Behavioral/Systems/Cognitive

Induction of cAMP Response Element-Binding Protein-Dependent Medium-Term Memory by Appetitive Gustatory Reinforcement in *Drosophila* Larvae

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The fruit fly *Drosophila melanogaster* has been successfully used as a model animal for the study of the genetic and molecular mechanisms of learning and memory. Although most of the *Drosophila* learning studies have used the adult fly, the relative complexity of its neural network hinders cellular and molecular studies at high resolution. In contrast, the *Drosophila* larva has a simple brain with uniquely identifiable neural networks, providing an opportunity of an attractive alternative system for elucidation of underlying mechanisms involved in learning and memory. In this paper, we describe a novel paradigm of larval associative learning with a single odor and a positive gustatory reinforcer, sucrose. Mutant analyses have suggested importance of cAMP signaling and potassium channel activities in larval learning as has been demonstrated with the adult fly. Intriguingly, larval memory produced by the appetitive conditioning lasts medium term and depends on both *amnesiac* and cAMP response element-binding protein (CREB). A significant part of memory was disrupted at very early phase by CREB blockade without affecting immediate learning performance. Moreover, we also show that synaptic output of larval mushroom body neurons is required for retrieval but not for acquisition and retention of the larval memory, including the CREB-dependent component.

Key words: amnesiac; *shibire*; *dunce*; *rutabaga*; mushroom bodies; olfactory memory

Introduction

Associative learning is one of the most essential functions of the brain enabling animals to predict vital environmental stimuli by experience. Because of its advanced genetics and molecular biology, *Drosophila* has been used as one of the successful model organisms for elucidation of underlying neural mechanisms. Systematic genetic studies have identified multiple phases of memory acquisition and consolidation (Quinn and Dudai, 1976; Tully et al., 1994b; Dubnau and Tully, 1998): acquisition or learning (LRN), short-term memory (STM), middle-term memory (MTM), anesthesia-resistant memory (ARM), and long-term memory (LTM). Whereas the cAMP signaling cascade plays an important role in LRN and STM (Byers et al., 1981; Livingstone et al., 1984; Drain et al., 1991; Levin et al., 1992), MTM is mediated by *amnesiac* (*amn*) (Quinn et al., 1979) and LTM by cAMP response element-binding protein (CREB)-dependent gene expression (Yin et al., 1994). Mutant analyses also indicate that memory processing is sequential from LRN to MTM but that consolidation of ARM and LTM might then occur in parallel (Isabel et al., 2004). Neuroanatomical studies have identified brain structures that are important to learning and memory in the adult fly (Heisenberg, 2003). In particular, neural output from mushroom body (MB) neurons is essential for memory retrieval but not for memory acquisition and retention (Dubnau et al., 2001; McGuire et al., 2001), suggesting that olfactory memory might be localized to MB neurons (Heisenberg, 2003; Gerber et al., 2004b).

Up to now, most of the *Drosophila* learning and memory studies have used adult flies with electric shocks (Quinn et al., 1974; Tully and Quinn 1985) (for review, see Dubnau and Tully, 1998; Waddell and Quinn, 2001). The adult paradigm enjoys robust behavioral responses, but the elaborate neuropil structures of its brain, although far more simpler than mammalian brains, still set an obstacle to unequivocal identification of the neural circuitry. In contrast, the *Drosophila* larva has a profoundly simple brain and is used in studies on a range of behaviors, such as foraging and social behaviors (Sokolowski, 1998; Wu et al., 2003; Xu et al., 2004). In addition, whereas the adult brain is formed through complex reorganization during metamorphosis, the larval brain is a simple extension of the embryonic axonal plan (Nassif et al., 2003). However, despite potential advantages, learning and memory studies with the *Drosophila* larva have been limited (Aceves-Piña and Quinn, 1979; Heisenberg et al., 1985; Tully et al., 1994a; Dukas, 1998), although a series of studies was reported

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recently (Scherer et al., 2003; Gerber et al., 2004a; Hendel et al., 2005; Michels et al., 2005; Neuser et al., 2005).

In this paper, we describe a novel single odor paradigm for olfactory associative learning with the *Drosophila* larva. We conditioned early third-instar larvae to associate a given odor with an appetitive gustatory reinforcer, sucrose (SUC). This larval conditioning induces MTM-like memory that depends on *amn* and CREB. Moreover, we show that synaptic output of larval MB neurons is required for retrieval but not for acquisition and retention of the larval memory, including the CREB-dependent component.

Materials and Methods

Fly stocks

The fly stocks used were as follows: wild-type Canton S (CS); STM mutants, *rutabaga* (*rut*)^{1} (Livingstone et al., 1984), *rut*^{2080} (Levin et al., 1992), and *dunce* (*dnc*)^{1} (Dudai et al., 1976; Byers et al., 1981); MTM mutant *amn*^{28A} (Moore et al., 1998); potassium channel mutants *ether a go-go* (*eag*)^{4PM} (Cowan and Siegel, 1984) and *Shaker* (*Sh*)^{rk0120} (Budnik et al., 1990); *hs-dCREB2-b* (line 17-2) (Yin et al., 1994); *UAS-shibire*^{ts1} (*shi*^{ts1}) inserted on chromosome III (Kitamoto, 2001); and MB-Gal4 enhancer trap line OK301 (Connolly et al., 1996) and 201Y (Yang et al., 1995). Stocks were kept at 25°C on a standard food consisting of 5.5 g/l agar, 40 g/l yeast extract, 90 g/l cornmeal, and 100 g/l glucose. Propionic acid (3 ml/l) and *n*-butyl-*p*-hydroxybenzoate (0.7 g/l) were added as fungicides.

Odorants and reinforcers

Chemicals of the highest grade were from Nacalai (Tokyo, Japan), except for acetic acid, hexylacetate, and heptylacetate (Wako, Tokyo, Japan) and isoamylacetate (Sigma-Aldrich, St Louis, MO).

Learning and memory experiments

Temperature. Unless otherwise noted, larval behavioral experiments were done at 25°C.

Preparation of larvae. Larvae were raised with the standard food described above without propionic acid because it affected larval olfactory response. Fly eggs were collected for 2 h on fresh food. To ensure homogenous animal population, staged larvae (72–76 h after egg laying), shortly after the second larval molt, were used in all experiments. Larvae were harvested out of food with 15% glucose solution and collected on a sieve (aperture, 500 μm). After three rinses with distilled water (DW), animals were picked up with a paint brush and transferred to training plates.

Olfactory training. Freshly prepared 2.5% agar plates (85 mm Petri dishes) were used. Reinforcer solution (1.0 ml of 1 M SUC or DW for control) was spread over agar so that the solution stays as liquid film on the agar surface. Over-dried plates were not used. Undiluted odor (10 μl) was spotted on a filter disk (55 mm in diameter) placed on the inside of the lid (see Fig. 1). Larvae were transferred with a paint brush on the training plate. Typically, several hundred animals are placed on a plate. The lid was closed immediately and kept for 30 min; thus, larvae were exposed to a given odor in conjunction with the reinforcer. After training, larvae were harvested with DW from the training plate, gently rinsed three times with DW in a 100 ml beaker to remove residual odorants and reinforcers, and transferred onto test agar plates.

Measurement of learning performance. Larval olfactory behavior was measured by a single odor preference test adapted from Heimbeck et al. (1999) using 85 mm Petri dishes filled with 2.5% agar. Small filter disks (10 mm) were placed on the opposite sides of the plate. A plastic lid of 1.5 ml Eppendorf tube was used as a support to avoid diffusion of the odorant through agar. For each test, 50–100 larvae were transferred to the center of the plate. Right after larval transfer, 2.5 μl of undiluted odorant was spotted on one of the filter disks and none on the opposite side (control). Then the lid was placed immediately, and the plate was kept for 3 min on the bench. For response index (RI) determination, animals that migrated within the 3 cm semicircular areas around the filter disks were counted (see Fig. 1). Under this criteria, 80–90% of animals applied on the test plate were counted: RI = (the numbers of animals in the odor area − the number of animals in the control area)/total number of animals counted. As an indicator of learning performance in association with SUC, ΔRI was calculated as the difference between the RIs of linalool LIN/SUC larvae and control LIN larvae, which are exposed to LIN in association with DW. For memory retention tests, larvae were trained as above, rinsed, transferred to a fresh 2.5% agar plate, and kept for the indicated time until olfactory preference test.

Temporal dissociation tests

For testing temporal association of odor and reinforcement, larvae were placed on a agar plate and subjected to the first treatment for 30 min. Animals were then harvested with DW, gently rinsed three times with DW in a 100 ml beaker, and transferred to another agar plate, on which they were subjected to the second treatment for 30 min. Larvae were then harvested with DW, rinsed, and transferred to test agar plates.

Induction of dCREB2-b with heat shock

For heat induction, staged early third-instar larvae (72–76 h after egg laying) were grown with 10 ml of food in small vials and were heat shocked for 30 min at 37.5°C. To ensure efficient and uniform heat shock, vials were submerged in a 37.5°C water bath up to the foam plug. After heat shock, the vials were returned to the bench (25°C) and kept for 30 min for recovery from heat sock. Larvae were then harvested from the vials as described above and used for associative training and test.

Inactivation of neural transmission with shi^{ts1}

The permissive and restrictive shi^{ts1} temperatures used in this work were 25°C and 30°C, respectively. Experiments were done in temperature-controlled rooms each adjusted to 25°C and 30°C. All instruments and reagents were prewarmed to the indicated temperature before experiments. First, larvae were trained as described above in the first temperature room. Then, we moved the training plates containing the larvae to the secondary temperature room. To ensure constant temperature, the plates were kept in a foam-polystyrene insulator box during room transfer. Larvae were then harvested from the training plate with DW (secondary temperature), gently rinsed three times with DW, harvested on a mesh, and transferred with a paint brush onto a fresh agar plate at the second temperature.

Olfactory response test

Larval olfactory response was measured using the same procedure used for RI determination in the learning assay. For each test, ~50–100 larvae were placed on the center of the test plate. Undiluted odorant (2.5 μl) was spotted on the test disk. RI was determined by counting larvae after 3 min.

Gustatory response test

Larval gustatory response was measured according to Lilly and Carlson (1990) with slight modifications. Plastic plates with a median separator (85 mm; Eiken, Tochigi, Japan) were used. The control half was filled with 0.5% agar in water and the test half with 0.5% agar in 1 M SUC. Plates were allowed to solidify for 2 h at room temperature and were used immediately to avoid diffusion of the test substance. Fifty to 100 larvae were lined along the separator and allowed to move on the agar surface for 5 min. Gustatory RI was calculated: RI = (the numbers of animals on the test half − the number of animals on the control half)/total number of the animals. Animals that left the gel surface (<10% of the applied) were not counted.

Statistics

For simplicity, data are presented based on parametric tests (Student's *t* test and ANOVA) in all figures. However, considering the small number of samples, we also examined the data with nonparametric tests (either the Mann–Whitney *U* test or the Kruskal–Wallis test) to further examine statistical significance. The conclusions were unaltered between the parametric and nonparametric tests. For multiple comparisons among relevant groups, the Dunnett's method and the Dunn's method were used in conjunction with ANOVA and the Kruskal–Wallis tests, respectively.

Histology

Third-instar larval brains were immunostained as described previously (Kurusu et al., 2002). The following antibodies were used: rabbit anti-

Table 1. Classification of odorants

Strong attractants	Diacetyl, methylacetate, butyricacid
Moderate attractants	GVA, LIN, PA, ethylacetate, propylacetate, 1-hexanol, isoamylacetate, propionic acid, acetic acid, 2-propanol, cyclohexanone, 1-octanol, geraniol, ethylpropionate, geranylacetate, acetophenone, ethylbutyrate, 3-octanol, 4-methylcyclohexanol, hexylacetate, heptylacetate, benzaldehyde, cyclohexanol
Moderate repellents	*N*-octylacetate, nonanol
Neutral or unperceived	Ethanol

Olfactory response criteria are as follows: moderate attractants, $0 < RI < 0.8$; moderate repellents, $0 > RI > -0.5$. Strong attractants attracted the majority of larvae (>90%) to the odor side. No strong repellent was found in this screen.

green fluorescent protein (GFP) (MBL, Nagoya, Japan) diluted 1:500; and Alexa-conjugated secondary antibody (Molecular Probes, Eugene, OR) diluted 1:400. Confocal images were captured with Zeiss (Oberkochen, Germany) LSM510.

Results

A single odor paradigm for larval olfactory associative learning

The larval olfactory system is significantly simpler than the adult system with only 21 odorant receptor neurons (Python and Stocker, 2002; Kreher et al., 2005; Ramaekers et al., 2005). To find chemicals that are suitable for larval learning assays, we first examined 30 odorants for naive larval chemotactic behavior and classified them in four groups based on their attractiveness (Table 1). We then examined the 19 moderate attractants for their effectiveness on larval appetitive olfactory conditioning (Fig. 1*A*). Larvae were exposed to an odor for 30 min in association with 1 M SUC spread on agar. After conditioning, larvae were gently rinsed with DW to remove SUC and tested for olfactory response on the test plate. For 10 of the 19 odorants, animals that received the odor with 1 M SUC showed enhanced migration to the conditioned odor with significantly higher RI than control larvae, which had been exposed to the same odor but in conjunction with DW (Fig. 1*B*). Among the odorants examined, we chose for the subsequent experiments LIN, Pentyl acetate (PA), and γ-valerolactone (GVA), which gave the largest RI increments in LIN/SUC conditioning.

Larval odor response changes are associative and odor specific

To examine whether the increase of RI after conditioning is attributable to associative learning, we performed a set of control experiments. Significant RI increase was observed only when larvae were trained with LIN in association with SUC (Fig. 2*A*, LIN/SUC); RI did not change significantly from naive larvae when larvae are trained with LIN in association with DW (LIN) or SUC alone (SUC). Notably, neither LIN nor SUC alone resulted in habituation of larval olfactory responses compared with naive animals. Similar results were obtained with PA, except that conditioning with PA in association with DW led to slight desensitization (Fig. 2*B*, PA). In contrast, conditioning with GVA in association with DW (Fig. 2*C*, GVA) led to strong desensitization. However, the associative conditioning with GVA/SUC overcame the suppression.

We then asked whether the enhancement of larval response requires simultaneous exposure to both the odor and the reinforcer. As a temporal dissociation control, larvae were successively exposed first to SUC and then to LIN or vise versa (Fig. 2*D*). Whereas simultaneous exposure to both LIN and SUC (conditioning 1) resulted in enhanced olfactory response as described above, the dissociation control (conditioning 3), in which larvae were first exposed to SUC and then to LIN, led to no enhancement compared with the odor alone control (conditioning 2). The requirement of temporal association between odor exposure and SUC reinforcement was further confirmed in another set of dissociation controls. Exposure to LIN (conditioning 5) led to slightly higher larval response than conditioning 2, which seems a nonassociative effect caused by the delay attributable to the 30 min mock treatment (for delayed nonassociative effects, see Fig. 4). Nonetheless, simultaneous exposure to LIN and 1 M SUC (conditioning 4) led to additional RI increment reproducing associative odor learning. In contrast, separate exposures to LIN and then 1 M SUC (conditioning 6) failed to do so.

We next asked whether the increased larval olfactory response was specific to the exposed odor. To address this question, we tested larval olfactory responses using odorants other than the one used for conditioning. When larvae were trained with LIN/SUC, PA/SUC, or GVA/SUC, only those trained with LIN/SUC showed significant RI increment in the olfactory test with LIN (Fig. 2*E*). Similarly, only larvae trained with PA/SUC showed significant RI increment in the olfactory test with PA (Fig. 2*F*). These results thus demonstrate that the enhanced larval response with SUC is specific to the conditioned odor and suggest that *Drosophila* larvae discriminate the three odors despite their limited olfactory system.

Whereas the above data emphasizes the importance of sucrose as a positive reinforcer, it is not clear whether RI stimulation is attributable to gustatory stimuli or attributable to higher osmotic pressure of 1 M sucrose than that of DW. To clarify this point, we trained larvae with LIN in association with 1 M D-sorbitol, a sugar that is tasteless to the flies (Dethier, 1976; Tempel et al., 1983). Conditioning with LIN in association with D-sorbitol failed to stimulate larval RI (0.48 ± 0.02; $n = 10$) compared with the control, in which larvae were exposed to LIN in association with DW (RI of 0.47 ± 0.03; $n = 20$).

Importance of the cAMP signaling pathway and K^+ channel in larval learning

Having established a single odor paradigm, we then examined larval responses of various mutants that are known to have deficits in adult olfactory learning and memory. We trained mutant larvae with LIN/SUC, LIN with DW, or SUC alone and measured RIs immediately after training (Fig. 3*A*). rut^1, defective in Ca/calmodulin-dependent adenylyl cyclase activity (Livingstone et al., 1984), failed to exhibit RI increment by the associative LIN/SUC training; no significant difference was detected compared with either control. This was further confirmed with another allele, rut^{2080}. Similarly, dnc^1, defective in cAMP phosphodiesterase activity (Dudai et al., 1976; Byers et al., 1981), showed no RI stimulation, confirming the previous results that *dnc* larvae showed no initial learning (Aceves-Piña and Quinn, 1979; Tully et al., 1994a). Thus, the requirement of both *rut* and *dnc* activities strongly argues for the importance of cAMP signaling for larval olfactory learning. Alternatively, cAMP signaling might also be important for maintenance of STM during training and olfactory test as proposed with adult flies (Dubnau and Tully, 1998; Waddell and Quinn, 2001). Because initial performance is completely lost in the *rut* and *dnc* mutants, our results do not allow distinction of the two possible mechanisms.

We then examined the potassium channel mutants eag^{4PM} and $Sh^{rk10120}$, which are deficient in courtship and olfactory learning behaviors in adult flies (Cowan and Siegel, 1984, 1986);

both eag^{4PM} and Sh^{rk0120} failed to exhibit RI increment by the associative training.

Conversely, amn^{28A}, defective for a gene encoding a putative neuropeptide that might act through adenylate cyclase to increase cAMP levels (Feany and Quinn, 1995; Moore et al., 1998), exhibited significant RI increment by the associative LIN/SUC training, although its increment (ΔRI) was significantly lower than that of wild type (Fig. 3*A*,*B*).

To control sensory integrity and locomotor activity of the mutant larvae, we measured larval olfactory responses for LIN using the same agar plate assay used for the learning tests. Normal LIN responses were recorded for all of the mutants (Table 2). The naive olfactory responses of dnc^{1} and amn^{28A} were slightly higher than wild type but not statistically significant. Gustatory response for SUC was also normal with the mutants, except for eag^{4PM}, which showed higher response. The gustatory responses of rut^{1} and rut^{2080} larvae were slightly lower than that of CS larvae, but the difference was not proved statistically significant by either Student's *t* test or the Mann–Whitney *U* test.

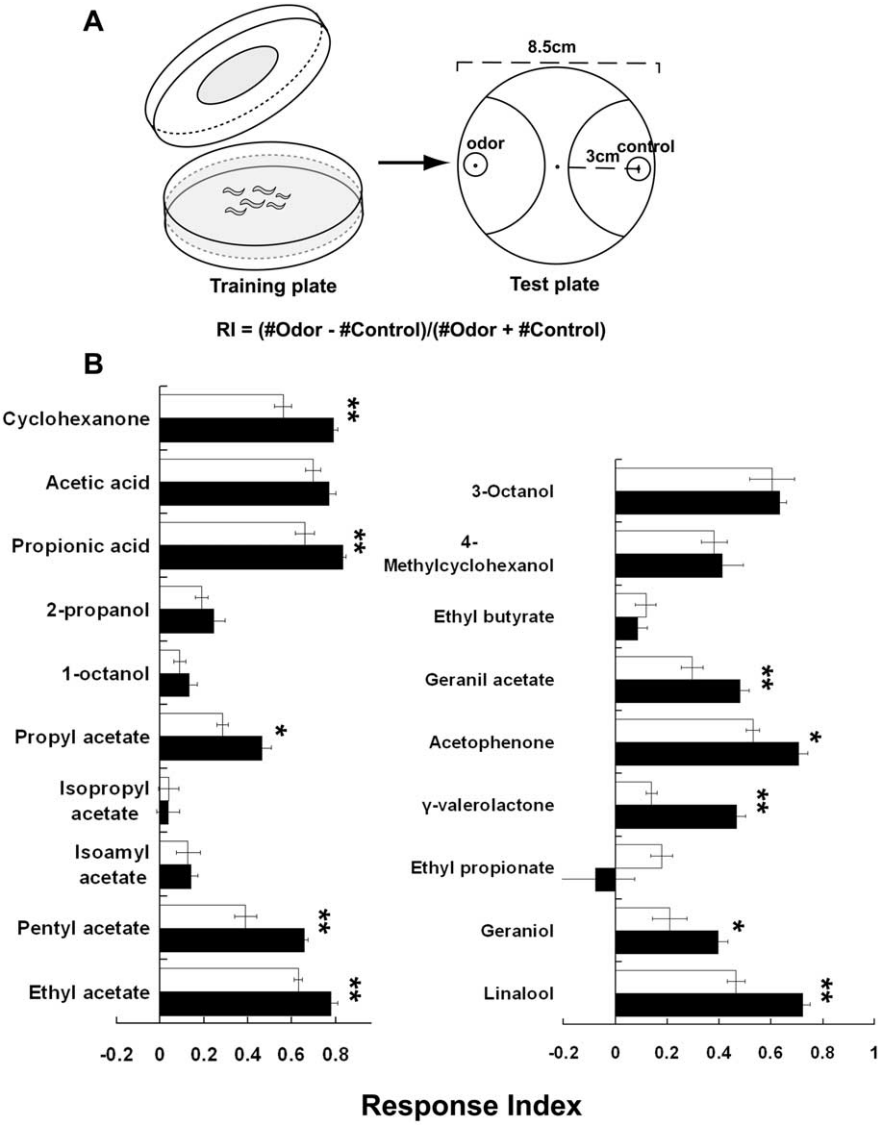

Figure 1. Training and test for larval olfactory associative learning. ***A***, Olfactory training and response test for larval associative learning. Training: staged early third-instar larvae (72–76 h after egg laying) are placed on 2.5% agar plates that are covered with thin layer of 1 M SUC solution (appetitive reinforcer) or DW (control). Odorant is spotted on a filter disk on the lid. After closing the lid, larvae are kept for 30 min and simultaneously exposed to the odorant and the reinforcer. Test: after training, larvae are gently rinsed with DW and transferred to the center of a 2.5% agar plate. A small filter disc, on which the test odorant is spotted, is placed on one side of the plate and a control disc on the opposite side. Typically, 50–100 larvae were used for the test. After 3 min, the numbers of animals moved in the indicated semicircular areas (80–90% population) are counted, and the RI was calculated as indicated. ***B***, Changes of larval odor attraction with various odorants. Larvae were trained with 1 M SUC or DW spread on agar plates and simultaneously exposed to the test odor. RIs with SUC (filled bar) and DW (open bar) were determined for each odorant. Appetitive training with SUC led to significant RI increment with the odors marked with asterisks (*$p < 0.05$, **$p < 0.01$ with Student's *t* test; $n = 8$–38). Significances were also confirmed by the Mann–Whitney *U* test.

Larval appetitive training produces medium-term memory that is dependent on *amn* and CREB

To study memory stability, we compared temporal changes of larval responses after LIN/SUC conditioning (Fig. 4*A*). Larval olfactory responses after the control conditioning (LIN) increased in 30 min and then gradually went down to the naive level, indicative of delayed nonassociative effect, which might be caused by either larval handling or odor exposure, although no effect was detected on the immediate larval responses (Fig. 2*A*). Larval responses after the associative LIN/SUC conditioning also showed similar initial RI increment (Fig. 4*A*). However, RI comparison between LIN/SUC and the control (LIN) showed significant memory performance (in ΔRI), which was retained beyond 60 min and gradually lost by 180 min (Fig. 4*A*,*F*, CS −HS).

Intrigued by this medium-term stability of the larval appetitive memory, we then asked whether *amn*, which is known to be essential for MTM formation in the adult fly, might be required for larval memory. In addition to the lower initial learning performance described above (Fig. 3), memory in amn^{28A} larvae was considerably short-lived and lost by 30 min (Fig. 4*E*,*F*), suggesting that the appetitive olfactory training generates *amn*-dependent memory in larvae.

To further characterize the larval memory, we also asked whether CREB, known to be essential to LTM formation in the adult fly (Yin et al., 1994), might be required for larval appetitive memory. For this, we induced dCREB2-b, a dominant-negative form of CREB (Yin et al., 1994; Davis et al., 1996), before conditioning. Notably, induction of dCREB2-b had no effect at time 0 min (Fig. 4*D*,*F*, dCREB2-b +HS), implying that CREB is not required for memory acquisition and/or STM. In contrast, memory retention was significantly impaired by the induction of dCREB2-b; memory trace in heat-shock-induced larvae became undetectable by 60 min. Either heat shock itself (Fig. 4*B*, CS +HS) or dCREB2-b without heat shock (Fig. 4*C*, dCREB2-b −HS) had no effect on immediate memory and its retention. Intriguingly, induction of dCREB2-b (dCREB2-b +HS) suppressed a large part of 30 min memory, suggesting very early

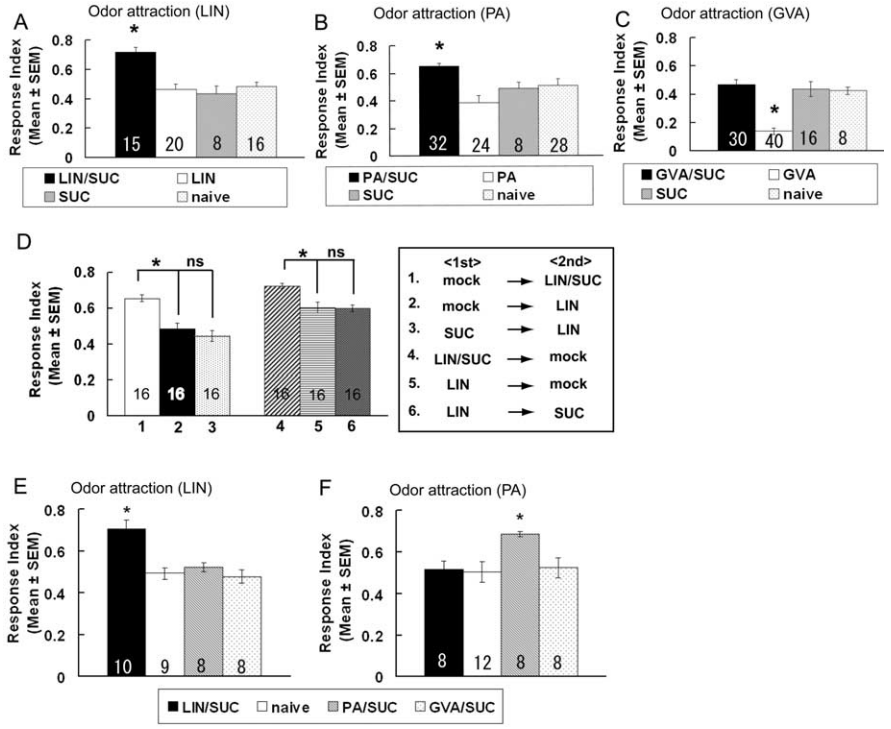

Figure 2. Characterization of appetitive olfactory conditioning in *Drosophila* larvae. ***A–C***, Larval olfactory conditioning with LIN (***A***), PA (***B***), and GVA (***C***). Type of larval conditioning is indicated in the box. SUC, 1 M SUC alone; LIN, exposure to LIN in association with DW; LIN/SUC, exposure to LIN in association with 1 M SUC. The same odorant was used in the olfactory conditioning and response test. With LIN and PA, significant increase of RI was observed only when larvae were exposed to both the odor and SUC; RI of the associative condition (LIN/SUC in ***A*** or PA/SUC in ***B***) was significantly higher than those of all the other conditions (in ***A*** and ***B***, $*p < 0.05$ with ANOVA compared with any of the other three conditions; also confirmed by Kruskal–Wallis test, $p < 0.005$). Conditioning with GVA in association with DW (GVA) resulted in desensitization, but the suppression was overcome with GVA/SUC conditioning (in ***C***, $*p < 0.05$ with ANOVA compared with any of the other three conditions; also confirmed by Kruskal–Wallis test, $p < 0.05$). ***D***, Temporal association and disassociation controls. Larvae were conditioned to LIN and SUC either simultaneously or successively. Type of larval conditioning is indicated in the box. 1, Simultaneously exposed to both LIN and 1 M SUC. 2, Exposed to LIN in association with DW. 3, Exposed to 1 M SUC without the odor and then exposed to LIN in association with DW. 4, Exposed to both LIN and 1 M SUC and then kept on a mock plate for 30 min. 5, Exposed to LIN in association with DW and then kept on a mock plate for 30 min. 6, Exposed to LIN in association with DW and then to 1 M SUC without the odor. Each exposure was for 30 min. Larvae were briefly rinsed after the first conditioning and transferred to another plate for the second conditioning. Note that the procedure resulted in dissociation by 1–2 min gap between the first and second treatments. $*p < 0.05$ with ANOVA; also confirmed by Kruskal–Wallis test; $n = 16$. ns, Not significant. ***E***, ***F***, Specificity of the larval olfactory response to the conditioned odor. ***E***, Olfactory response tests with LIN. Larvae were conditioned to LIN, PA, or GVA in association with SUC and tested with LIN. ***F***, Olfactory response tests with PA. Larvae were conditioned to LIN, PA, or GVA in association with SUC and tested with PA. Note the larval RI was significantly stimulated only to the odor that was paired with SUC. RI of the test odor conditioning (LIN/SUC in ***E*** and PA/SUC in ***F***) was significantly higher than those of all the other conditionings. In ***E***, $*p < 0.05$, ANOVA, compared with any of the other three conditionings; also confirmed by Kruskal–Wallis test, $p < 0.05$. In ***F***, $*p < 0.05$, ANOVA, compared with any of the other three conditionings; also confirmed by Kruskal–Wallis test, $p < 0.05$). Number of each sample is indicated in the bars.

requirement of CREB activity for retention of the larval memory. Conversely, the olfactory and gustatory responses of dCREB-2-b larvae were normal compared with wild type and not altered by heat-shock treatment (Table 2).

Synaptic output of larval MB neurons is required for retrieval of larval memory

Whereas MBs have been shown as centers for learning and memory in the adult fly (de Belle and Heisenberg, 1994; Connolly et al., 1996; Dubnau et al., 2001; McGuire et al., 2001; Schwaerzel et al., 2002; Heisenberg, 2003; Gerber et al., 2004b), neural networks involved in larval learning and memory has yet to be described. To investigate roles of the larval MBs in learning and memory, we temporarily inactivated neural output of larval MB neurons by expressing *UAS-shi*^{ts1} (Kitamoto, 2001). The *shi*^{ts1} gene encodes a temperature-sensitive form of dynamin, which is essential for endocytosis and synaptic vesicle recycling and thereby allows rapid and reversible inactivation of synaptic transmission at a restrictive temperature. As expression drivers, we used two Gal4 lines, 201Y (Yang et al., 1995) and OK301 (Connolly et al., 1996), both of which drive strong expression in the larval MBs (Fig. 5*A*,*B*): 201Y in the majority of the larval MB neurons (Kurusu et al., 2002) and OK301 in a subset of MB neurons. The two lines are also expressed weakly in scattered hemisphere neurons but with different patterns.

Both *201Y/shi*^{ts1} and *OK301/shi*^{ts1} larvae exhibited normal 30 min memory at the permissive temperature (Fig. 5*D*). Memory performance was also unchanged when larvae were trained and kept at the restrictive temperature and shifted to the permissive temperature 10 min before the test (Fig. 5*E*). However, when larvae were trained and kept at the permissive temperature and shifted to the restrictive temperature 10 min before the test, memory performance was significantly impaired in *201Y/shi*^{ts1} and *OK301/shi*^{ts1} but not in the control +/*shi*^{ts1} (Fig. 5*F*).

To exclusively determine the requirement of MB output for the CREB-dependent memory component, we then examined memory performance 90 min after conditioning. An independent set of experiments confirmed that the entire larval memory at 90 min was indeed CREB dependent (Fig. 5*C*). With *shi*^{ts1}, we detected no memory deficit at the permissive temperature in all of the genotypes examined (Fig. 5*G*). Memory performance was also normal in all of the genotypes when larvae were trained and kept at the restrictive temperature and shifted to the permissive temperature 10 min before the test (Fig. 5*H*). However, when larvae were trained and kept at the permissive temperature and shifted to the restrictive temperature 10 min before the test, memory performance was abolished in *201Y/shi*^{ts1} and *OK301/shi*^{ts1} larvae but not in +/*shi*^{ts1} larvae (Fig. 5*I*). These results thus suggest that synaptic output of larval MB neurons is necessary for retrieval but not for acquisition and retention of the CREB-dependent larval memory.

The naive olfactory response of *OK301/shi* larvae, but not of *201Y/shi*, was higher than wild type, but none of the temperature manipulations significantly altered olfactory and gustatory acuities of the *shi* larvae (Table 2).

Discussion

Associative learning in the *Drosophila* larva was first described by Aceves-Piña and Quinn (1979) and investigated by others with modified schemes (Heisenberg et al., 1985; Tully et al., 1994a; Dukas, 1998). However, despite the recent progress in the study of behavioral plasticity in the adult fly, the larval learning and

memory has been left remarkably unexplored. Recently, Garber has described individual assays in which odorants or light/dark are alternately presented to larvae with gustatory reinforcers (Scherer et al., 2003; Gerber et al., 2004a; Michels et al., 2005). They also described an appetitive conditioning assay with fructose (Hendel et al., 2005; Neuser et al., 2005). In this paper, we described an alternative paradigm for larval associative learning using a single odor in association with SUC. We discuss the salient features of our paradigm in the light of the previous larval studies as well as the novel aspects of larval memory disclosed by our paradigm.

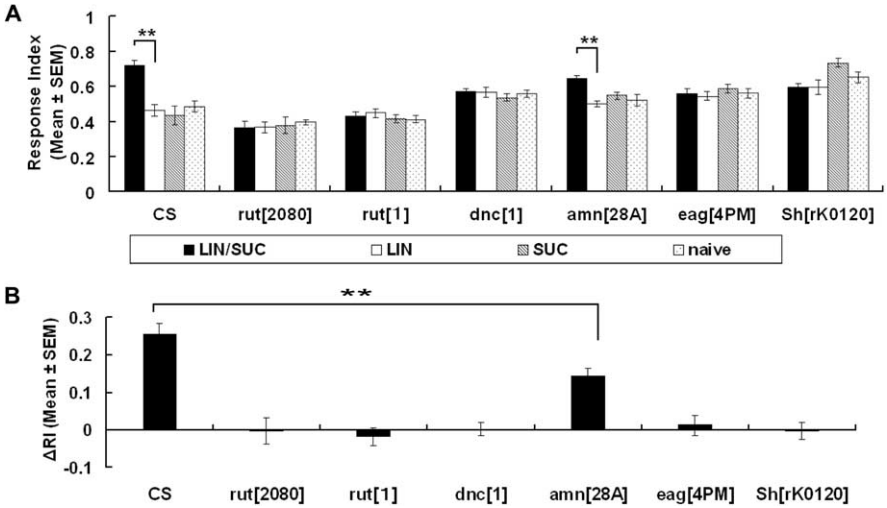

Figure 3. Olfactory learning in mutant larvae. ***A***, Comparisons of the larval RIs after LIN/SUC and control conditionings. Type of larval conditioning is indicated in the box. SUC, 1 M SUC alone; LIN, exposure to LIN in association with DW; LIN/SUC, exposure to LIN in association with 1 M SUC. $^{**}p < 0.001$ by Student's *t* test; also confirmed by Mann–Whitney *U* test, $p < 0.001$. Comparison was LIN/SUC versus LIN. $n = 13$–24. ***B***, RI increments in wild-type and mutant larvae in association with SUC. ΔRI = RI (LIN/SUC) − RI (LIN). The associative LIN/SUC training lead to significant ΔRI for wild-type larvae (CS). The ΔRI of *amn* larvae was significantly lower than that of wild type (in ***B***, $^{**}p < 0.001$ with Student's *t* test; confirmed by Mann–Whitney *U* test, $p < 0.05$; comparison was *amn* vs CS; $n = 24$ for CS and $n = 15$ for *amn*). rut^1, rut^{2080}, dnc^1, eag^{s160}, and Sh^{s263} showed no RI increment with the associative LIN/SUC training.

Larval conditioning and performance test with a single odor

Most studies on *Drosophila* associative learning have used reciprocal and symmetrical experimental paradigms with two odors (Quinn et al., 1974; Tully and Quinn, 1985) (for review, see Dubnau and Tully, 1998; Waddell and Quinn, 2001). In contrast, our paradigm uses only a single odor for conditioning and test. Consequently, this asymmetric nature calls for parallel controls to rule out nonassociative learning such as habituation and sensitization. Nonetheless, our paradigm resulted in significant learning only by the associative conditioning, in which both an odor and SUC were simultaneously presented to larvae (Fig. 2*A–D*); enhanced larval olfactory response was specific to the odor paired with SUC (Fig. 2*E*,*F*), excluding nonassociative sensitization to a broad range of odors. Conversely, it should be noted that strong desensitization was observed for certain odors such as GVA (Fig. 2*C*). Even with LIN, which showed no desensitization in immediate learning (Fig. 2*A*), delayed nonassociative effects on larval olfactory response were detected (Fig. 4*A*), emphasizing the importance of odor choice and careful data interpretation.

Because different sets of larvae are used for control experiments for the stimuli involved, reproducibility of larval responses is critical to our paradigm. At this point, we used select odorants screened for larval olfactory learning. Thus, of 30 chemicals, we chose several odorants that produced significant RI increment with SUC (Fig. 1*B*). Many odorants, such as 1-octanol and 4-methylcyclohexanol, which have been used in adult studies, failed to produce significant RI increment (Fig. 1*B*). The fact that larvae and adult flies exhibit different olfactory responses (Rodrigues, 1980; Lilly and Carlson, 1990) also highlights the importance of odorant choice for larval experiments.

Despite its asymmetric design, several points are of note as to our paradigm. First, the simple experimental design minimizes stress on larvae, which could affect learning performance. Second, our paradigm generates MTM that lasts up to 3 h (Fig. 4), longer than the duration by Neuser et al. (2005) (90 min) and Aceves-Piña and Quinn (1979) (30 min). Third, our paradigm is free from odor discriminative task. Because the larval olfactory system is considerably simpler than the adult system (Python and Stocker, 2002; Kreher et al., 2005; Ramaekers et al., 2005), simultaneous discrimination of different odors could complicate animal responses, although other studies used two-odor paradigms (Aceves-Piña and Quinn, 1979; Heisenberg et al., 1985; Tully et al., 1994a; Dukas, 1998; Scherer et al., 2003; Gerber et al., 2004a; Hendel et al., 2005; Michels et al., 2005; Neuser et al., 2005). Finally, because only a single odor is applied to larvae during training, the simple design of our paradigm may be of use in imaging of neural representation of the conditioned odor in the brain during learning and memory.

Table 2. Sensory acuities

	Olfactory response (LIN)	Gustatory response (1 M SUC)
Wild type (CS)	0.48 ± 0.05	0.44 ± 0.03
rut^{2080}	0.41 ± 0.01	0.36 ± 0.04
rut^1	0.49 ± 0.03	0.37 ± 0.05
dnc^1	0.55 ± 0.05	0.40 ± 0.04
amn^{28A}	0.55 ± 0.04	0.44 ± 0.06
eag^{4PM}	0.57 ± 0.04	0.56 ± 0.04*
Sh^{rK0120}	0.67 ± 0.05	0.49 ± 0.04
dCREB2-b −HS	0.48 ± 0.04	0.43 ± 0.05
dCREB2-b +HS	0.43 ± 0.04	0.45 ± 0.0
+/*shi* (25°C)	0.65 ± 0.03	ND
+/*shi* (30°C)	0.75 ± 0.05	ND
OK301/*shi* (25°C)	0.68 ± 0.05	0.43 ± 0.07
OK301/*shi* (30°C)	0.79 ± 0.03	0.47 ± 0.04
201Y/*shi* (25°C)	0.52 ± 0.04	0.40 ± 0.06
201Y/*shi* (30°C)	0.56 ± 0.05	0.41 ± 0.04

Olfactory and gustatory responses were examined as described in Materials and Methods. Averages of at least eight experiments. No significant difference in olfactory response was observed between wild type and the mutants presented in Figure 3 (by Student's *t* test and Mann–Whitney *U* test). Gustatory responses toward 1 M SUC were also normal with the Figure 3 mutants, except for eag^{4PM} ($^*p < 0.05$ by Student's *t* test and $p < 0.05$ by Mann–Whitney *U* test). The olfactory responses of dCREB2-b (either −HS or +HS) larvae were normal compared with wild type. The naive olfactory responses of +/*shi* and OK301/*shi* larvae were higher than wild type, but none of the temperature manipulations of the *shi* larvae significantly impaired olfactory and gustatory acuities. Errors are SEM. ND, Not determined.

Requirement for *amn* in larval learning and memory

Adult flies with *amn* mutations show a reduction in immediate memory as well as a more profound reduction in MTM (Quinn et al., 1979; DeZazzo et al., 1999; Waddell et al., 2000). However, previous results about *amn* requirement in larvae are inconsis-

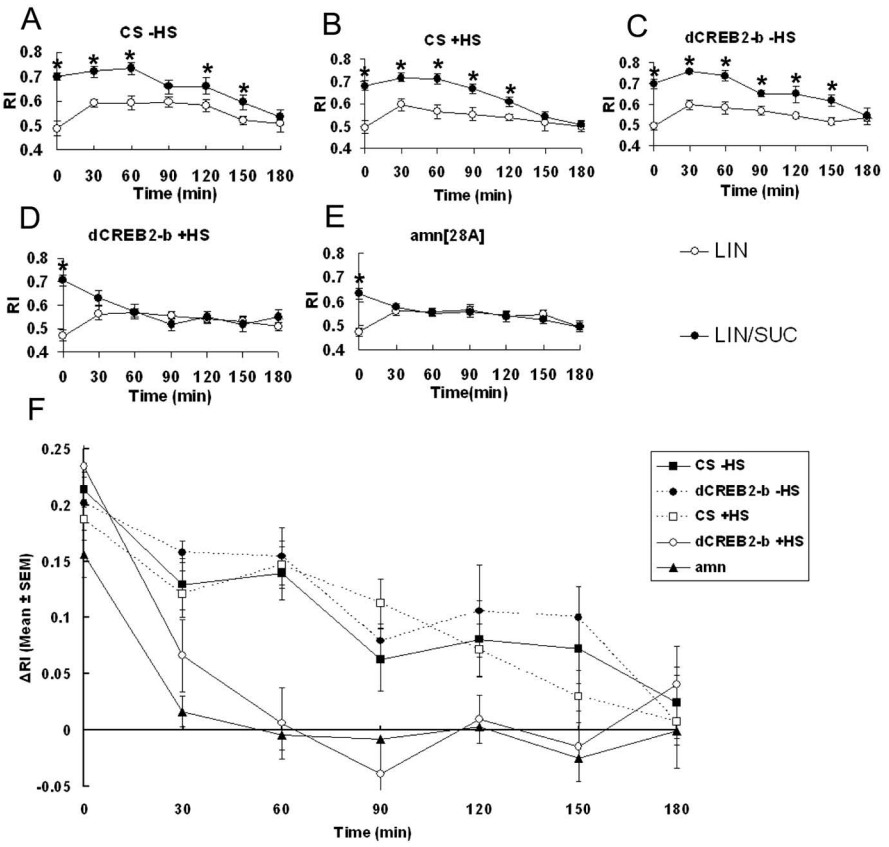

Figure 4. Retention of appetitive memory in wild-type and mutant larvae. ***A–E***, Temporal changes of larval olfactory responses after appetitive training with LIN/SUC and control training with LIN. LIN, Exposure to LIN in association with DW; LIN/SUC, exposure to LIN in association with 1 M SUC. ***A***, Wild type without heat shock (CS −HS); ***B***, wild type with heat sock (CS +HS); ***C***, dCREB2-b without heat shock (dCREB2-b −HS); ***D***, dCREB2-b with heat shock (dCREB2-b +HS); ***E***, *amn* ^{28A}. In ***B*** and ***D***, larvae were heat shocked for 30 min at 37.5°C before training. $*p < 0.05$ with Student's test, comparison between LIN/SUC and LIN. ***F***, Olfactory memory performances in wild-type and mutant larvae plotted in ΔRI. Medium-term larval memory was detected for wild-type (CS −HS and CS +HS) and non-heat shocked dCREB2-b (dCREB2-b −HS) larvae. Memory in heat shocked dCREB2-b larvae (dCREB2-b +HS) became undetectable by 60 min, and memory in *amn* ^{28A} larvae became undetectable by 30 min. Each data point represents RI of independent animal groups (average of 14–22 experiments).

tent; using electric shocks as aversive reinforcement, Aceves-Piña and Quinn (1979) described near-normal 15 min memory, whereas Tully et al. (1994a) detected no initial learning. In our paradigm, *amn* larvae showed reduced but significant immediate learning/memory (Figs. 3, 4), which is lost later by 30 min (Fig. 4). The reason for these discrepancies is unknown, but different ways of reinforcement could differently modulate memory formation and retention. Moreover, Keene et al. (2004) described that *amn* requirement is different with different odors. In the adult brain, the AMN peptide is expressed in dorsal paired medial (DPM) neurons that are situated medially to MBs and ramify throughout the MB lobes (Waddell et al., 2000). In contrast, little is known about the network of the DPM neurons and the AMN expression pattern in the larval brain.

Early requirement of CREB activity for Larval MTM

Studies with *Aplysia*, mice, and adult *Drosophila* flies show that CREB-dependent transcription is required for cellular events underlying LTM (for review, see Abel et al., 1998; Silva et al., 1998; Mayford and Kandel, 1999; Lonze and Ginty, 2002). These studies have shown that CREB functions as a conserved molecular switch for LTM, which is thought to be induced several hours after training. Moreover, intervals between trainings or stimulations are generally required to produce CREB-dependent long-term effects (Tully et al., 1994b; Silva et al., 1998; Menzel, 2001).

In contrast, the larval CREB-dependent memory is stable for only medium term (Fig. 4). Moreover, our paradigm continuously exposes larvae to an odor and SUC during training, a condition similar to massed training of adult flies (Tully et al., 1994b). Intriguingly, CREB is recruited shortly after learning in larvae; a significant portion of 30 min memory was disrupted by the CREB blocker, whereas immediate learning was not (Fig. 4*F*). If the larval MTM is induced after STM as in the adult fly (Tully et al., 1994b), this very early CREB requirement might imply fast transition of memory phases. Alternatively, the CREB-dependent memory might also be generated independently. Intriguingly, it has been proposed that CREB can be activated independent of STM in long-term synaptic facilitation in *Aplysia* (Emptage and Carew, 1993; Casadio et al., 1999; Purcell et al., 2003). In addition, although memory performance becomes undetectable in 3 h, the requirement of CREB activity suggests neural mechanisms that are in part shared with LTM in the adult fly. In fact, memory decay after CREB blockade is somewhat slower than in *amn* mutants (Fig. 4). Furthermore, whereas Yin et al. (1994) showed that the CREB blocker suppressed 1 and 7 d memories, whether the blockade has more immediate effects is not known, leaving the possibility that CREB could be recruited early in the adult fly as well. Notably, memory performance in the adult fly tends to be higher with spaced training than with massed training already at several hours (Tully et al., 1994b).

Biochemically, CREB is activated by phosphorylation in response to diverged extra cellular stimuli (Lonze and Ginty, 2002). Among them, the protein kinase A (PKA) plays a central role in phosphorylation of CREB1-a, the catalytic subunit. Because the larval memory is completely disrupted in *dnc* and *rut* mutants (Fig. 3), the cAMP–PKA pathway might be involved in the early activation of CREB in larvae. Alternatively, intracellular pathways other than PKA could also be recruited to mediate CREB activation. Intriguingly, increase of intracellular cAMP is known to activate mitogen-activated protein kinase in *Aplysia*, which in turn phosphorylates CREB2-b, the regulatory subunit, allowing transcriptional activation by the catalytic CREB isoform in the nuclei (Abel et al., 1998; Lonze and Ginty, 2002).

Importance of larval MBs for learning and memory

The adult MBs are highly complex structures with three sets of lobes, each of which might participates in different memory traces (Zars et al., 2000; Pascual and Preat, 2001; Heisenberg, 2003; Isabel et al., 2004). In contrast, the larval MBs exhibit a remarkably simple projection pattern with only a single set of lobes (Tettamanti et al., 1997; Armstrong et al., 1998; Kurusu et

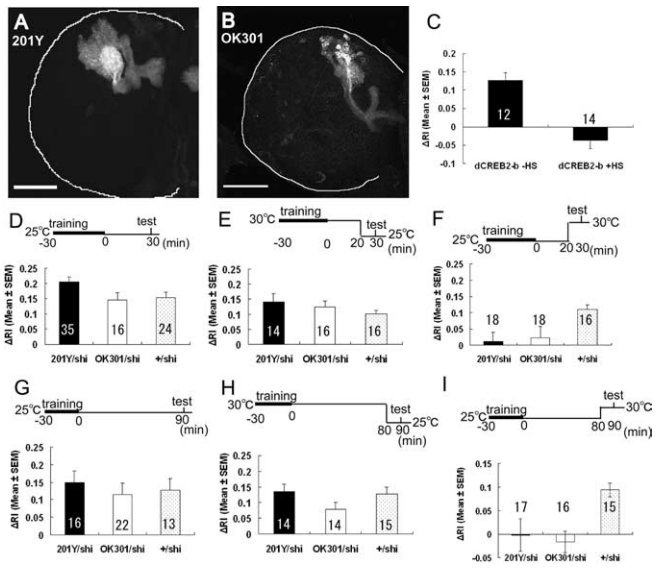

Figure 5. Synaptic output of larval MB neurons is required for retrieval of larval memory. ***A***, ***B***, Expression patterns of the MB-Gal4 lines 201Y (***A***) and OK301 (***B***) monitored with UAS-mCD8::GFP. Confocal images. Brain hemisphere is outlined. ***C***, CREB dependence of 90 min memory. Different set of experiments from those in Figure 4. Memory at 90 min produced by LIN/SUC training was completely abolished by heat-shock induction of dCREB2-b (Student's *t* test, $p < 0.001$; also confirmed by Mann–Whitney *U* test, $p < 0.001$). ***D–F***, Disruption of neurotransmission through larval MB neurons abolished retrieval of 30 min memory. Larvae were trained with LIN/SUC, and their memory performance was tested 30 min after training. Expression of *UAS-shi*^{ts1} was induced with the indicated MB-Gal4 driver. Temperature shift protocol is indicated above each graph. The permissive temperature was 25°C, and the restrictive temperature was 30°C. The 30 min memory in *shi*^{ts1}/201Y and *shi*^{ts1}/OK301 larvae was normal in ***D*** (ANOVA, $p > 0.05$; confirmed by Kruskal–Wallis test, $p > 0.1$) and ***E*** (ANOVA, $p > 0.3$; confirmed by Kruskal–Wallis test, $p > 0.1$) but significantly impaired in ***F*** (ANOVA, $p < 0.05$; confirmed by Kruskal–Wallis test, $p < 0.05$). ***G–I***, Disruption of neurotransmission in MB neurons abolished retrieval of 90 min appetitive memory. Larvae were trained with LIN/SUC, and their memory performance was tested 90 min after training. The 90 min memory in *shi*^{ts1}/201Y and *shi*^{ts1}/OK301 larvae was normal in ***G*** (ANOVA, $p > 0.5$; confirmed by Kruskal–Wallis test, $p > 0.5$) and ***H*** (ANOVA, $p > 0.1$; confirmed by Kruskal–Wallis test, $p > 0.2$) but significantly impaired in ***I*** (ANOVA, $p < 0.05$; confirmed by Kruskal–Wallis test, $p < 0.05$). Number of each sample is indicated in the bars.

al., 2002). In addition, recent studies have revealed straightforward organization of the larval olfactory system with only 21 olfactory receptor neurons targeting the 21 antennal lobe glomeruli, from which projection neurons target the larval MB calyx that consists of ~28 glomeruli (Python and Stocker, 2002; Kreher et al., 2005; Ramaekers et al., 2005).

Our finding that larval MB output is essential for memory retrieval discloses functional importance of the larval MBs and directly demonstrates anatomical commonality of memory networks between the larval and adult brains. Furthermore, the results that larval MB output is not required for memory acquisition and retention suggest that larval olfactory memory is localized upstream of larval MB synapses, in either MB neurons themselves or upstream circuits such as antennal lobes. Combined with the recent advances in functional neural imaging, the simple and identifiable neural network of the larval olfactory system will help further elucidation of the cellular basis of learning and memory in the brain.

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