Behavioral/Systems/Cognitive

Dopamine and Octopamine Differentiate between Aversive and Appetitive Olfactory Memories in *Drosophila*

Martin Schwaerzel,[1] Maria Monastirioti,[2] Henrike Scholz,[1] Florence Friggi-Grelin,[3] Serge Birman,[3] and Martin Heisenberg[1]

[1]Lehrstuhl für Genetik und Neurobiologie, Biozentrum, Am Hubland, D-97074 Würzburg, Germany, [2]Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology, 71110 Heraklion, Greece, and [3]Developmental Biology Institute of Marseille, F-13288 Marseille Cedex 9, France.

The catecholamines play a major role in the regulation of behavior. Here we investigate, in the fly *Drosophila melanogaster*, the role of dopamine and octopamine (the presumed arthropod homolog of norepinephrine) during the formation of appetitive and aversive olfactory memories. We find that for the formation of both types of memories, cAMP signaling is necessary and sufficient within the same subpopulation of mushroom-body intrinsic neurons. On the other hand, memory formation can be distinguished by the requirement for different catecholamines, dopamine for aversive and octopamine for appetitive conditioning. Our results suggest that in associative conditioning, different memories are formed of the same odor under different circumstances, and that they are linked to the respective motivational systems by their specific modulatory pathways.

Key words: **learning and memory; dopamine; octopamine; reinforcement; *Drosophila*; memory trace; synaptic plasticity**

Introduction

One of the central tenets of cellular learning models is that cAMP signaling is involved in synaptic plasticity and associative memory formation (Yin and Tully, 1996; Kandel, 2001; Roman and Davis, 2001; Antonov et al., 2003). It has been proposed that the conditioned stimuli (CSs) and unconditioned stimuli (USs) converge at the level of the adenylyl cyclase, one signaled via Ca^{2+} and calmodulin, the other via heterotrimeric G-protein (Dudai et al., 1988). This presynaptic aspect of synaptic and behavioral plasticity has been documented for a variety of organisms throughout the animal kingdom using different kinds of reinforcers (Yin and Tully, 1996; Kandel, 2001). A question that up to now has received little attention is how brains are organized to allow the same CS to be associated with different USs, the implicit assumption being that, for instance, appetitive and aversive memories might be stored at different brain sites.

In the fly *Drosophila melanogaster*, odor learning has been extensively studied, mostly using electric shock as an aversive US. Yet *Drosophila* has been shown to remember odors also as signals for food, egg-laying sites, and mates (Tempel et al., 1983; McBride et al., 1999; Mery and Kawecki, 2002). Thus, even in the small fly brain, an odor can be associated with a variety of reinforcers.

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Correspondence should be addressed to Dr. Martin Heisenberg, Lehrstuhl für Genetik und Neurobiologie, Biozentrum, Am Hubland, D-97074 Würzburg, Germany. E-mail: heisenberg@biozentrum.uni-wuerzburg.de.

M. Schwaerzel's present address: University of Missouri-Columbia, Biological Sciences, Lefevre Hall, Columbia, MO 65211.

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We show here that distinct memory traces are formed in appetitive and aversive conditioning for the same odor, both in the same set of ~700 mushroom-body intrinsic neurons (Kenyon cells). Moreover, the results reveal the specific requirement of octopamine (OA) in appetitive and dopamine (DA) in aversive reinforcement.

Materials and Methods

Fly care. All flies were raised on standard cornmeal–molasses food (Guo et al., 1996) in a 14/10 hr light/dark cycle at 25°C and 60% relative humidity. Experimental flies were transferred to fresh food vials for up to 48 hr before the behavioral tests. Before experiments flies were starved for 18 hr in empty vials equipped with moist filter paper to prevent desiccation. For heat shock, flies were placed into empty vials at 37°C for 30 min. This treatment was applied twice, with a 6 hr interval, during the starvation period. To minimize nonspecific effects, flies were allowed to recover for 12 hr before testing. For behavioral experiments we used 3- to 5-d-old males and females in mixed groups. All behavioral experiments were performed either in dim red light or complete darkness at 80% relative humidity. Only responses to sugar were tested in daylight and uncontrolled humidity conditions.

Canton-S (Würzburg) was used as a wild-type control for the rut^{2080}-lines: The UAS-rut^{+} transgene (Zars et al., 2000a) and the 247-Gal4 transposon (Zars et al., 2000b) were recombined onto the same third chromosome and crossed into wild-type Canton-S and rut^{2080} mutant background for at least 10 generations. The control lines rut^{2080}; +; UAS-rut^{+} and rut^{2080}; +; 247-Gal4 were handled similarly. Behavioral experiments were performed with animals from these homozygous lines or with progeny of crosses between homozygous lines. For temperature-dependent blockade of synaptic transmission, we used progeny of crosses between the homozygous parental lines UAS-shi^{ts1} (as virgin females) and the Gal4-lines 247-Gal4 (Zars et al., 2000b) and TH-Gal4 (Friggi-Grelin et al., 2003) (as males). The line UAS-shi^{ts1} contains multiple inserts on the X and third chromosomes (Kitamoto, 2001). Because the original TβH^{M18} stock (Monastirioti et al., 1996) carried an additional mutation in the *white* (*w*) gene, we crossed it to Canton-S and isolated

recombinant TβH^{M18} lines with the w^+ allele. Nonrecombinant w^+ lines were kept as controls. Because of the sterility in females of the TβH^{M18} mutation, it was balanced over FM7. Homozygous and hemizygous TβH^{M18} flies were tested in behavioral experiments regardless of sex. To generate tyramine-β-hydroxylase (TβH) inducible flies, a 3 kb *Eco*RI fragment containing TβH cDNA was cloned downstream of the heat shock protein 70 (hsp70) promoter of the pCaSpeR-hs transformation vector (Thummel and Pirrotta, 1992), and transgenic flies were generated by standard procedures. A transformant carrying the insert on the third chromosome was brought into TβH^{M18} mutant background by standard crosses.

Sensory assays. Reactivity to sugar was tested in vertical tubes (80 ml) carrying a ring of filter paper (width, 10 mm) at half their height, soaked in either 2 M sucrose solution or water. We recorded the time that starved flies spent on the filter paper (t_{filter}) during a total period of 30 sec (t_{total}), starting from the moment the fly initially touched the paper. We calculated a quantitative response index (RI) as $RI^S = [t_{filter}/t_{total}] \times 100$. The RI^S can vary between (nearly) zero (no time spent on the filter paper) and 100 (total experimental time spent on the filter paper).

For reactivity to electric shock, groups of ~100 flies were tested in a T-maze assay, giving them 1 min to choose between an electrified (12 pulses of 130 V and 1.3 sec duration at 5 sec intervals) and a nonelectrified tube, both equipped with copper wires. From each experiment we counted the number of flies choosing the electrified (N_{shock}) and the nonelectrified tube ($N_{no\ shock}$), and calculated a response index as $RI^E = [(N_{shock} - N_{no\ shock})/(N_{shock} + N_{no\ shock})] \times 100$.

Spontaneous responses to odors were tested again in the T-maze assay giving the flies 2 min to choose between two airstreams (750 ml/min), one scented with the test odor and the other unscented (laboratory air). An RI was calculated from the number of flies choosing the scented airstream (N_{odor}) and the unscented one (N_{air}). $RI^O = [(N_{odor} - N_{air})/(N_{odor} + N_{air})] \times 100$.

Conditioning experiments. Pavlovian training procedures in a T-maze apparatus were applied according to the procedure of Tully and Quinn (1985). Either sucrose or electric shock were used as reinforcers. We modified the original apparatus to handle four groups of animals simultaneously. Starved flies were trained and tested in groups of ~100.

For sugar learning, two training trials were applied if not stated otherwise. During each trial, flies were allowed to feed on 2 M sucrose solution for 30 sec. The sugar was spread onto a filter paper covering the entire training tube. Before and during exposure to the sugar, the first odor (CS^+) was sucked through the tube (flow speed, 750 ml/min). Immediately afterward, flies were transferred for 30 sec to another tube containing a filter paper soaked in water and exposed to a second odor (CS^-).

The memory test started 100 sec after the last training trial (if not stated otherwise). Flies were placed between two air streams (750 ml/min), one scented with the formerly rewarded odor and the other with the nonrewarded odor, and were given 2 min to choose one of them. During a reciprocal experiment with a different group of flies, the first and second odors were exchanged. For each of the two experiments, we counted the number of flies choosing the rewarded (N_{CS+}) and unrewarded odor (N_{CS-}) and calculated the performance index as $PI_{1/2} = [(N_{CS+} - N_{CS-})/(N_{CS+} + N_{CS-})] \times 100$. To rule out nonassociative effects, the PIs of the first and the reciprocal experiment were averaged [$PI = (PI_1 + PI_2)/2$]. Test odorants were diluted 36-fold in paraffin oil (Fluka, Neu-Ulm, Germany) and presented in 15-mm-diameter [ethyl acetate (EA)] and 16-mm-diameter [isoamyl acetate (IA)] cups in the air stream. Under these conditions, naive flies showed no preference between the two odors in the choice test.

For electric shock learning, flies were given 12 current pulses of 130 V and 1.3 sec duration during the 1 min that the tube was scented with the first odor (CS^+). After 45 sec of fresh air, the tube was scented for an additional minute with the second odor but without electric shock (CS^-), followed by another period of 45 sec of air. Test and calculation of PIs were the same as in sugar learning. Contrary to previous publications, we present PIs for electric shock learning as negative values to indicate the avoidance of the CS^+.

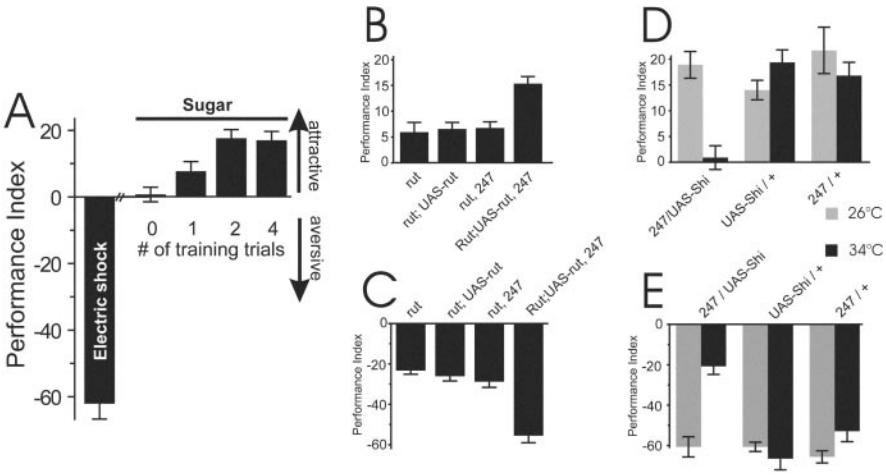

Figure 1. Localizing aversive and attractive memory formation. After Pavlovian training with either sugar or electric shock reinforcement, the same olfactory cues elicit attraction (PI > 0) or repulsion (PI < 0) in the 3 min memory test. *A*, Wild-type Canton-S acquires a strong aversive memory after a single trial of electric shock reinforcement, whereas at least two training trials of sugar reward are required for a significant positive memory score ($p < 0.05$). However, a total of four training trials does not result in an additional increase in performance ($p > 0.05$). Therefore, in all of the following experiments on sugar learning, two training trials are used. In sugar (*B*) and electric shock learning (*C*), mutant *rut*^{2080} flies (including *rut*;UAS-rut+ and *rut*;247-Gal4 flies) show only ~40% of wild-type memory scores [$p < 0.001$, compared with *rut* rescued flies (*rut*;UAS-rut+;247-Gal4) or wild-type in *A*]. Expression of the rut$^+$ cDNA in ~700 Kenyon cells of the 247-Gal4 driver line (*rut*-rescue) is sufficient to restore performance of sugar and electric shock memory to wild-type levels ($p > 0.05$). *D*, Restrictive temperature throughout the experiment (black columns) completely abolishes sugar memory in flies expressing the UAS-*shi*^{ts1} transgene exclusively in the Kenyon cells of the MBs (247-Gal4/UAS-*shi*^{ts1}; $p < 0.001$). No temperature-dependent decrease is found in genetic control flies heterozygous for each of the transgenes alone (247-Gal4/+ and UAS-*shi*^{ts1}/+; $p > 0.05$). At the permissive temperature, all genotypes show normal memory ($p > 0.05$). *E*, Electric shock memory is strongly reduced at the restrictive temperature in flies expressing the Shi-transgene in the Kenyon cells (247-Gal4/UAS-*shi*^{ts1}) compared with genetic controls at either permissive or restrictive temperature ($p < 0.001$). Data are the means ± SEM of at least six experiments.

Results

Olfactory learning was studied in *Drosophila* using either an appetitive (sugar) or an aversive US (electric shock). To make the comparison as stringent as possible, sugar reward learning (Tempel et al., 1983) was adapted to the apparatus of Tully and Quinn (1985), which originally was designed for aversive electric shock learning. For conditioned stimuli (CS^+ and CS^-), the same two odors (EA and IA) were used throughout, and memory performance was always tested in the same binary choice assay using starved flies. The main difference between the two paradigms was that in one case the flies received a sugar reward and, in the other, electric shocks during training.

In the memory test (Fig. 1*A*), wild-type Canton-S flies avoided an odor after having experienced it together with electric shocks [PI = −62.1 ± 4.6 (electric shock memory)], whereas they were attracted by the same odor if it had been combined once with sugar [PI = 7.8 ± 2.4 (sugar memory)]. Because the one-trial PI for sugar learning was inconveniently small, we repeated the training cycle to yield a PI of 17.7 ± 2.6. Because no additional increase was observed with two additional trials, all results on sugar memory used the two-trial training procedure.

Appetitive olfactory memory is located in the mushroom bodies

Both forms of odor learning require the mushroom bodies (MBs), a second-order neuropil of the insect olfactory pathway (Heisenberg et al., 1985), and depend on cAMP signaling as revealed by the impairment in mutants such as *dunce*, *rutabaga* (*rut*), and *amnesiac* (*amn*) affecting different steps in cAMP metabolism (Tempel et al., 1983). Disruption of the cAMP signaling pathway exclusively in the Kenyon cells of the MBs abolishes olfactory learning (Connolly et al., 1996). On the other hand, MB-specific expression of the wild-type form of the type 1 adenylyl cyclase (AC) *rut* in an otherwise *rut* mutant brain rescues performance of olfactory memory (Zars et al., 2000b). Thus, the Kenyon cells of the MBs represent the sufficient set of cells in which cAMP-dependent synaptic plasticity supports memory formation after electric shock reinforcement. To determine whether sugar-dependent memory can also be localized to the MBs, we tested memory performance in genetic mosaics, exclusively restoring *rut* function within ~700 Kenyon cells of the MBs (Zars et al., 2000b; Schwaerzel et al., 2002).

Mutant rut^{2080} flies are impaired in sugar memory (37% of wild-type performance) (Fig. 1*A,B*) and in electric shock memory (42% of wild-type performance) (Fig. 1*C*) to approximately the same extent. With wild-type *rut*-cDNA expressed in the MBs (rut^{2080}; 247-Gal4/UAS-rut^{+}), they show normal sugar and electric shock memory performance (Fig. 1*B,C*), indicating that formation of sugar and electric shock memories is *rut*-dependent and located within the same set of ~700 Kenyon cells (Schwaerzel et al., 2002).

Synaptic output from the same cells is also necessary for memory performance, as shown using the same driver line 247-Gal4 combined with a temperature-sensitive blocker of synaptic transmission [$Shibire^{ts1}$, UAS-shi^{ts1} (Kitamoto, 2001)]. Both sugar and electric shock learning are normal at the permissive temperature (26°C) in 247-Gal4/UAS-shi^{ts1} flies (Fig. 1*D,E*, white bars) but severely impaired at the restrictive temperature (34°C) (Fig. 1*D,E*, black bars). The genetic controls do not show any corresponding decrement in performance. In agreement with previous results (Schwaerzel et al., 2002), suppression of electric shock memory at the restrictive temperature is not complete using the UAS-shi^{ts1} effector gene (Fig. 1*E*), whereas sugar memory is abolished completely. This difference between the two learning paradigms might reflect the different signal-to-noise ratios in the two memory scores.

It is a hallmark of electric shock learning that the output of chemical synapses from the Kenyon cells is required only during retrieval but not during acquisition (Dubnau et al., 2001; McGuire et al., 2001; Schwaerzel et al., 2002), suggesting that memory acquisition does not require sustained neural activity in circuits downstream of the MBs or cross talk between Kenyon cells. To test for the role of synaptic output from Kenyon cells in sugar memory, neurotransmitter release was blocked during either training or testing in 247-Gal4/UAS-shi^{ts1} flies. Figure 2 shows that blocking Kenyon cell ouptput impairs sugar memory only during retrieval but not during acquisition. This finding indicates that, as with electric shock (Schwaerzel et al., 2002, data not shown), sugar reinforcement (Fig. 2) can modulate the Kenyon cells while their output is turned off (electrical synapses have not yet been reported for Kenyon cells). Together, these experiments show that in electric shock and sugar learning, the olfactory memories are *rut*-dependent and localized within the same group of 700 Kenyon cells. To further characterize these memories, we investigated the putative modulatory transmitters of the US pathways in electric shock and sugar learning.

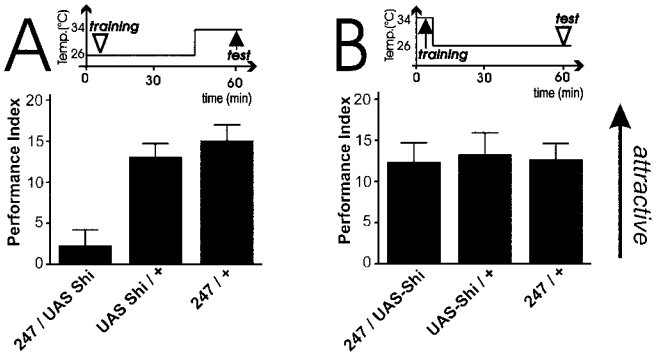

Figure 2. Sugar memory formation is independent of synaptic output from Kenyon cells during acquisition. Synaptic output from the Kenyon cells is selectively blocked during either acquisition or retrieval of memory in 247-Gal4/UAS-shi^{ts1} flies. *A*, When 247-Gal4/UAS-shi^{ts1} flies are trained at the permissive temperature (26°C) and tested 60 min later at the restrictive temperature (34°C), performance is decreased compared with the heterozygous control groups UAS-shi^{ts1}/+ and 247-Gal4/+ ($p < 0.001$). *B*, In contrast, when 247-Gal4/UAS-shi^{ts1} flies are trained at the restrictive and tested at the permissive temperature, memory is not affected compared with the genetic controls ($p > 0.05$). Temperature is shifted to 34°C 15 min before the training. Means and SEMs of six experiments are shown. The same temperature regimen has been applied to electric shock learning with very similar results (data not shown), confirming previous results (Dubnau et al., 2001; McGuire et al., 2001; Schwaerzel et al., 2002).

Octopamine is required for sugar learning

In the honeybee, OA mediates at least some of the reinforcing capacity of sugar reward in an associative olfactory discrimination task (Hammer and Menzel, 1998; Menzel et al., 1999). Therefore, we investigated the role of OA in sugar and electric shock learning in *Drosophila* using the TβH-deficient mutant, $T\beta H^{M18}$. The biosynthetic pathway to OA is blocked in this mutant, and it has no detectable levels of OA (Monastirioti et al., 1996). When tested for electric shock memory, mutant $T\beta H^{M18}$ flies performed like the wild-type controls (Fig. 3*A*), but when tested for sugar memory (Fig. 3*B*), the mutant was severely impaired, showing PIs near zero. Thus, blocking OA synthesis does not cause a general learning deficit but specifically interferes with sugar learning. This phenotype could be rescued by a transgene containing the wild-type *TβH* cDNA downstream of the hsp70 promoter. With heat shock, these flies ($T\beta H^{M18}$; hsp70-*TβH* HS^{+}) showed wild-type performance in sugar memory (Fig. 3*C*). The heat shock itself had no memory-enhancing effect in mutant $T\beta H^{M18}$ flies, supporting the implicit assumption that OA levels can be rescued by restoring enzymatic function.

Besides lacking OA, the mutant $T\beta H^{M18}$ accumulates tyramine, its direct precursor (Monastirioti et al., 1996) and a functional neurotransmitter (Nagaya et al., 2002). To test whether the increase in tyramine or the absence of OA causes the phenotype, $T\beta H^{M18}$ mutant flies were fed OA (10 mg/ml) for either 1 or 18 hr before training. OA-fed mutant $T\beta H^{M18}$ flies performed like wild-type flies (Fig. 3*D*). Surprisingly, a feeding period of as little as 1 hr was sufficient to restore the learning–memory defect, indicating that OA is taken up by the neurons via a rapid mechanism. This is in line with several reports in bees, in which the feeding of OA increases levels of this neurotransmitter in the brain (Schulz and Robinson, 2001; Barron et al., 2002) and behavioral effects occur within the range of minutes after uptake (Pankiw and Page, 2003).

To distinguish between an effect of OA during acquisition and

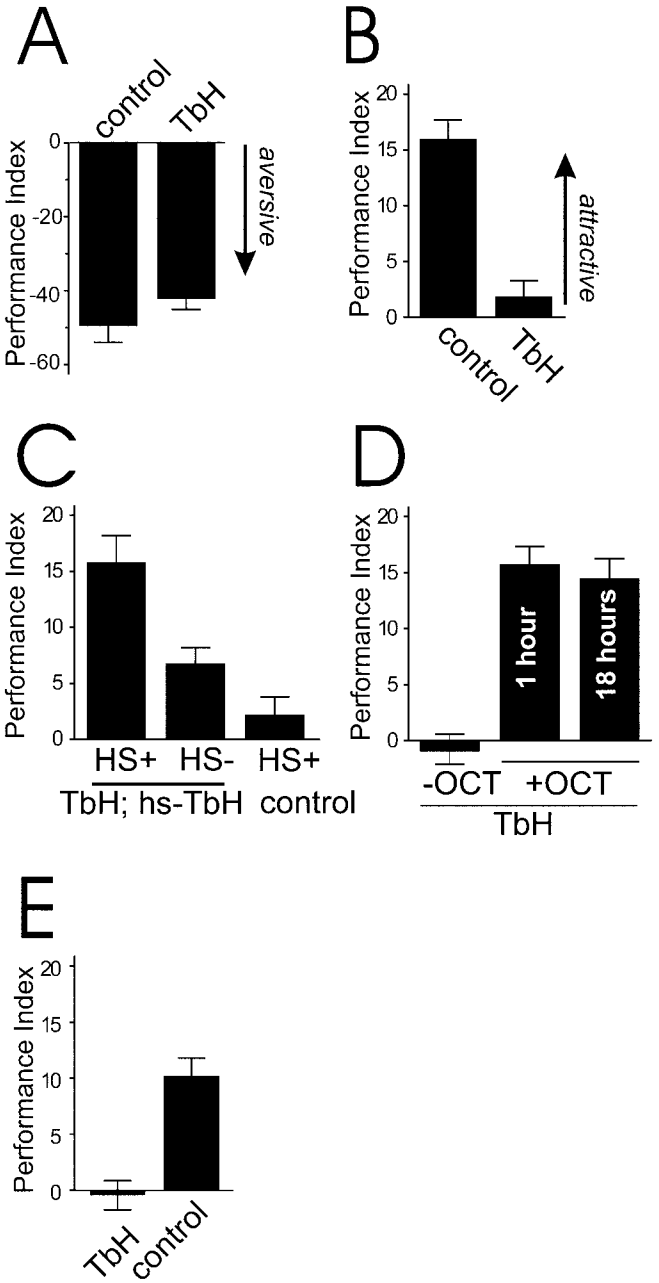

Figure 3. Octopamine is necessary for the acquisition of sugar memory. *A*, Flies lacking octopamine caused by a mutation in the mutant $T\beta H^{M18}$ show normal electric shock memory (3 min memory; $p > 0.05$ compared with control lines described in Materials and Methods; note that electric shock memory of the $T\beta H$ mutant and control lines is slightly lower than that of our CS wild-type). *B*, In contrast, no sugar memory is detected in $T\beta H$ mutant flies ($p < 0.001$). *C*, Mutant $T\beta H^{M18}$ flies with a heat-shock inducible $T\beta H^{+}$ cDNA ($T\beta H$; hs1) show normal sugar memory after heat shock (HS^{+}) ($p < 0.05$). Neither the hs1-construct alone ($T\beta H$; hs1 − HS^{-}) nor the heat shock itself have a significant effect compared with the $T\beta H^{M18}$ mutant ($p > 0.05$) *D*, Mutant $T\beta H^{M18}$ flies show normal sugar memory after feeding on octopamine (10 mg/ml) for 1 or 18 hr before the experiment ($p < 0.001$). *E*, Octopamine is required during acquisition. If $T\beta H^{M18}$ flies are fed octopamine for 1 hr starting right after the training, no memory is detected, although the feeding itself does not abolish 1 hr memory in the control flies ($p < 0.001$). Data are means and SEMs of six experiments (except for 12 experiments on the $T\beta H^{M18}$ mutant in *A* and *B*).

retrieval, OA was fed to the mutant flies just after training, and memory was tested 1 hr later. No rescue of performance was found in these flies, whereas in control flies the same OA feeding regimen had no deleterious effect (Fig. 3*E*). Therefore, we conclude that OA is required during acquisition. Whether it is also needed during retrieval cannot be decided.

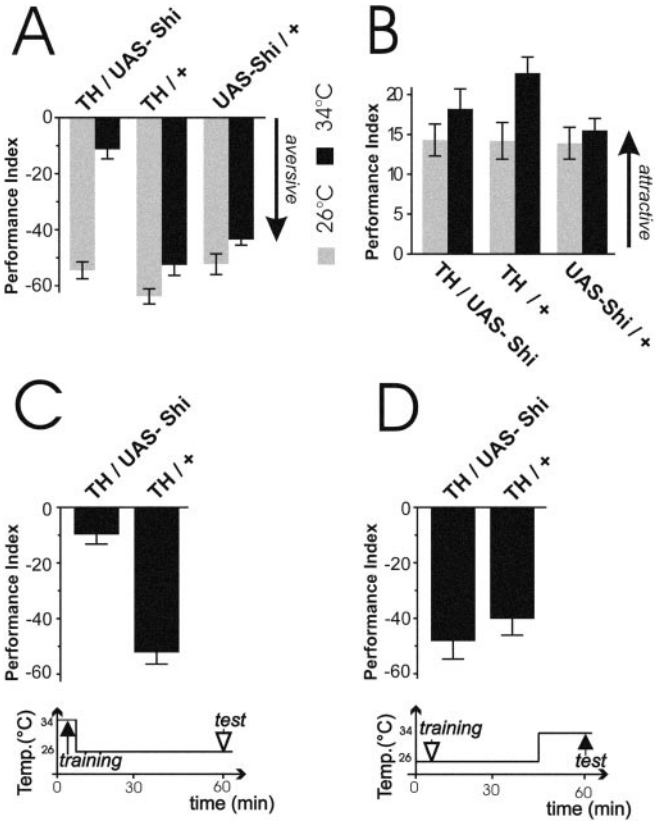

Figure 4. DA is necessary for acquisition but not retrieval of electric shock memory. *A*, Blockade of transmission from (putatively) dopaminergic neurons in TH-Gal4/UAS-shi^{ts1} flies at 34°C severely disturbs electric shock 3 min memory ($p < 0.001$). This temperature-dependent decrease in performance is absent in the genetic controls (TH-Gal4/+ and UAS-shi^{ts1}/+) ($p > 0.05$). *B*, In contrast, no temperature-dependent decrease in sugar memory is observed in any of the groups (TH-Gal4/UAS-shi^{ts1}, TH-Gal4/+, UAS-shi^{ts1}/+) ($p > 0.05$, except for TH-Gal4/+). Here, performance is significantly increased at the restrictive temperature: $p < 0.05$). *C*, One hour memory in electric shock learning is strongly decreased if transmission in dopaminergic neurons is blocked during acquisition only ($p < 0.001$). Flies are transferred to a restrictive temperature 15 min before the experiment. *D*, Memory is not affected if the neurons are blocked only during retrieval ($p > 0.05$). Data are means and SEMs of six experiments.

Dopamine neurons are involved in electric shock learning

Previous experiments with flies carrying temperature-sensitive alleles of the dopa-decarboxylase gene involved in the biosynthesis of both dopamine and serotonin had indicated a role for one or both of these monoamines in electric shock olfactory learning (Tempel et al., 1984). Performance in olfactory memory correlated with the concentrations of these substances in head homogenates. However, this effect could not be reproduced a few years later (Tully, 1987). Here, we focused exclusively on the role of DA in olfactory learning using the transgenic line TH-Gal4 carrying Gal4 under the control of the regulatory region of the tyrosine hydroxylase (*TH*) gene (Friggi-Grelin et al., 2003). TH catalyzes the first step in DA biosynthesis, and the *TH* gene is selectively expressed in most or all dopaminergic neurons in the CNS. Thus, the TH-Gal4 line provides specific experimental access to dopaminergic neurons (Friggi-Grelin et al., 2003). To block chemical synapses in these cells, the TH-Gal4 driver was combined with the UAS-shi^{ts1} effector gene above. Using electric shock as reinforcement, olfactory memory in TH-Gal4/UAS-shi^{ts1} flies was severely

Table 1. Sensory acuity tests

Genotype	Shock avoidance	Odorant avoidance				Sugar reactivity
		EA		IAA		
		1:36	1:6	1:36	1:6	
Rut^{2080}	68.6 ± 3.1	13.8 ± 5.9	16.9 ± 5.3	9.4 ± 3.4	76.6 ± 3.9	78.0 ± 6.0
Rut^{2080};UAS-rut^{+}	76.6 ± 3.0	8.5 ± 4.7	23.4 ± 5.6	14.8 ± 5.8	70.6 ± 1.7	81.0 ± 5.7
Rut^{2080};247	72.6 ± 3.0	15.3 ± 4.0	36.8 ± 8.2	4.8 ± 1.7	64.2 ± 5.1	74.5 ± 6.3
Rut-rescue	71.7 ± 3.8	7.2 ± 3.0	35.0 ± 7.0	16.9 ± 6.9	72.9 ± 5.6	85.8 ± 4.0
247/UAS-shi (26°C)	76.2 ± 2.9	−3.0 ± 4.0	36.0 ± 4.8	11.5 ± 4.6	60.8 ± 2.2	83.2 ± 7.9
247/UAS-shi (34°C)	78.2 ± 2.1	−8.1 ± 8.2	41.7 ± 6.7	16.3 ± 2.0	44.6 ± 8.4	72.3 ± 7.9
$T\beta H^{+}$ control	ND	ND	ND	ND	ND	81.8 ± 5.1
$T\beta H^{M18}$	ND	ND	ND	ND	ND	80.8 ± 5.6
TH/UAS-shi (26°C)	79.0 ± 4.4	ND	ND	ND	ND	ND
TH/UAS-shi (34°C)	77.0 ± 6.7	ND	ND	ND	ND	ND

Electric shock, sugar, and olfactory sensitivities of experimental and control animals. Odors were tested at the normal (dilution 1:36) and a sixfold higher concentration. Mutant $T\beta H^{M18}$ flies were tested only for sugar sensitivity and TH/UAS-shi^{ts1} flies only for sensitivity to electric shock, because they had normal memory scores in the alternative learning assays. No significant differences ($p > 0.05$) in any of the assays were detected between experimental and control flies. For each experiment, the means of six (and, in the case of sugar, the means of at least 20 experiments, except for 40 experiments on the $T\beta H^{M18}$ mutant) are shown. Errors are SEMs. ND, Not determined.

impaired at the restrictive temperature, whereas the genetic control flies showed normal performance (Fig. 4*A*, black bars). The same experiment at the permissive temperature resulted in normal memory performance in all genotypes (Fig. 4*A*, gray bars). In contrast, memory performance in sugar reward learning in TH-Gal4/UAS-shi^{ts1} flies was not impaired at the restrictive temperature (Fig. 4*B*). These experiments show that synaptic output from dopaminergic neurons is important in electroshock learning but is dispensable for learning–memory with sugar reinforcement. This is the opposite result from that obtained by OA depletion above (Fig. 3), suggesting that the two catecholamines are differentially involved in the two reinforcement pathways for sugar reward and electric shock.

To test this hypothesis further, flies were subjected to the restrictive temperature only during either acquisition or retrieval. When trained at the restrictive and tested at the permissive temperature, TH-Gal4/UAS-shi^{ts1} flies showed very little electric shock memory, whereas control flies were not affected by this temperature regime (Fig. 4*C*). High temperature during retrieval has no deleterious effect on electric shock memory in TH-Gal4/UAS-shi^{ts1} flies (Fig. 4*D*).

All genotypes showing a memory deficit and the appropriate controls were tested for spontaneous responses to odors, sugar, and electric shock. As documented in Table 1, *rut* mutant flies with and without the rescue constructs perform similarly in these assays. In flies expressing the UAS-shi^{ts1} transgene either in the MBs (247-Gal4/UAS-shi^{ts1}) or in the DA-positive cells (TH-Gal4/UAS-shi^{ts1}), the different temperatures used throughout the memory tests had no negative effect on the detection of relevant cues. Also, loss of OA in $T\beta H^{M18}$ mutant flies did not affect sugar detection. In the case of the DA- and OA-affected flies, the reinforcer-specific nature of the memory defects explicitly excluded a defect in olfactory acuity. Thus, the changes in olfactory memories are likely not to be caused by changes in sensory processing.

Discussion

Our results support three major conclusions. First, during the association of an olfactory cue with either a sugar reward or an electric shock punishment, both forms of olfactory memories require cAMP signaling within the same 700 Kenyon cells of the MBs. Second, for memory retrieval but not acquisition with either of the two reinforcers, output from this same set of cells is required. Hence, the memory must be formed and stored upstream of this synaptic level. Third, sugar and electric shock reinforcement are mediated by different modulatory neurotransmitters, DA in case of electric shock and OA in case of sugar reward. These findings confirm and extend previous work, concluding that output synapses of Kenyon cells are the site of olfactory memory (summarized in Heisenberg, 2003).

Appetitive and aversive olfactory memories are localized to the same neuropil

Associative behavioral adaptations are mediated by the plasticity of synapses within neural circuits (Kandel, 2001). But what are the smallest units of memory? Do they correspond to the modulation of a single synapse or to the concerted change of many or all synapses in a circuit? Attempts to localize olfactory memory in the *Drosophila* brain (Heisenberg et al., 1985; de Belle and Heisenberg, 1994; Connolly et al., 1996; Zars et al., 2000a,b; Dubnau et al., 2001; McGuire et al., 2001; Schwaerzel et al., 2002) have provided partial answers to these questions.

In many species, including *Aplysia*, mouse, and *Drosophila*, the type-1 AC has been shown to be critical in synaptic plasticity (Lechner and Byrne, 1998; Villacres et al., 1998; Kandel, 2001; Antonov et al., 2003). No cases of cAMP-independent associative synaptic or behavioral plasticity have yet been reported conclusively. In *Drosophila*, one of the corresponding mutants, *rut*, shows abnormal performance in every learning paradigm tested so far. By identifying the minimally sufficient set of neurons that in a *rut* mutant brain need to express a wild-type form of the RUT protein to restore a particular memory performance, one can localize the memory trace of the corresponding behavioral adaptation. This approach was successfully applied to two types of memory in *Drosophila*, heat box memory (Zars et al., 2000a) and olfactory memory (Zars et al., 2000b).

Using the same approach in a side-by-side comparison between sugar and electric shock reinforcement, our results show that wild-type *rut*-AC expression in ~700 Kenyon cells (25–30% of total) rescues memory performance for both kinds of reinforcement. Thus, aversive and appetitive olfactory memories are both mediated by synaptic plasticity in the same group of cells.

Attributing the rescue to an effect on synaptic plasticity in the adult Kenyon cells disregards the possibility that the genetic manipulation might rescue a developmental function of *rut*-AC, necessary later in the adult for memory. Several lines of evidence argue for an adult function, but only recently has a new genetic manipulation been designed that definitely rules out a developmental effect. Use of a temperature-sensitive Gal80, a suppressor of Gal4, ensured that wild-type *rut*-AC was expressed only during

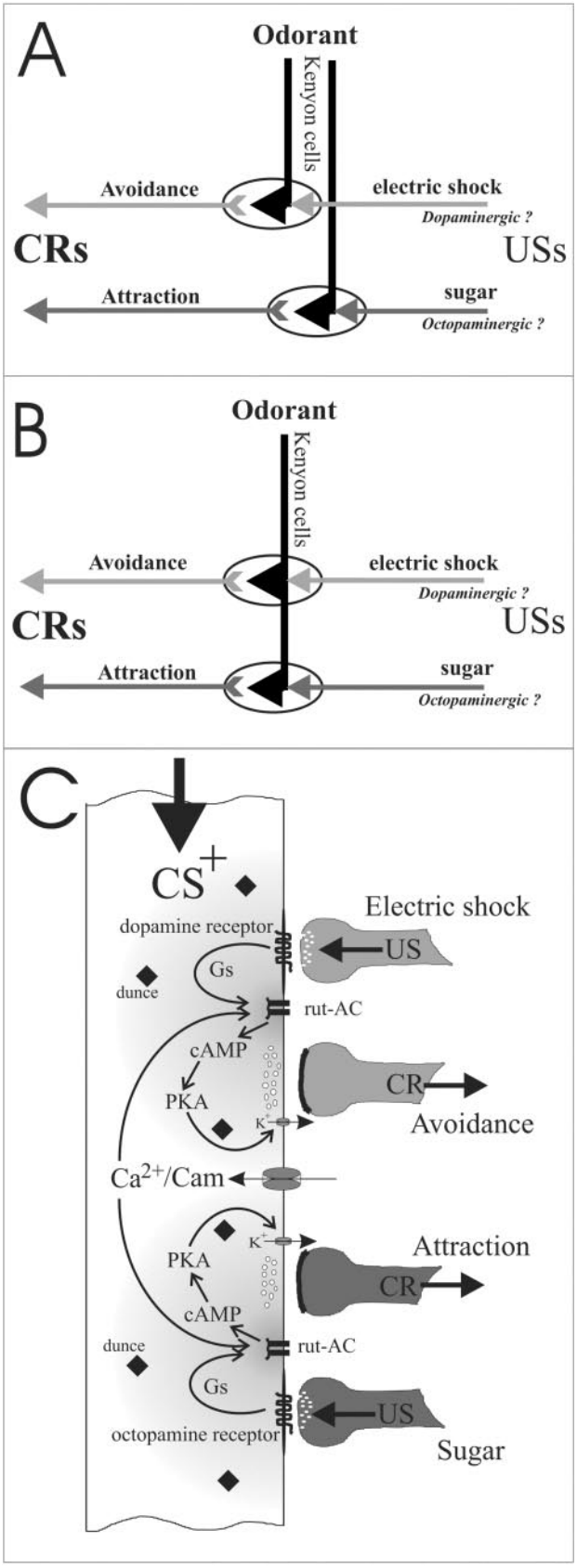

Figure 5. Alternative representations of olfactory memory traces. In the MBs, modulatory neurons representing specific USs (e.g., electric shock or sugar) have synaptic input to Kenyon cells representing the fly's odor space (all perceivable odors). Each modulatory neuron is the functional companion of an MB output neuron (CR neuron), which can mediate a conditioned response. A CR neuron will be recruited to respond to a particular odorant if the companion US and the odorant coincide. *A*, If the odor space is represented in the MBs several times in parallel (i.e., by separate sets of Kenyon cells), each set could be connected to just one US–CR pair (schematized as circles). *B*, If the odor space is represented in the MBs only once, the Kenyon cells would have to be connected to several US–CR pairs. *C*, In this case, different memory traces would be stored in the same set of Kenyon cells at different occasions using the same molecular mechanism independently at different locations along the axon.

adulthood (S. E. McGuire, P. T. Le, R. D. Davis, personal communication).

Although our experiments do not specify where in the Kenyon cells cAMP signaling is required, the existing evidence suggests a presynaptic mechanism at Kenyon cell output synapses. At the *Drosophila* larval neuromuscular junction, cAMP signaling is presynaptically involved in plasticity (Zhong and Wu, 1991). For the sensory-motor synapses mediating classical conditioning of the gill withdrawal reflex in *Aplysia* (one of the best-studied preparations for this problem), it has been firmly established that the cAMP cascade is involved presynaptically (Antonov et al., 2003). Again, to our knowledge, no conclusive example of a postsynaptic contribution of cAMP signaling has been reported.

In the *Aplysia* synapses above, plasticity has a postsynaptic component based on a mechanism resembling the NMDA receptor and long-term potentiation in mammals. In *Drosophila* olfactory conditioning, a similar postsynaptic contribution is unlikely to play a role during the first 3 hr, because this effect would require neurotransmitter release from the presynapse, which can be blocked during acquisition and memory retention without a deleterious effect on memory, using shi^{ts1}, a conditional blocker of synaptic transmission (McGuire et al., 2001; Dubnau et al., 2001; Schwaerzel et al., 2002).

The tentative presynaptic effect of cAMP signaling locates the synaptic plasticity underlying olfactory memory to the synapses connecting Kenyon cells to MB output neurons. These are found in the MB lobes, including the rostral peduncle and spur (Schürmann, 1987; Yasuyama et al., 2002). Additional support for cAMP signaling to occur in the lobes rather than calyx is derived from the "memory" gene *amn*, which has been shown to be involved in cAMP regulation (Feany and Quinn, 1995). The putative AMN neuropeptide is required exclusively in two prominent neurons, the so-called dorsal paired medial neurons, that profusely innervate the MB lobes (Waddell et al., 2000). Other components of the cAMP pathway such as *rut* and receptors for DA and OA [summarized by Crittenden et al., 1998; for dDA1 (Kim et al., 2003)], all are predominantly expressed in the adult MB lobes.

OA and DA differentiate between sugar and electric shock reinforcement

Associative synaptic plasticity depends on the convergence between impulses from two signals, the CS and the US. Considering the proposed role of *rut*-AC as a molecular coincidence detector (Dudai et al., 1988), one can assume that the MB input neurons carrying the US for sugar and electric shock should also connect to the lobes, although their direct anatomical identification is pending.

Our results show that acquisition of an olfactory memory with electric shock is dependent on the dopaminergic system, whereas acquisition with sugar depends on the octopaminergic system. OA as neurotransmitter in sugar learning seems to be conserved between *Drosophila* and the honeybee. The bee VUMmx1 neuron, an unpaired neuron localized in the subesophageal ganglion, appears to be octopaminergic and has been shown to carry some of the reinforcing properties of the US. It innervates the calices,

antennal lobes, and lateral protocerebrum but not the MB lobes (Hammer and Menzel, 1998). Nevertheless, the learning paradigms used [individual conditioning of the proboscis extension reflex in bees vs the population-based conditioned osmotaxis in *Drosophila* (Tully, 1986)] are different; therefore, it might be too early to compare the sugar memories in the bee and *Drosophila* with respect to its organization on a circuit level. Unfortunately, the role of the monoamines in aversive conditioning has not been tested in bees.

These findings raise the question of whether the effects of the two catecholamines on electric shock and sugar learning can be generalized to other appetitive and aversive reinforcers and to positive and negative behavioral modulation in general. In the monkey, midbrain dopaminergic neurons have been described that carry the reinforcing properties of a US in appetitive but not aversive conditioning (Mirenowicz and Schultz, 1996). It will be interesting to see whether a similar dissociation between modulatory systems for appetitive and aversive conditioning, with the contingency between good–bad and monoamines exchanged, also applies to the monkey, and, potentially, to humans.

Models of separate odor memories in the mushroom body

Separate memory traces for electric shock and sugar conditioning had been suggested previously, because these have different kinetics of consolidation and decay (Tempel et al., 1983). The distinct effects of the two catecholamines in the reinforcement pathways discovered here underline this notion. Surprisingly, however, our localization experiments assign the two memories to the same neuropil structure, a subset of 700 Kenyon cells.

Based on the functional anatomy of the olfactory pathway, odors are assumed to be represented in the MBs by specific sets of Kenyon cells (Heisenberg, 2003). For an odorant to become predictive of a given reinforcing event (e.g., sugar or electric shock), the output synapses of this set of Kenyon cells should be modified to drive an MB output neuron mediating the conditioned response (e.g., approach or avoidance). MB input neurons representing the appropriate reinforcers (e.g., sugar or electric shock) should provide the modulatory input. At present it is still not known whether the identified monoamines, OA and DA, are the modulatory neurotransmitters at the site of synaptic plasticity or act further upstream in the respective US pathway. The former alternative is supported by the observation that the MB lobes are equipped with DA and OA receptors that can couple to AC of the *rut* type via Gs protein (Han et al., 1996; Han et al., 1998, Crittenden et al., 1998; Kim et al., 2003). In addition, the neurons relevant for electric shock learning send TH-Gal4-positive fibers to the MB neuropil at the level of the spur and the vertical lobes (supplementary Fig. 1, available at www.jneurosci.org).

As mentioned above, the respective output neurons are prespecified to announce sugar or electric shock and so are the modulatory neurons. Hence, these form specific pairs (US–CR pairs) that are functionally linked to adapt the CR neuron to one of many odors in the conditioning events. Two schemes can be proposed of how the US–CR pairs and sets of Kenyon cells might be interconnected. The two diagrams (Fig. 5*A*,*B*) differ with respect to the organization of odor representations in the MBs. If the same odors were represented by several sets of Kenyon cells, each set could be connected with just one US–CR pair (Fig. 5*A*). In this case, sugar and electric shock memories could be formed in different sets, both specifically responding to the same odorant, but one being modulated by OA, the other by DA. Both these sets would be contained within the set of 700 Kenyon cells of the 247-Gal4 driver line. Alternatively, if each odor is represented by only one set, the Kenyon cells should be responsive to multiple modulatory inputs (Fig. 5*B*). In this case, both memories would be formed within the same cells. The synapses of the US–CR pairs with the Kenyon cells should be closely associated, and these synaptic domains would have to be independently modulated by cAMP signaling (Fig. 5*C*). Because *Drosophila* can associate many events (US) with odors, Kenyon cells may accommodate many such US–CR pairs. A requirement for space at the Kenyon cells may then explain the stalk-like structure of MBs. At present it is not possible to distinguish between these two alternatives.

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