 2003) prompted us to investigate the role of dDA1 in olfactory conditioning. When subjected to aversive conditioning using odorants BA and OCT as conditioned stimuli and electric shock as a US, *dumb*^{1} homozygous mutants showed severely impaired performance immediately after training (Fig. 2*A*). Performance of *dumb*^{1} did not decline at 1 h after training, suggesting that *dumb*^{1} is defective in learning rather than memory. *dumb*^{1} has two break points caused by inversion. Thus, to investigate the lesion accountable for poor performance of *dumb*^{1}, we used two deficiency lines, *Df(3L)AC1* and *Df(3R)su(Hw)7*, having deletion between chromosomes 67A2 and 67D13 and chromosomes 88A9 and 88B2, respectively, which include each break point (Pauli et al., 1995; Deak et al., 1997). Similar to *dumb*^{1}, *dumb*^{1}/*Df(3R)su(Hw)7* trans-heterozygous mutants exhibited poor performance immediately or 1 h after training (Fig. 2*A*). In contrast, performance of *dumb*^{1}/*Df(3L)AC1* was comparable to that of *Canton-S* and *dumb*^{1}/+ or *Df(3R)su(Hw)7*/+ heterozygous flies immediately after training. These data indicate that the lesion in chromosome 88A is responsible for poor learning of *dumb*^{1} mutants. The flies heterozygous for both deficiency chromosomes had slightly lower performance scores compared with those of *Canton-S* and *dumb*^{1}/+ at 1 h after training (Fig. 2*A*). This could be attributable to putative memory genes in the deleted chromosomes.

We next asked whether the *dumb*^{1} phenotype is linked to the lesion in dDA1 by examining the independent *dumb* allele *dumb*^{2} and *dumb*^{1}/*dumb*^{2} trans-heterozygous mutants in aversive conditioning. Like *dumb*^{1}, both genotypes had negligible performance scores immediately after or 1 h after training (Fig. 2*B*), supporting the potential role of dDA1 in punishment-mediated olfactory learning. *dumb*^{1} and *dumb*^{2} heterozygous flies exhibited normal performance (Fig. 2*A*,*B*); thus, a single copy of dDA1 may be sufficient for mediating this process.

To test whether *dumb* mutants could learn better with different conditioned stimuli, we used other odorants in electric shock-mediated olfactory conditioning. When trained with EA and IAA as conditioned stimuli, *dumb*^{1} mutants also displayed severely impaired learning (PI of *dumb*^{1}, 4.2 ± 1.3; PI of CS, 59.3 ± 2.4; $n = 6$; two-tailed Student's *t* test, $p < 0.0001$). This suggests that dDA1 is involved in aversive learning induced by diverse odor inputs. The impaired performance of *dumb* mutants is not attributable to anomalous sensory modalities because all *dumb* alleles and the control *Canton-S* and w^{1118} flies showed comparable avoidance of the CS odors and electric shock presented at two different concentrations or intensities, respectively (Tables 1–3). Thus, poor learning of *dumb* mutants is likely attributable to their inability to associate CS+ with US.

Aversive learning requires dDA1 in the MB

Synaptic output of dopamine neurons was previously shown to be required during training for aversive learning (Schwaerzel et al., 2003), implicating the similar requirement of dDA1 at the time of learning. To test this, we used the pan-neuronal driver Elav-GAL4 and GAL80^{ts}, which allows the temporal control of GAL4 activities (McGuire et al., 2003). GAL80 binds to GAL4 to sequester it from activating upstream activating sequence (UAS). The temperature-sensitive GAL80^{ts} can no longer bind to GAL4 at 30°C, allowing it to act on UAS to induce downstream gene expression. The piggyBac inserted at the first intron of the dDA1 gene in *dumb*^{2} has UAS (Thibault et al., 2004). Although the piggyBac insertion itself interferes with endogenous dDA1 ex-



pression in *dumb*^{2}, UAS in piggyBac, after binding to GAL4, may induce dDA1 transcription from the second exon containing the 5′ untranslated sequence and the start codon. Thus, we crossed *dumb*^{2} with *dumb*^{1} carrying Elav-GAL4 and GAL80^{ts} to generate *Elav-GAL4,GAL80*^{ts}*/+;dumb*^{1}*/dumb*^{2} flies. The *Elav-GAL4,GAL80*^{ts}*/+;dumb*^{1}*/dumb*^{2} kept at room temperature did not have any detectable dDA1 induction (Fig. 3*A*); however, when the flies were reared at 30°C for 3 d, conspicuous dDA1 IR was visible in the MB lobes and pedunculi, the central complex, and other brain areas including antennal lobes (Fig. 3*B*,*C* and data not shown). Whereas Elav-GAL4 is expressed in all neurons (Ito et al., 1998), membrane-bound GFP reporters driven by Elav-GAL4 are enriched in certain brain areas including the aforementioned structures (data not shown). Therefore, the temporal manipulation of GAL80^{ts} and Elav-GAL4 activities was effective in restricting dDA1 expression at the adult stage in *dumb* mutants.

When *Elav-GAL4,GAL80*^{ts}*/+;dumb*^{1}*/dumb*^{2} flies reared at room temperature were subjected to electric shock-mediated conditioning, they showed poor learning; however, their performance was dramatically improved after temperature shift to 30°C (Fig. 4*A*). The performance score of *Elav-GAL4,GAL80*^{ts}*/+;dumb*^{1}*/dumb*^{2} with the restored dDA1 expression was slightly lower than that of *Canton-S*; nonetheless, it was not significantly different from that of *Canton-S* treated with the same temperature shift but was different from that of uninduced *Elav-GAL4,GAL80*^{ts}*/+;dumb*^{1}*/dumb*^{2} ($p = 0.0009$). Therefore, dDA1 is required in the adult neurons, presumably at the time of training, for aversive memory formation. Notably, the same manipulation in the *dumb*^{2} heterozygous background (*Elav-GAL4,GAL80*^{ts}*/+;dumb*^{2}*/+*) did not alter the performance scores after brief training (2 pulses of electric shock) or regular training (12 pulses of electric shock) (Fig. 4*B*). This indicates that the ectopically expressed dDA1 has a negligible effect on normal learning of the heterozygous flies and thus unlikely contribute to the reinstated performance of *dumb*^{1}*/dumb*^{2} mutants.

We next addressed whether the learning phenotype of *dumb* mutants is attributable to deficient dDA1 function in the MB rather than in the central complex or other neurons. MB247-GAL4 contains 247 bp of dMEF2 regulatory sequence that allows GAL4 expression rather specifically in a subset of the MB neurons projecting to the α/β lobes and the gamma lobes, but not the α'/β' lobes (Schulz et al., 1996; Schwaerzel et al., 2002; Krashes et al., 2007). When *MB247-GAL4/UAS-GFP* in the wild-type background was stained with the dDA1 antibody, the GFP-labeled (thus MB247-GAL4-expressing) MB neurons were positive for

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Figure 2. The learning phenotype of *dumb* mutants in aversive olfactory conditioning. ***A***, ***B***, Flies were trained with BA and OCT as CS and tested immediately after (acquisition) or 1 h after training (1 h memory). ***A***, *dumb*^{1} homozygous and *dumb*^{1}*/Df(3R)su(Hw)7* trans-heterozygous mutants exhibited severely impaired learning, whereas *dumb*^{1}*/+*, *Df(3R)su(Hw)7/+*, and *dumb*^{1}*/Df(3L)AC1*, which have one copy of the dDA1 gene, showed performance similar to that of *Canton-S* (AVOVA; $F_{(5,35)} = 35.9$; $p < 0.0001$; $n = 6$ for all groups; asterisks indicate significant difference by *post hoc* Tukey–Kramer tests). ***B***, At 1 h after training, *dumb*^{1}, *dumb*^{1}*/Df(3R)su(Hw)7*, *Df(3R)su(Hw)7/+*, and *dumb*^{1}*/Df(3L)AC1* showed defective performance compared with *Canton-S* and *dumb1/+* (ANOVA; $F_{(5,35)} = 26.04$; $p < 0.0001$; $n = 6$; asterisks indicate significant difference compared with *Canton-S* by *post hoc* Student's *t* test). ***C***, *dumb*^{2} homozygous and *dumb*^{1}*/dumb*^{2} transheterozygous mutants showed no trace of learning and 1 h memory, whereas learning or 1 h memory performance of *dumb*^{2} heterozygous flies (*dumb*^{2}*/+*) was similar to that of the genetic control line w^{1118} (*w*) (acquisition ANOVA: $F_{(3,23)} = 55.3$, $p < 0.0001$; 1 h memory ANOVA: $F_{(3,23)} = 21.6$, $p < 0.0001$; $n = 6$; asterisks indicate significant difference by Tukey–Kramer tests). Error bars indicate SEM.

dDA1 IRs although the relative intensities of GFP and dDA1 signals varied in the different MB lobes (Fig. 3*D*). Thus, MB247-GAL4 was used to reinstate dDA1 expression in the MB of *dumb* mutants. After staining with anti-dDA1 antibody, dDA1 expression was apparent in the MB lobes (Fig. 3*E*) and pedunculi (Fig. 3*F*) but not in other neural structures (Fig. 3*E*,*F* and data not shown) of *MB247-GAL4/+; dumb^1/dumb^2*. When subjected to electric shock-mediated conditioning, *MB247-GAL4/+;dumb^1/dumb^2* or *MB247-GAL4/+;dumb^2/dumb^2* had the learning scores comparable to those of *Canton-S* (Fig. 4*C*). Moreover, fully reinstated performance was observed in *MB247-GAL4/+; GAL80^{ts},dumb^2/dumb^1* reared at 30°C for 3 d before training but not in *MB247-GAL4/+;GAL80^{ts},dumb^2/dumb^1* reared at room temperature (Fig. 4*D*). Therefore, dDA1 expressed only in the subset of the adult MB neurons is necessary and sufficient to rescue the *dumb* mutant's impaired learning, indicating the indispensable role of the MB dDA1 in aversive memory formation.

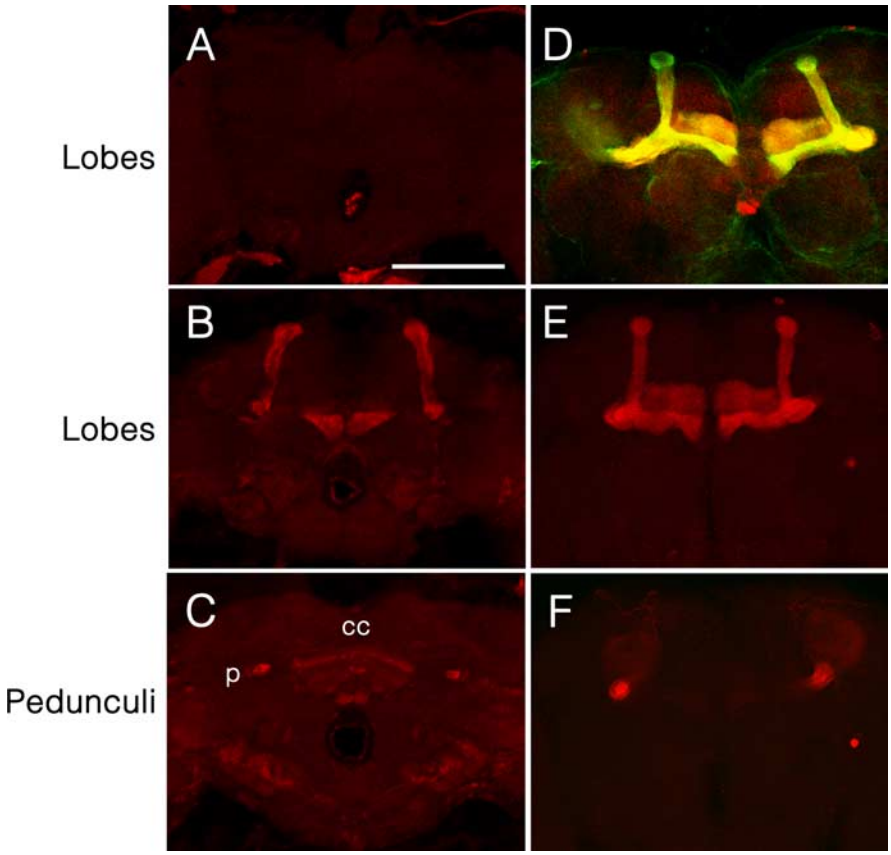

Figure 3. Restored dDA1 expression in *dumb* transgenic mutants. ***A–C***, *dumb^1/dumb^2* trans-heterozygous mutants carrying Elav-GAL4 and GAL80^{ts} reared at room temperature (uninduced) had no detectable dDA1 expression (***A***); however, when they were incubated at 30°C for 3 d (induced), dDA1 IRs were visible in the MB lobes (***B***) and pedunculi, the central complex, and other brain structures (***C***). ***D***, GFP driven by MB247-GAL4 in the wild-type genetic background was visible in most, if not all, dDA1-positive MB neurons. ***E***, ***F***, *dumb^1/dumb^2* carrying MB247-GAL4 had conspicuous dDA1 expression in the MB lobes (***E***) and pedunculi (***F***) but not in the central complex (***F***). p, Pedunculus; cc, central complex. All images are at the same magnification. Scale bar, 100 μm.

Appetitive learning requires dDA1 in the MB

Dopamine is crucial in appetitive learning in mammals; however, the previous study (Schwaerzel et al., 2003) of *TH-GAL4/UAS-Shi^{ts}* flies suggests that this is not the case in *Drosophila*. To investigate this further, we tested *dumb* mutants in sugar-mediated olfactory conditioning. To our surprise, both *dumb^1* and *dumb^2* mutants exhibited poor performance immediately after training (Fig. 5*A*). Although *dumb* mutants' performance in appetitive learning was not as severely impaired as in aversive conditioning, it was significantly different from that of *Canton-S* (Fig. 5*A*) or *w^{1118}* (data not shown). As in electric shock-mediated conditioning, *dumb* mutants' performance did not decline at 1 h after training, indicating a crucial role of dDA1 in acquisition, as opposed to short-term memory, of appetitive conditioning. Moreover, *dumb^2* homozygous or *dumb^1/dumb^2* trans-heterozygous mutants carrying MB247-GAL4 displayed fully reinstated learning in sugar-mediated conditioning (Fig. 5*B*). These data indicate that dDA1 is required in the same subset of the MB neurons for aversive and appetitive learning.

Discussion

dDA1 in aversive learning

Previous research on olfactory conditioning in *Drosophila* has primarily focused on the intracellular components, many of which are involved in learning and/or memory processes in the MB (McGuire et al., 2005). Although those studies have revealed important insights, the receptors that initiate signaling cascades into motion in the MB are unknown. The findings presented here provide the first demonstration of a MB receptor essential for aversive learning. The role of dDA1 in this behavioral plasticity is physiological, rather than developmental. This is consistent with the observations that synaptic output of dopaminergic neurons is necessary during training (Schwaerzel et al., 2003), and the learning phenotype of *rut* mutants is rescued by the restricted expression of rut-AC, a potential dDA1 effector, in the adult MB (McGuire et al., 2003; Mao et al., 2004). Moreover, the dopaminergic processes projecting to the MB gamma lobe strongly respond to electric shock (US) and show altered activities after CS+ exposure
(Riemensperger et al., 2005). Together, these strongly implicate dDA1 as a receptor conveying aversive US information in the MB lobes for memory formation.

Two additional neuromodulator systems are previously implicated in aversive olfactory conditioning in *Drosophila*. One neuromodulator system is the glutamate NMDA receptor composed of dNR1 and dNR2 subunits. Flies with decreased dNR1 expression show diminished performance in aversive conditioning (Xia et al., 2005). Although dNR1 in the MB is crucial for anesthesia-resistant and midterm memories, dNR1-dependent learning occurs outside of the MB (Lin, 2005). Another putative modulator involved in olfactory learning is Amn, which has sequence homology with mammalian neuropeptide PACAP. Although *amn* mutants are mostly defective in midterm memory, they are mildly impaired in learning when BA is used as CS+ and learning of BA depends on synaptic output of Amn-expressing DPM neurons projecting to the MB lobes (Keene et al., 2004).

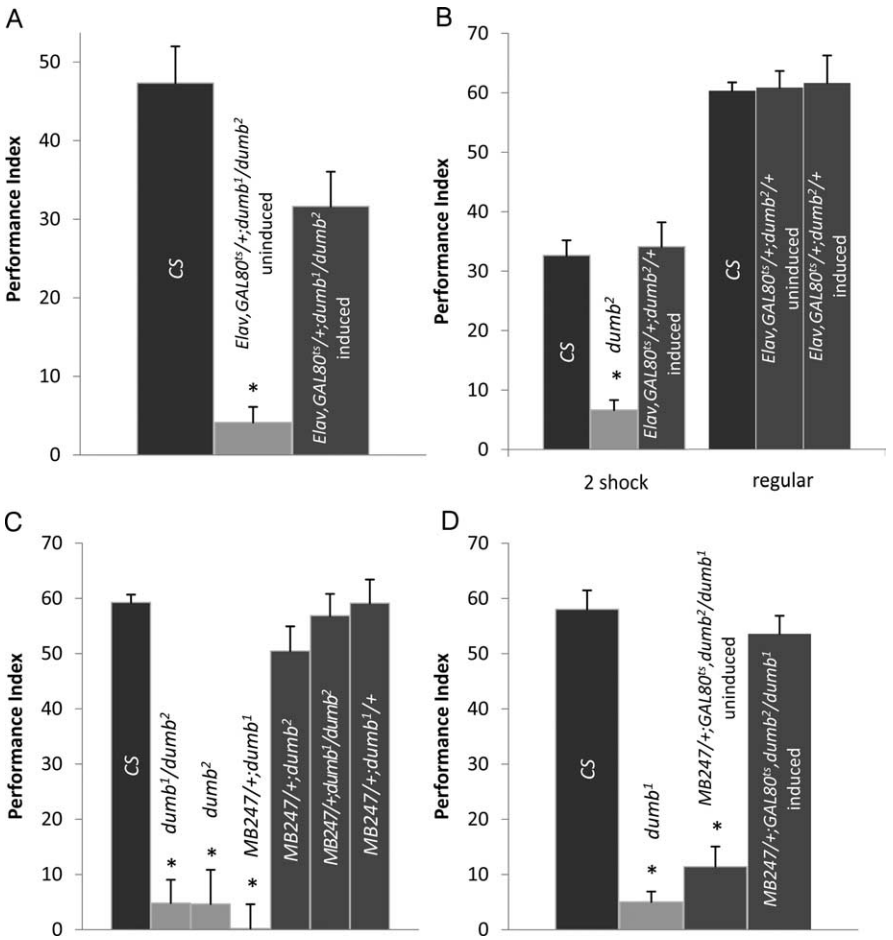

Figure 4. Rescue of the *dumb* mutant's phenotype in aversive learning. ***A***, The restricted dDA1 expression in the adult nervous system rescued the learning phenotype of *dumb* mutants. $dumb^1/dumb^2$ carrying Elav-GAL4 and GAL80^{ts} reared at room temperature (*Elav,GAL80^{ts}/+;$dumb^1/dumb^2$* uninduced) showed poor performance immediately after training; however, performance of the same genotype reared at 30°C for 3 d (*Elav,GAL80^{ts}/+;$dumb^1/dumb^2$* induced) was not significantly different from that of *Canton-S* (Kruskal–Wallis test, $p = 0.0009$; $n = 6$; the asterisk indicates significant difference by Mann–Whitney tests). ***B***, When subjected to brief (submaximal) training with two pulses of electric shock (2 shocks), $dumb^2$ heterozygous flies carrying Elav-GAL4 and GAL80^{ts} that were reared at 30°C for 3 d to induce ectopic dDA1 expression (*Elav,GAL80^{ts}/+;$dumb^1/dumb^2$* induced) had the learning score comparable with that of *Canton-S*, whereas $dumb^2$ homozygous mutants showed impaired learning (ANOVA; $F_{(2,17)} = 27.2$; $p < 0.0001$; $n = 6$; the asterisk indicates significant difference by Tukey–Kramer tests). Likewise, the $dumb^2$ heterozygous flies with ectopic dDA1 expression (*Elav,GAL80^{ts}/+;$dumb^1/dumb^2$* induced) had performance comparable with that of *Canton-S* and the same genotype without heat treatment (*Elav,GAL80^{ts}/+;$dumb^1/dumb^2$* uninduced) when subjected to regular training with 12 pulses of electric shock (regular) (ANOVA; $F_{(2,17)} = 0.04$; $p = 0.96$; $n = 6$). ***C***, The *dumb* transgenic mutants expressing dDA1 in the MB lobes (*MB247/+;$dumb^2$* and *MB247/+;$dumb^1/dumb^2$*) had the learning scores similar to those of *Canton-S* and *MB247/+;$dumb^1$/+*, whereas all three lines with deficient dDA1 expression ($dumb^2$, *$dumb^1$/$dumb^2$*, and *MB247/+;$dumb^1$*) had significantly low learning scores (ANOVA; $F_{(6,41)} = 43.6$; $p < 0.0001$; $n = 6$; asterisks indicate significant difference by Tukey–Kramer tests). ***D***, The temporally induced dDA1 expression only in the adult MB rescued the learning phenotype of *dumb* mutants. $dumb^1/dumb^2$ carrying MB247-GAL4 and GAL80^{ts} that were reared at 30°C for 3 d (*MB247/+;GAL80^{ts},$dumb^2/dumb^1$* induced) had the learning score comparable with that of *Canton-S*, whereas the same genotype reared at room temperature (*MB247/+;GAL80^{ts},$dumb^2/dumb^1$* uninduced) and $dumb^1$ homozygous mutants had significantly low learning scores (ANOVA; $F_{(3,23)} = 77.6$; $p < 0.0001$; $n = 6$; asterisks indicate significant difference by Tukey–Kramer tests). Error bars indicate SEM.

Thus, it has been suggested that putative *amn*-encoded neuropeptides, by binding to their receptor(s) in the MB neuropil, may mediate memory formation; however, the predicted Amn neuropeptides or their receptors remain unidentified. Therefore, dDA1 represents the only MB receptor identified to date that is essential for aversive learning. Notably, *dumb* mutants, similar to MB-less flies, show negligible learning (de Belle and Heisenberg, 1994). This indicates that the MB neurons absolutely require dDA1 for aversive memory formation.

dDA1 in appetitive learning

The data presented here demonstrate the crucial role of dDA1 in sugar-mediated olfactory learning as well. Interestingly, *dumb* mutants have diminished, yet significant, performance scores, implicating an additional receptor(s) for this type of learning. Indeed, *tβh* mutants lacking octopamine show severe impairment in appetitive conditioning, which is rescued by feeding octopamine before, but not after, training (Schwaerzel et al., 2003). Thus, octopamine represents another neuromodulator crucial for appetitive learning. Because the MB is a primary neural substrate for appetitive conditioning, reward memory formation is likely mediated by dDA1 and an octopamine receptor(s) in the MB.

The previous study of *TH-GAL4/UAS-Shi^{ts}* flies, in which endocytosis of the dopamine neurons expressing TH-GAL4 can be temporally controlled by dominant-negative dynamin Shi^{ts}, suggests that dopamine is not involved in appetitive conditioning (Schwaerzel et al., 2003; Kim et al., 2007). This is contrary to the learning phenotype of D_1 dopamine receptor mutants *dumb*. Nonetheless, the discrepancy may be reconciled by several reasonable possibilities. First, TH-GAL4 used in the previous study to drive Shi^{ts} may not be expressed, or expressed at low levels, in a subset of the dopamine neurons critical for appetitive learning. Second, dopamine neuronal output conveying sugar information may not be completely inhibited by Shi^{ts}. Third, dopamine crucial for appetitive learning may be secreted by a dynamin-independent pathway. These possibilities may be tested by investigating *pale* mutants that are unable to synthesize dopamine; however, such flies die during development because of an essential role of dopamine in cuticle formation (Budnik and White, 1987). Future studies on conditional *pale* mutants should help resolve this issue.

Punishment and reward signals activated by dDA1 in the MB

We have shown here that dDA1 expression driven by MB247-GAL4 fully rescues the learning phenotypes of *dumb* mutants in electric shock- as well as sugar-mediated conditioning. This indicates that appetitive and aversive memory formations are mediated by dDA1 in the same subset of the MB neurons (~30% of all MB neurons). This poses an intriguing question as to how those MB neurons distinguish punishment versus reward information delivered by dDA1 to generate avoidance versus preference behavioral output. The key to answering this question may be intracellular effectors in the MB neuropil. *rut*-AC is crucial in the MB247-GAL4-expressing MB neurons for both aversive and appetitive learning (Schwaerzel et al., 2003).

Notably, *rut* mutants retain some learning capacities in electric shock- and sugar-mediated conditioning, whereas MB-less flies or the flies with inhibited MB synaptic output exhibit no trace of learning in both assays (de Belle and Heisenberg, 1994; Krashes et al., 2007). This implicates additional cellular components crucial for aversive and appetitive memory formation. Because *dumb* mutants are rather completely impaired in aversive learning, dDA1 may activate *rut*-AC and other cellular components in the MB to process punishment information.

G-protein-coupled receptors including dopamine receptors can recruit multiple effector systems through heteromeric G-proteins or through cross-interactions of diverse signaling components (Lidow et al., 2001; Pierce et al., 2002). Thus, for aversive learning, dDA1 activated by electric shock US input may recruit the mitogen-activated protein (MAP) kinase cascade in addition to the cAMP pathway. The activated protein kinase A and MAP kinases may act on ion channels or cell adhesion molecules such as integrin and fasII to modify MB synaptic output, leading to avoidance behavior (Yoshihara et al., 2000; Berke and Wu, 2002; Koh et al., 2002; Selcher et al., 2002). Consistently, the flies defective in 14-3-3 and S6KII, which are involved in the MAP kinase cascade, and *α-integrin* and *fasII* mutants are poor learners in electric shock-mediated conditioning (Skoulakis and Davis, 1996; Grotewiel et al., 1998; Cheng et al., 2001; Putz et al., 2004).

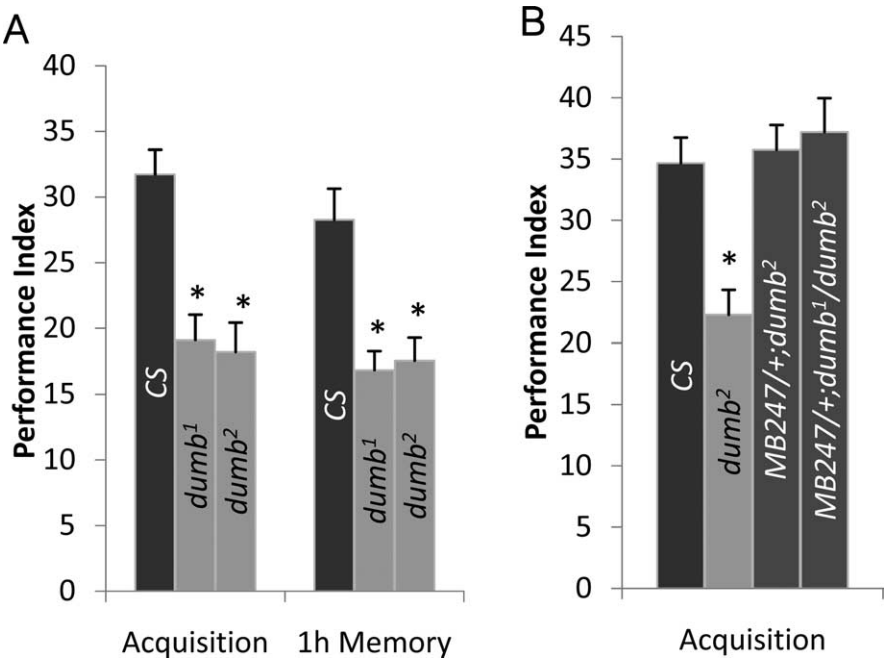

Figure 5. Impaired learning of *dumb* mutants in appetitive conditioning and rescue by reinstated dDA1 expression in the MB. ***A***, Both *dumb*^{1} and *dumb*^{2} mutants were moderately impaired in acquisition (ANOVA; $F_{(2,17)} = 14.2$; $p < 0.0001$; $n = 6$) and 1 h memory (ANOVA; $F_{(2,17)} = 11.5$; $p < 0.005$; $n = 6$) of sugar-mediated olfactory conditioning. ***B***, The dDA1 expression in the MB driven by MB247-GAL4 was sufficient to rescue the learning phenotype of *dumb*^{2} homozygous (*MB247/+;dumb*^{2}) and *dumb*^{1}/*dumb*^{2} transheterozygous (*MB247/+;dumb*^{1}/*dumb*^{2}) mutants (ANOVA; $F_{(3,50)} = 10.2$; $p < 0.0001$; $n = 6$ for *MB247/+;dumb*^{1}/*dumb*^{2}; $n = 15$ for other groups). Asterisks denote significant difference by Tukey–Kramer tests. Error bars indicate SEM.

For reward-mediated learning, reward US input may impinge on at least two receptors, dDA1 and an octopamine receptor, in the MB. Their simultaneous activities may recruit multiple effectors that possibly include rut-AC, MAP kinases, protein kinase C, and CaM kinase II. The biochemical changes collectively activated by these effectors may alter MB synaptic output to generate preference behavior. Interestingly, OAMB (octopamine receptor) activates the increases in intracellular calcium as well as cAMP (Han et al., 1998) and is a good candidate that can turn on the aforementioned effectors for processing reward information in the MB. The punishment and reward effectors may be at work in separate areas of the same MB neuropil or in different MB neurons or neuropils, which are differentially innervated by dopaminergic axons conveying electric shock input or by dopaminergic and octopaminergic axons conveying sugar input. At present, there is limited information on intracellular components involved in appetitive learning. Future studies in this venue will help attest this model. Together, concurrent CS+ and US received during training may activate dDA1 (for punishment US) or dDA1 and an octopamine receptor (for reward US) to induce distinctive biochemical changes, leading to avoidance or preference behavior, respectively.

D_1 dopamine receptor in pavlovian conditioning

Multiple lines of evidence indicate that dopamine in the amygdala, the nucleus accumbens, and the medial prefrontal cortex in mammals is crucial for acquisition, expression, and/or extinction in aversive pavlovian conditioning (for review, see Pezze and Feldon, 2004). However, the receptors mediating the functions of dopamine are unclear. Studies using D_1/D_5 dopamine receptor antagonist *R*(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride (SCH23390) in rats suggest the significant roles of the D_1-type receptor in the amygdala and the nucleus accumbens during acquisition in fear conditioning and conditioned taste aversion (CTA), respectively (Guarraci et al., 1999; Fenu et al., 2001). However, the mice lacking D_1 or D_5 receptor show normal acquisition in fear conditioning (El-Ghundi et al., 2001; Holmes et al., 2001). Likewise, D_1-deficient mice show normal learning of CTA to salt (CS) paired with LiCl (US), although they do not develop CTA to sucrose (Cannon et al., 2005). The discrepant findings of the pharmacological and genetic studies may be attributable to either other receptor types affected by SCH23390 or compensatory adaptations in D_1 or D_5 knock-out mice. The studies reported here support the latter and clarify the indispensable role of D_1 receptor in aversive pavlovian conditioning. Additionally, pharmacological studies reveal the significant role of D_1-type receptors in appetitive pavlovian conditioning in mammals (Schroeder and Packard, 2000; Baker et al., 2003; Eyny and Horvitz, 2003; Dalley et al., 2005) and possibly in *Aplysia* (Reyes et al., 2005), although no information is available on D_1 or D_5 knock-out mice in this type of behavioral plasticity. Therefore, the studies described here elucidate, for the first time, the critical role of D_1 receptor in appetitive pavlovian conditioning.

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