Behavioral/Systems/Cognitive

Distinctive Neuronal Networks and Biochemical Pathways for Appetitive and Aversive Memory in *Drosophila* Larvae

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Associative strength between conditioned stimulus (CS) and unconditioned stimulus (US) is thought to determine learning efficacy in classical conditioning. Elucidation of the neuronal mechanism that underlies the association between CS and US in the brain is thus critical to understand the principle of memory formation. With a simple brain organization, the *Drosophila* larva provides an attractive model system to investigate learning at the neurocircuitry level. Previously, we described a single-odor paradigm for larval associative learning using sucrose as a reward, and showed that larval appetitive memory lasts longer than 2 h. In this work, we describe behavioral and genetic characterization of larval aversive olfactory memory formed in our paradigm, and compare its stability and neurocircuitry with those of appetitive memory. Despite identical training paradigms, larval olfactory memory formed with quinine or NaCl is short-lived to be lost in 20 min. As with appetitive memory, larval aversive memory produced in this paradigm depends on intact cAMP signaling, but neither mutation of *amnesiac* nor suppression of CREB activity affects its kinetics. Neurocircuitry analyses suggest that aversive memory is stored before the presynaptic termini of the larval mushroom body neurons as is the case with appetitive memory. However, synaptic output of octopaminergic and dopaminergic neurons, which exhibit distinctive innervation patterns on the larval mushroom body and antennal lobe, is differentially required for the acquisition of appetitive and aversive memory, respectively. These results as a whole suggest that the genetically programmed memory circuitries might provide predisposition in the efficacy of inducing longer-lived memory components in associative learning.

Key words: dopamine; octopamine; *shibire*; *dunce*; *rutabaga*; mushroom body

Introduction

In Pavlovian conditioning, animals learn association between a conditioned stimulus (CS) and an unconditioned stimulus (US) through training. Because associative strength between CS and US is thought to determine learning efficacy, elucidation of the neuronal mechanism that underlies CS–US association in the brain is critical to understand the principle of memory formation. In the past decades, studies in *Drosophila* have uncovered seminal aspects of associative memory including signaling molecules and memory phases (Davis, 2005; Margulies et al., 2005; Keene and Waddell, 2007). Anatomically, mushroom bodies (MBs) have been characterized as centers for olfactory memory in the fly brain (Heisenberg, 2003). MBs receive olfactory information from the antennal lobe (AL) via the projection neurons. During memory formation, MBs also receive recurrent signals from dorsal paired medial neurons, the activity of which is essential to induce middle-term memory (MTM) with both aversive and appetitive US cues (Waddell et al., 2000; Keene et al., 2004, 2006; Yu et al., 2005; Krashes et al., 2007). In addition, pharmacological and molecular studies in honeybees (Hammer and Menzel, 1998) and fruit flies (Yu et al., 2004; Thum et al., 2007) suggest that AL functions as another neural structure involved in memory formation.

Studies in *Drosophila* have also provided insights into differential properties of appetitive and aversive memory. In a classic study, Tempel et al. (1983) suggested different memory kinetics of electric shock and sugar-reward memories. As for neural modulators, octopamine (OA) and dopamine (DA) have differential roles in acquisition of appetitive and aversive memory, respectively (Schwaerzel et al., 2003; Schroll et al., 2006), although it is also shown that mutations of the D_1–DA receptor (dDA1) significantly suppress aversive olfactory learning, and moderately impair sugar-mediated learning (Kim et al., 2007). In addition, an overlapping set of MB neurons might be involved in retrieval of both appetitive and aversive memory (Schwaerzel et al., 2003; Krashes et al., 2007; Thum et al., 2007).

To dissect the neurocircuitry of olfactory memory, the *Drosophila* larva provides an excellent model system. The larval brain is much simpler than the adult brain, and the basic design of its olfactory system is highly straightforward without redundancy (Ramaekers et al., 2005; Gerber and Stocker, 2007; Vosshall and Stocker, 2007). We have previously described that a sugar-reward training of *Drosophila* larvae produce olfactory memory that is retained beyond 120 min and depends on both *amnesiac* (*amn*)

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and cAMP response element-binding protein (CREB) (Honjo and Furukubo-Tokunaga, 2005).

In this work, we have extended our larval study using aversive reinforcers. We show that larval memory formed with quinine hemisulfate (QH) is short-lived and suggest that it is stored before the synaptic termini of MB. We also show that synaptic output of larval OA and DA neurons is differentially required for the acquisition of appetitive and aversive memory, respectively. These monoaminergic pathways exhibit discrete convergence patterns with the odor CS pathway in the larval brain, suggesting the importance of genetically programmed circuits in efficient induction of longer-lived memory components in associative learning.

Materials and Methods

Fly stocks. The following fly stocks were used: wild-type Canton-S; short-term memory (STM) mutants *rutabaga* (*rut*) ^{1} (Livingstone et al., 1984) and *dunce* (*dnc*) ^{1} (Dudai et al., 1976); MTM mutant *amn* ^{28A} (DeZazzo et al., 1999); *hs-dCREB2-b* transgenic fly (line 17–2) (Yin et al., 1994); *UAS-shibire* ^{ts1} inserted on chromosome III (Kitamoto, 2001); MB-GAL4 enhancer trap line *OK301* (Connolly et al., 1996; Honjo and Furukubo-Tokunaga, 2005) and *201Y* (Yang et al., 1995); *TH-GAL4* (Friggi-Grelin et al., 2003); *TDC2-GAL4* (Cole et al., 2005); and *UAS-mCD8::GFP* (Lee and Luo, 1999). Stocks were kept at 25°C on a standard food as described previously (Honjo and Furukubo-Tokunaga, 2005).

Larval conditioning experiments. Larval conditioning experiments were performed as described previously (Honjo and Furukubo-Tokunaga, 2005). Larvae were raised with standard food without propionic acid. Staged early third instar larvae (72–76 h after egg laying) were used for all experiments. Unless otherwise noted, larval behavioral experiments were performed at 25°C. For training, larvae were placed on the surface of a 2.5% agar plate (diameter 85 mm), on which 1 ml reinforcers were spread shortly before experiments. Undiluted odor (10 μl), linalool (LIN) (Nacalai), or pentyl acetate (PA) (Nacalai) was spotted on a filter disk (55 mm in diameter) placed on the inside of the lid, and larvae were exposed to the odor for 30 min in conjunction with the reinforcer solution. The reinforcers used were 1 M sucrose (SUC) (Nacalai), 0.1% QH (Sigma), or 1 M NaCl (Wako). Distilled water (DW) was used for the control. Typically, several hundreds animals are placed on a plate and conditioned *en masse*. During conditioning, larvae crawl around over the wet gel surface taking up the US substance as liquid. The majority of the animals ingested the reinforcer during the 30 min training (94% for 1 M SUC, 90% for DW, and 88% for 0.1% QH, by uptake tests with 0.2 mg/ml Acid Red 52). In the temporal dissociation test, animals were successively exposed to odor and reinforcer for 30 min each as described previously (Honjo and Furukubo-Tokunaga, 2005).

Larval olfactory response was measured by a single odor preference test adapted from Heimbeck et al. (1999). After associative training, larvae were harvested with DW, gently rinsed three times with DW in a 100 ml beaker to remove residual odorant and reinforcer, and transferred onto test agar plates. For each test, the Response Index (RI) was calculated as RI = (the numbers of animals in odor area minus the number of animals in control area)/(total number of animals counted). For an indicator of memory performance, ΔRI was calculated as RI (odor/reinforcer) minus RI (odor/DW). For memory retention tests, larvae were trained as above, then transferred to another agar plate and kept for the indicated time before the memory test.

The experiments with *UAS-shibire* ^{ts1} were performed in temperature-controlled rooms adjusted at the permissive (25°C) or the restrictive (30°C or 31°C) temperatures. All instruments and reagents were stabilized to the indicated temperature before experiments. For temperature shift, larvae trained in the first temperature room were moved to the secondary temperature room. The plates were kept in a foam-polystyrene insulator box during the transfer between the different temperature rooms.

Olfactory and gustatory response tests. Larval olfactory and gustatory responses were determined as described previously (Honjo and Furukubo-Tokunaga, 2005). Fifty to 100 larvae were used per single experiment. RI was determined after 3 min. Gustatory response was measured using plastic plates with a median separator (85 mm, Eiken). The control half was filled with 0.5% agar and the test half with 0.5% agar containing 1 M sucrose or 0.1% QH. Plates were solidified for 2 h at 25°C or 31°C, and 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 as RI = (the numbers of animals on the test half minus the number of animals on the control half)/(total number of the animals). Animals that left the gel surface were not counted.

Induction of dCREB2-b with heat shock. Heat shock treatment was performed as described previously (Honjo and Furukubo-Tokunaga, 2005). Food vials containing early third instar larvae were submerged in a 37.5°C water bath. The vials were then returned on the bench (25°C) and kept for 30 min for larval recovery from the heat shock. Larvae were then collected and used for conditioning.

Statistics. Data are presented based on parametric tests (Student's *t* test and ANOVA) in all figures for simplicity. 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. For multiple comparison among relevant groups, Dunnett's method and Dunn's method were used in conjunction with ANOVA and the Kruskal–Wallis test, respectively. In either test, $p < 0.05$ was considered statistically significant.

Immunohistochemistry. Immunological staining of the larval and adult brains was performed as described previously (Kurusu et al., 2002). The antibodies used were as follows: Rat anti-CD8α (Caltag) diluted 1:50; rabbit anti-TRIO diluted 1:1000 (Awasaki et al., 2000); rabbit anti-octopamine (Advanced Targeting Systems); mouse anti-choline acetyl transferase (ChAt 4B1) (Takagawa and Salvattera, 1996) diluted 1:1000; mouse anti-FAS II (1D4) (Grenningloh et al., 1991) diluted 1:5; and Alexa-conjugated secondary antibodies (Invitrogen) diluted 1:1000. Confocal images were captured with a Zeiss LSM510 and processed using Adobe Photoshop.

Double-labeling study with fluorescent in situ *hybridization and green fluorescent protein.* Fluorescent *in situ* hybridization was performed as described previously (Kobayashi et al., 2006) with slight modifications. Digoxigenin-labeled RNA probes against *tyramine β-hydroxylase* were prepared with EST clones RH19793 and RH48375 obtained from the *Drosophila* Genomics Resource Center (Bloomington, IN). Hybridization was performed at 55°C overnight. Hybridization signals were detected with anti-DIG-POD (Roche Applied Science) and amplified by TSA Biotin System (PerkinElmer) using Streptavidin-Alexa 546 (Invitrogen). Green fluorescent protein (GFP) signals were enhanced with rabbit anti-GFP (Medical & Biological Laboratories).

Results

Aversive olfactory conditioning in *Drosophila* larvae

To study aversive memory in larvae, we used our larval training protocol (Honjo and Furukubo-Tokunaga, 2005) with LIN as a CS odor and 0.1% QH as an aversive US (LIN/QH). Associative training with LIN/QH, but not with either LIN alone or with QH alone, caused significant suppression in larval RI (Fig. 1*A*). This suppression was not specific to LIN as a significant RI decrement was also confirmed with another odorant, PA (Fig. 1*B*). Furthermore, suppression of larval RI required simultaneous exposure to the odor and QH as demonstrated in a temporal dissociation test (Fig. 1*C*), in which animals were successively exposed first to LIN and second to QH, or vice versa. Exposure to LIN before QH (conditioning 5) led to slightly higher response possibly due to the latency between the odor exposure and the memory test, which causes a delayed nonassociative effect described previously (Honjo and Furukubo-Tokunaga, 2005). The difference was not significant ($p > 0.1$) although when compared with any of the other controls (conditions 2, 3, 4 and 6). However, only simultaneous exposure to LIN and QH (conditioning 1) reproduced significant suppression of larval olfactory response compared with any of the other conditionings ($p < 0.05$).

To examine nonspecific RI suppression by aversive US exposure, we tested larval olfactory response using a second odorant that was not used for training. When larvae were trained with PA/QH or LIN/QH, only LIN/QH larvae showed significant RI suppression when tested with LIN (Fig. 1*D*). Similarly, only larvae trained with PA/QH showed RI suppression when tested with PA (Fig. 1*E*). Furthermore, in both tests, LIN/QH and PA/QH larvae exhibited significant difference in their response to the test odor ($p < 0.01$). Thus, these results argue that the observed decrement in larval response was not attributable to general olfactory suppression but rather represents discriminative and specific alteration in the response to the CS odor.

Using electric shock as a reinforcer, it has been shown that *dnc* mutations abolish larval olfactory learning (Aceves-Piña and Quinn, 1979; Tully et al., 1994a). In addition, we have shown that larval appetitive learning depends on cAMP signaling (Honjo and Furukubo-Tokunaga, 2005). To determine whether cAMP signaling was similarly involved in the formation of aversive memory in our paradigm, we examined mutant larvae of *rut*^{1} and *dnc*^{1}, and found that both mutants failed to show significant RI decrement after aversive LIN/QH conditioning (Fig. 1*F*). To control integrity of sensory-motor responses, we measured larval olfactory response for LIN using the same agar plate assay used for learning tests (supplemental Table 1, available at www.jneurosci.org as supplemental material). The naive olfactory response of *rut*^{1} larvae and the quinine reactivity of *dnc*^{1} larvae were slightly lower than those of wild-type (Canton-S) but the difference was not significant for both cases. Larval olfactory and gustatory responses were otherwise normal with these mutants. These results thus suggest that larval aversive memory induced by our paradigm depends on the cAMP signaling.

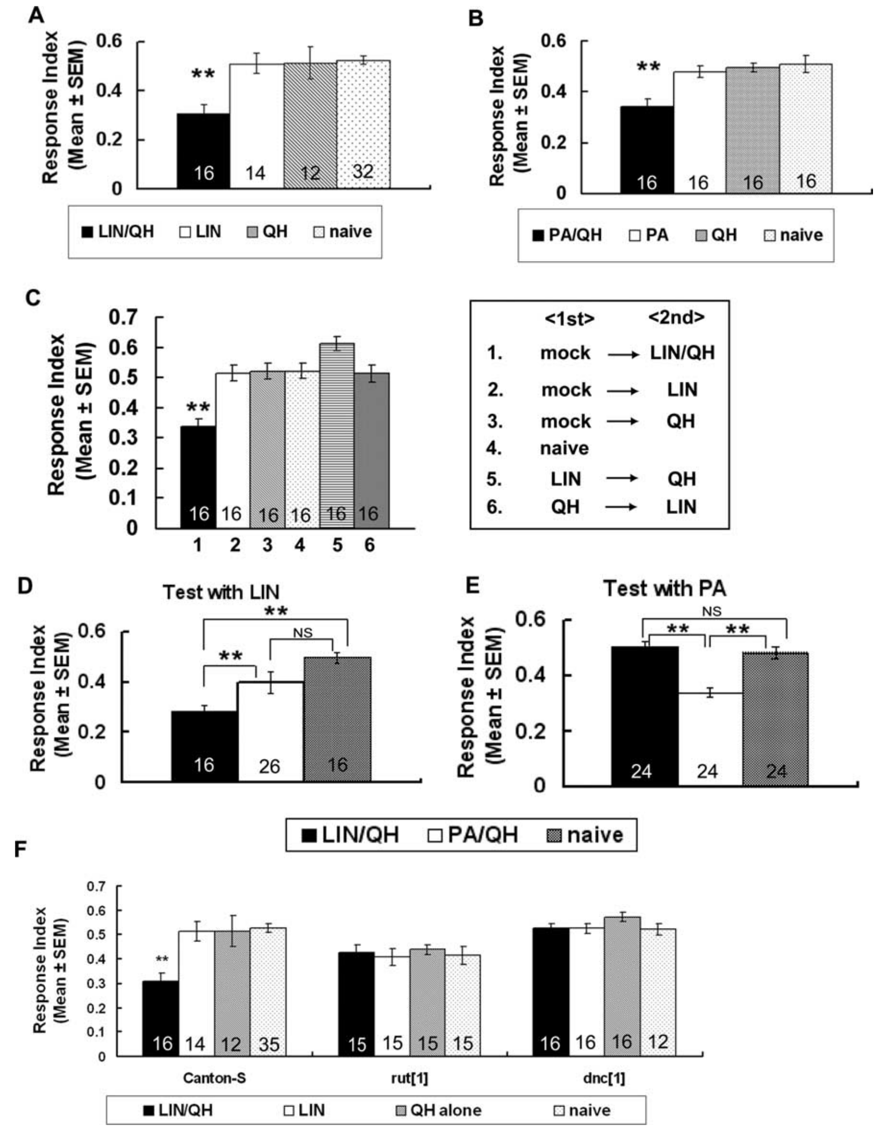

Figure 1. Characterization of aversive olfactory conditioning in *Drosophila* larvae. ***A***, ***B***, Larval aversive olfactory conditioning with LIN (***A***) and PA (***B***). Type of larval conditioning is indicated in the box: LIN/QH, exposure to LIN in association with 0.1% QH; LIN, exposure to LIN in association with DW; QH, 0.1% QH alone. The same odorant was used in the olfactory conditioning and test. Significant RI decrement was observed only when larvae were exposed to the odor in contingent with QH (LIN/QH in ***A*** and PA/QH in ***B***) (**$p < 0.01$ by the Kruskal–Wallis test and Dunn's *post hoc* test, compared with any of the other three conditions; also confirmed by ANOVA). ***C***, Temporal dissociation controls. Larvae were conditioned by LIN and QH either simultaneously or successively. Type of larval conditioning is indicated in the box. 1: mock and exposure to LIN in association with QH. 2: mock and exposure to LIN in association with DW. 3: mock and QH alone. 4: no conditioning (naive). 5: first exposed to LIN in association with DW, and then to 0.1% QH without the odor. 6: first exposed to 0.1% QH without the odor and then to LIN in association with DW. Each exposure time was 30 min. Larvae were briefly rinsed after the first conditioning and transferred to another plate for the second conditioning. Note the procedure resulted in dissociation by a 1–2 min gap between the first and second treatments. Only temporally associative conditioning resulted in significant decrement of RI (**$p < 0.05$ by the Kruskal–Wallis test followed by Dunn's *post hoc* test, compared with any of the other three conditions; also confirmed by ANOVA). ***D***, ***E***, Specificity of the larval olfactory response to the conditioned odor. ***D***, Tests with LIN. Larvae were conditioned either with LIN or PA in association with QH and tested with LIN. ***E***, Tests with PA. Larvae were conditioned either with LIN or PA in association with QH and tested with PA. Note the larval RI was significantly decreased only to the odor that was paired with QH (**$p < 0.01$, by the Kruskal–Wallis test followed by Dunn's *post hoc* test, compared with any of the other three conditions; also confirmed by ANOVA; NS, not significant). ***F***, Aversive memory in cAMP mutants. Type of larval conditioning is indicated in the box. The associative LIN/QH conditioning led to significant RI decrement for wild-type, but not for mutant, larvae. The number of each sample is indicated within the bar. (**$p < 0.01$ by the Mann–Whitney *U* test, also confirmed by Student's *t* test).

Stability of the aversive memory induced with LIN/QH

Previously, we showed that larval appetitive memory generated with LIN and SUC (LIN/SUC) lasted longer than 2 h (Honjo and Furukubo-Tokunaga, 2005). To determine the stability of the aversive memory induced by the LIN/QH training described above, we examined the temporal change of the larval olfactory response after training (Fig. 2). Despite the identical training procedure involving same odor (LIN) and same exposure time (30 min), larval memory generated with the LIN/QH conditioning was lost in 20 min (Fig. 2*A*,*F*).

Because we used different US substances between LIN/SUC and LIN/QH trainings, we then compared larval gustatory re-

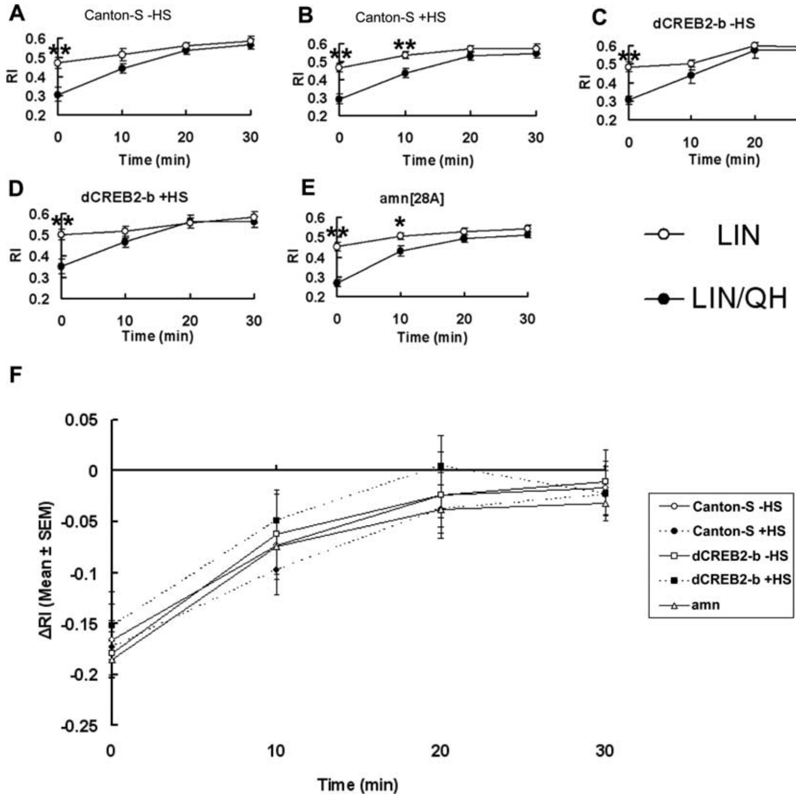

Figure 2. Larval aversive memory is short term. ***A–E***, Temporal changes of larval olfactory response after LIN/QH training. ***A***, Wild type without heat shock; ***B***, wild type with heat shock; ***C***, dCREB2-b without heat shock; ***D***, dCREB2-b with heat shock; and ***E***, *amn* ^{28A}. $*p < 0.05$, $**p < 0.01$ by the Mann–Whitney *U* test between LIN/QH and LIN, also confirmed by Student's test. In ***B*** and ***D***, larvae were heat shocked at 37.5°C for 30 min before training. ***F***, Olfactory memory performances in wild-type and mutant larvae plotted in ΔRI. No significant difference was found among the memory scores of all experimental animals at all time points ($p > 0.65$ for all points by the Kruskal–Wallis test followed by Dunn's *post hoc* test; also confirmed by ANOVA). Each data point represents RI of independent animal groups (average of 14–18 experiments).

sponses elicited by 1 M SUC and 0.1% QH by agar plate assay. Although larvae showed opposite behaviors, response indices for the two US compounds were of similar magnitude at the concentrations used in training (supplemental Table 1, Canton-S, available at www.jneurosci.org as supplemental material), suggesting that the reinforcing power of the aversive QH stimuli was comparable with that of the appetitive SUC stimuli at least by the behavioral criteria. Moreover, initial memory scores are comparable between the LIN/SUC and LIN/QH conditionings (compare Fig. 2*F* with Fig. 4*F* of Honjo and Furukubo-Tokunaga, 2005), although the small suppression of initial memory in *amn* larvae observed after the appetitive LIN/SUC conditioning (Honjo and Furukubo-Tokunaga, 2005) was not detected after the aversive LIN/QH conditioning. These results suggest that the observed instability in the aversive memory induced with LIN/QH was unlikely to be caused by suboptimal memory induction. In addition, we found that aversive memory formed with 1 M NaCl was also short lived (supplemental Fig. 1, available at www.jneurosci.org as supplemental material).

Larval appetitive memory formed with LIN/SUC consists of short-term and medium-term components, the latter of which is lost by either *amn* mutation or suppression of CREB activity (Honjo and Furukubo-Tokunaga, 2005). *amn* mutant larvae exhibit lower initial memory and form only STM. Although initial memory performance is intact, appetitive memory in heat-shocked dCREB2-b larvae is lost within 30 min (Honjo and Furukubo-Tokunaga, 2005). Whereas *amn* and CREB are involved in the formation of MTM and long-term memory (LTM), respectively, in the adult fly (Yin et al., 1994; DeZazzo et al., 1999; Waddell et al., 2000; Keene et al., 2004, 2006; Perazzona et al., 2004), memory components produced by each of these genes are yet to be dissociated in larvae.

In contrast to the appetitive memory induced with the LIN/SUC training, neither the initial memory nor its retention was affected in *amn* mutant larvae when larvae were aversively trained with LIN/QH (Fig. 2*B–F*). Likewise, no difference was detected for aversive memory in heat induced dCREB2-b larvae. Examination of larval sensory-motor activities indicated that *amn* larvae showed normal olfactory and gustatory responses in the agar plate tests (supplemental Table 1, available at www.jneurosci.org as supplemental material). Similarly, olfactory response of dCREB2-b larvae was not altered by heat-shock treatment (supplemental Table 1, available at www.jneurosci.org as supplemental material). Gustatory response of dCREB2-b larvae was slightly higher with heat shock but the difference was not significant (supplemental Table 1, available at www.jneurosci.org as supplemental material). These results suggest that aversive memory formed with LIN/QH by this paradigm involves neither *amn*- nor CREB-dependent components, a feature consistent with its short-term stability described above.

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

In the adult fly, different subsets of MB neurons are used sequentially during odor memory processing, in which neurotransmission from the α'/β' neurons is required to acquire and stabilize both aversive and appetitive memory (Krashes et al., 2007; Krashes and Waddell, 2008), whereas neural transmission from α/β neurons is required for memory retrieval (Dubnau et al., 2001; McGuire et al., 2001; Schwaerzel et al., 2002, 2003; Krashes et al., 2007; Krashes and Waddell, 2008). In contrast to the multiple-lobe organization of the adult MB, the larval MB, whose neurons are classified into a single type that corresponds to the γ group in the adult, exhibits a morphologically homogeneous projection pattern with only one dorsal lobe and one medial lobe, although concentric layer organization is found internally (Kurusu et al., 2002).

Despite the simple organization, synaptic output of larval MB neurons has been shown to be required for retrieval, but not for acquisition and retention, of larval appetitive memory formed with LIN/SUC (Honjo and Furukubo-Tokunaga, 2005). To determine the neural circuits for larval aversive memory induced with LIN/QH, we temporarily inactivated synaptic transmission of larval MB neurons by expressing *UAS-shi* ^{ts1} (Kitamoto, 2001) under MB-GAL4 drivers, *201Y*, and *OK301*, both of which drive specific MB expression in the larval brain (Kurusu et al., 2002; Honjo and Furukubo-Tokunaga, 2005). Most of the larval MB neurons are labeled with

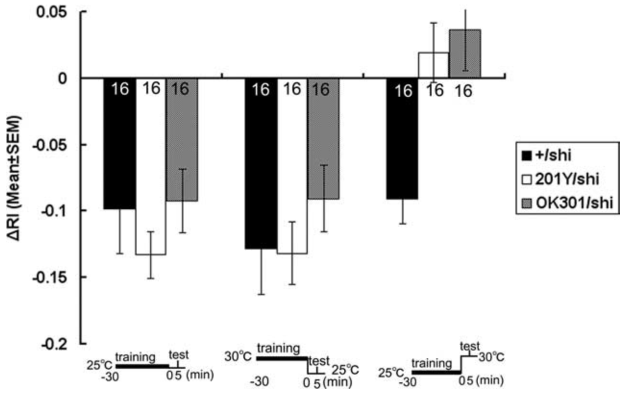

Figure 3. Synaptic output of MB neurons is required for retrieval of larval aversive memory. Larval memory performance was tested at 5 min after aversive LIN/QH conditioning. *UAS-shi*^{ts1} was expressed by either *201Y* or *OK301* GAL4 drivers. The temperature shift protocol is indicated below each set of the graphs. No difference was detected in memory performance for *201Y/shi*^{ts1} and *OK301/shi*^{ts1} larvae when both training and test were performed at 25°C or when training was performed at 30°C and test was performed at 25°C ($p > 0.5$ for both types of larvae, compared with $+$*/shi*^{ts1} by the Kruskal–Wallis test followed by Dunn's *post hoc* test, also confirmed by ANOVA). In contrast, olfactory memory was completely impaired in *201Y/shi*^{ts1} and *OK301/shi*^{ts1} larvae when trained at 25°C and tested at 30°C ($p < 0.05$ for both types of larvae, compared with $+$*/shi*^{ts1} by the Kruskal–Wallis test and Dunn's *post hoc* test, also confirmed by ANOVA).

201Y, whereas a more restricted subset of MB neurons is labeled by OK301 (Honjo and Furukubo-Tokunaga, 2005).

Larvae expressing *UAS-shi*^{ts1} by either MB driver showed normal 5 min memory when both training and test were performed at the permissive temperature (25°C) (Fig. 3). Memory performance was also unaffected in *201Y/shi*^{ts1} and *OK301/shi*^{ts1} larvae when trained at the restrictive temperature (30°C) and tested at the permissive temperature. In contrast, when trained at the permissive temperature and tested at the restrictive temperature, aversive memory performance was lost in both *201Y/shi*^{ts1} and *OK301/shi*^{ts1}, but not in the control $+$*/shi*^{ts1}, larvae.

Control experiments for sensory acuities showed that the naive olfactory response of *OK301/shi* ^{ts1} was higher at the restrictive temperature ($p < 0.05$ by the Mann–Whiney *U* test) (supplemental Table 1, available at www.jneurosci.org as supplemental material). The quinine reactivity of *201Y/shi*^{ts1} larvae was slightly lower at 30°C but the difference was not significant ($p > 0.5$). Otherwise, the olfactory and gustatory responses of *201Y/shi*^{ts1}, *OK301/shi*^{ts1} and the control $+$*/shi*^{ts1} larvae were not altered significantly between the permissive and the restrictive temperatures (supplemental Table 1, available at www.jneurosci.org as supplemental material).

These results demonstrate that, as with the larval appetitive memory induced with LIN/SUC, synaptic output of larval MB neurons is required for the retrieval, but not for the acquisition, of the larval aversive memory induced with LIN/QH. The result that the retrieval of appetitive and aversive memory is similarly blocked in *OK301/shi*^{ts1} larvae suggest that both types of memory traces might be localized to or mediated by an overlapping subset of MB neurons labeled by this driver of limited MB expression, although elucidation of the exact usage of different subsets of larval MB neurons awaits further behavioral and anatomical investigations.

Synaptic output of DA and OA/TA neurons is differentially required for acquisition of larval aversive and appetitive memory

Biogenic amines have critical functions in mediating the reinforcing effects of US in associative learning in insects (Hammer, 1993; Hammer and Menzel, 1998; Schwaerzel et al., 2003; Unoki et al., 2005, 2006; Kim et al., 2007; Vergoz et al., 2007). In the adult fly, OA is necessary for the formation of sugar reward memory whereas synaptic output of DA neurons is required for acquisition, but not retrieval, of aversive memory induced with electric shock (Schwaerzel et al., 2003). Mutations of the dDA1 significantly suppress aversive olfactory learning, and moderately impair sugar-mediated learning (Kim et al., 2007). It is also shown that light-induced activation of larval DA and OA/tyraminergic (TA) neurons triggers aversive and appetitive learning, respectively, in *Drosophila* larvae (Schroll et al., 2006). However, whether the DA and OA/TA neurons are differentially required for larval aversive or appetitive olfactory learning remains to be demonstrated.

To identify the modulatory neurons involved in larval associative learning, we expressed *UAS-shi*^{ts1} in larval DA and OA/TA neurons using GAL4 drivers, *TH-GAL4* (Friggi-Grelin et al., 2003) and *TDC2-GAL4 (*Cole et al., 2005), respectively. *TH-GAL4* is expressed in larval DA neurons under the promoter of the *tyrosine hydroxylase* (*TH*) gene, which is involved in DA synthesis. *TH-GAL4* recapitulates most of the expression pattern of the *tyrosine hydroxylase* gene in the larval brain (supplemental Fig. 2*A*, available at www.jneurosci.org as supplemental material) (Friggi-Grelin et al., 2003). However, *TDC2-GAL4* is expressed in larval OA/TA neurons under the promoter of the *tyrosine decarboxylase* (*TDC*) gene. Double-labeling experiments confirmed that most of the *TDC2-GAL4* neurons express *tyramine β-hydroxylase* mRNA (supplemental Fig. 2*B*, available at www.jneurosci.org as supplemental material), which converts TA to OA. Indeed, double labeling with an anti-OA antibody confirmed that *TDC2-GAL4* soma, in particular, those in the subesophageal ganglia (SOG), and their extensions are immunoreactive for OA (supplemental Fig. 3, available at www.jneurosci.org as supplemental material).

When the *TH/shi*^{ts1} or *TDC2/shi*^{ts1} larvae were aversively trained with QH and tested at the permissive temperature (25°C), both genotypes of larvae showed normal memory scores (Fig. 4*A*). However, when these larvae were trained at the restrictive temperature (31°C) and tested at the permissive temperature, memory performance was abolished in *TH/shi*^{ts1}, but not in *TDC2/shi*^{ts1}, larvae ($p < 0.01$ compared with $+$*/shi*^{ts1} larvae). However, *TH/shi*^{ts1} larvae exhibited significant memory performance when trained at the permissive temperature and tested at the restrictive temperature. Memory performance of *TDC2/shi*^{ts1} was also significant in the 25–31°C shift experiment (supplemental Table 2, available at www.jneurosci.org as supplemental material) ($p < 0.01$ comparison between LIN/SUC and LIN alone conditionings by the Mann–Whitney *U* test), and indistinguishable from that of the control $+$*/shi*^{ts1} larvae.

$+$*/shi*^{ts1}larvae showed lower performance in the 25–31°C shift compared with their performances in the other temperature shift experiments (not significant by the Kruskal–Wallis test, $p = 0.05$) (also see supplemental Fig. 4*A*, available at www.jneurosci.org as supplemental material). However, *TH/shi*^{ts1} showed better performance in the 25–31°C shift experiment compared with their performances in the 25–25°C shift experiment (not significant, $p > 0.2$ by the Kruskal–Wallis test) (supplemental Fig. 4*A*, available at www.jneurosci.org as supplemental material), resulting a better performance when compared with the control $+$*/shi*^{ts1}larvae ($p < 0.05$ by the Kruskal–Wallis test followed by Dunn's *post hoc* test).

However, when *TH/shi*^{ts1} or *TDC2/shi*^{ts1} larvae were appetitively trained with SUC at restrictive temperature and tested at

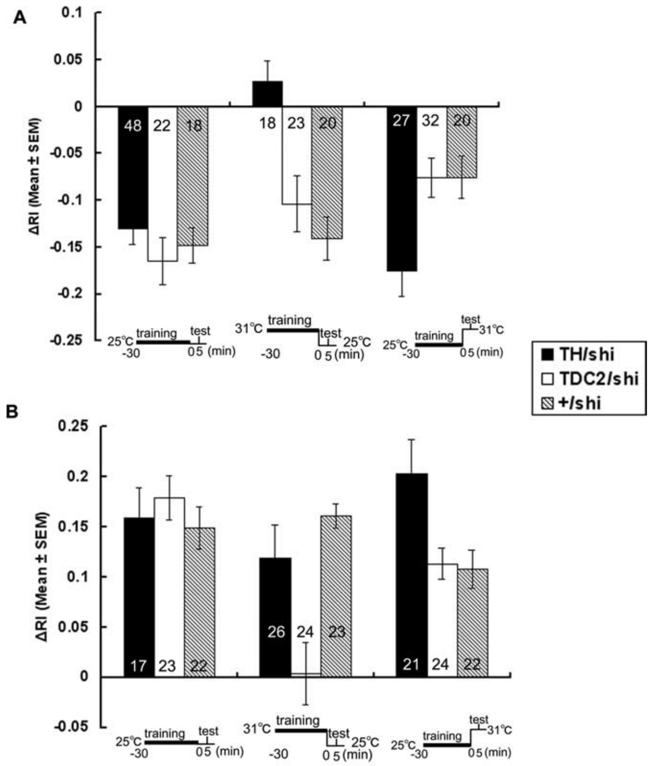

Figure 4. Synaptic output of monoaminergic neurons is differentially required for acquisition of appetitive and aversive memory. *UAS-shi*^{ts1} was driven by *TH-GAL4* or *TDC2-GAL4* to block synaptic transmission from DA or OA/TA neurons, respectively. The temperature shift protocol is indicated below each set of graphs. ***A***, Aversive 5 min memory induced with LIN/QH. *TH/shi*^{ts1}, but not *TDC2/shi*^{ts1}, larvae exhibited significant memory impairment when trained at 31°C and tested at 25°C ($p < 0.01$ compared with the $+$/*shi*^{ts1}by the Kruskal–Wallis test with Dunn's *post hoc* test, also confirmed by ANOVA). In the 25–31°C shift experiment, memory performance of *TH/shi*^{ts1} larvae was higher than that of $+$/*shi*^{ts1} larvae ($p < 0.05$ by the Kruskal–Wallis test followed by Dunn's *post hoc* test) but not adversely altered. No difference was found between *TDC2/shi*^{ts1} and $+$/*shi*^{ts1} larvae in the 25–31°C shift experiment ($p > 0.5$ by the Kruskal–Wallis test followed by Dunn's *post hoc* test, also confirmed by ANOVA), and both *TDC2/shi*^{ts1} and $+$/*shi*^{ts1} larvae exhibited significant memory performance (see supplemental Table 2, available at www.jneurosci.org as supplemental material). However, neither *TH/shi*^{ts1} nor *TDC2/shi*^{ts1} showed memory defect in the 25–25°C shift experiment ($p > 0.5$ compared with $+$/*shi*^{ts1} by the Kruskal–Wallis test followed by Dunn's *post hoc* test also confirmed by ANOVA). ***B***, Appetitive 5 min memory induced with LIN/SUC. *TDC2/shi*^{ts1}, but not *TH/shi*^{ts1}, larvae showed significant memory impairment when trained at 31°C and tested at 25°C ($p < 0.01$ compared with the $+$/*shi*^{ts1} by the Kruskal–Wallis test followed by Dunn's *post hoc* test, also confirmed by ANOVA). In the 25–31°C shift experiment, memory performance of *TH/shi*^{ts1} larvae was higher than that of $+$/*shi*^{ts1} larvae ($p < 0.05$ by the Kruskal–Wallis test with Dunn's *post hoc* test) but not adversely altered. No difference was found between *TDC2/shi*^{ts1} and $+$/*shi*^{ts1} larvae in the 25–31°C shift experiment ($p > 0.5$ by the Kruskal–Wallis test with Dunn's *post hoc* test, also confirmed by ANOVA), and both *TDC2/shi*^{ts1} and $+$/*shi*^{ts1} types of larvae exhibited significant memory performance (see supplemental Table 2, available at www.jneurosci.org as supplemental material). However, neither *TH/shi*^{ts1} nor *TDC2/shi*^{ts1} showed memory defect in the 25–25°C shift experiment ($p > 0.5$ compared with $+$/*shi*^{ts1} by the Kruskal–Wallis test with Dunn's *post hoc* test also confirmed by ANOVA).

permissive temperature, memory performance was abolished in *TDC2/shi*^{ts1}, but not in *TH/shi*^{ts1}, larvae ($p < 0.01$, compared with $+$/*shi*^{ts1} larvae) (Fig. 4*B*). Normal memory performance was observed in both *TH/shi*^{ts1} and *TDC2/shi*^{ts1} larvae when trained at 25°C and tested also at 25°C ($p > 0.5$, compared with $+$/*shi*^{ts1} larvae). *TH/shi*^{ts1} larvae exhibited significant memory performance when trained at 25°C and tested at 31°C (supplemental Table 3, comparison between LIN/SUC and LIN alone conditionings by the Mann–Whitney *U* test, available at www.jneurosci.org as supplemental material) ($p < 0.01$). Memory performance of *TDC2/shi*^{ts1} was also significant in the 25–31°C shift experiment (supplemental Table 3, available at www.jneurosci.org as supplemental material) ($p < 0.01$ comparison between LIN/SUC and LIN alone conditionings by the Mann–Whitney *U* test), and indistinguishable from that of the control $+$/*shi*^{ts1} larvae.

As with the aversive memory performance, $+$/*shi*^{ts1}larvae showed lower performance in the 25–31°C shift experiment with appetitive US (not significant, $p > 0.1$, compared with their performances in the other temperature shift experiments by the Kruskal–Wallis test) (supplemental Fig. 4*B*, available at www.jneurosci.org as supplemental material). However, *TH/shi*^{ts1} larvae again showed better performance in the 25–31°C shift experiment compared with their performances in the other temperature shift experiments (not significant, $p > 0.5$ by the Kruskal–Wallis test) (supplemental Fig. 4*B*, available at www.jneurosci.org as supplemental material), resulting a higher performance when compared with the control $+$/*shi*^{ts1}larvae ($p < 0.05$ by the Kruskal–Wallis test followed by Dunn's *post hoc* test).

As described, *TH/shi*^{ts1} larvae exhibited better performance in the 25–31°C shift experiment when compared with the $+$/*shi*^{ts1} control in both aversive and appetitive memory tests. To examine whether the ΔRI values of the *TH/shi*^{ts1} larvae in the 25°C-31°C shift experiment represent nonassociative components, we trained *TH/shi*^{ts1} larvae at 25°C by successively exposing them to the US and the CS odor in a temporally dissociated manner, and examined their memory performance at 31°C. However, we failed to detect significant memory performance in this way with either aversive or appetitive US ($\Delta RI = -0.05 \pm 0.03$ with QH; $\Delta RI = +0.01 \pm 0.03$ with SUC) (supplemental Tables 2 and 3, available at www.jneurosci.org as supplemental material) ($p > 0.5$, comparison between LIN/SUC and LIN alone by the Mann–Whitney *U* test), suggesting that most, if not all, of the memory scores of the *TH/shi*^{ts1} larvae in the 25–31°C shift experiment represent an associative component. We also examined whether the increased memory score of the *TH/shi*^{ts1} larvae was caused by the genetic background of the *TH-GAL4* chromosome, but found that the heterozygous $+$/*TH* control larvae exhibited memory scores that were comparable with that of $+$/*shi*^{ts1} larvae ($\Delta RI = -0.10 \pm 0.03$ with QH; $\Delta RI = +0.09 \pm 0.03$ with SUC) (supplemental Tables 2 and 3, available at www.jneurosci.org as supplemental material).

Although these data argue for that the higher memory score with *TH/shi*^{ts1} larvae is mostly caused by associative learning, it is an unexplained effect that is not understood. We therefore should be cautious in interpreting the *TH/shi*^{ts1} results in the 25–31°C shift experiment as an enhanced memory performance. As described above, the ΔRI value of *TH/shi*^{ts1} larvae in the 25–31°C shift experiment was not increased significantly from their ΔRI value in the 25–25°C control for either aversive or appetitive memory (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). Furthermore, although the olfactory response of *TH/shi*^{ts1} larvae was significantly altered after both types of the associative training, *TH/shi*^{ts1} larvae exhibited lower olfactory response for LIN in the 25–31°C shift experiment (supplemental Tables 2 and 3, available at www.jneurosci.org as supplemental material), thus suggesting irregularity in their sensory-motor activities specifically induced in the temperature shift experiment. Indeed, *TH/shi*^{ts1} larvae exhibited complex response to LIN in the memory test under the 25–31°C temperature shift; they were initially repelled shortly but then attracted by the test odor, whereas they showed straightforward response to the odor at the other temperature conditions as with the other larvae including +/*shi*^{ts1}.

In other respects, olfactory responses of the *TH/shi*^{ts1}, *TDC2/shi*^{ts1} and +/*shi*^{ts1}larvae did not differ between the permissive and restrictive temperatures (supplemental Table 1, available at www.jneurosci.org as supplemental material). Both *TH/shi*^{ts1} and *TDC2/shi*^{ts1} larvae showed higher sugar response at the restrictive temperature, but gustatory reactivity of *TH/shi*^{ts1}, *TDC2/shi*^{ts1} and +/*shi*^{ts1}larvae was not altered adversely at the restrictive temperature (supplemental Table 1, available at www.jneurosci.org as supplemental material). These results thus indicate that the memory deficits of *TH/shi*^{ts1} and *TDC2/shi*^{ts1} larvae observed in the acquisition blockade (31–25°C shift experiment) are unlikely to be caused by sensory-motor defects.

As a whole, these results confirm that the monoamines DA and OA/TA indeed play important roles in mediating the US signals in larval associative learning, and demonstrate that neural transmission of DA and OA/TA neurons is differentially required for acquisition of aversive and appetitive larval memory, respectively. It should be noted, however, that our results do not exclude the involvement of other transmitters that might be coreleased by the DA or OA/TA neurons targeted by the GAL4 drivers.

DA and OA/TA neurons exhibit spatially distinct innervation patterns on larval MB and AL

The fact that synaptic transmission of MB neurons is required to retrieve both aversive and appetitive memory suggests that both types of memory might be localized to larval MB neurons or upstream circuits such as AL neurons. Although our results indicated that synaptic output of DA and OA/TA neurons were differentially required for the acquisition of aversive and appetitive larval memory, respectively, the underlying mechanisms by which these monoaminergic neurons convey the US information into the memory circuitry remained to be elucidated. As a way to answer this question, we investigated the local projection patterns of the DA and OA/TA neurons in MB and AL.

In the adult brain, DA neurons innervated the α/α'-lobes, the heel, parts of the β/γ-lobes, and less densely the calyx (supplemental Fig. 5*A*,*B*, available at www.jneurosci.org as supplemental material) (see also Riemensperger et al., 2005; Zhang et al., 2007). In addition, DA neurons exhibited interglomerulus innervation in ALs (supplemental Fig. 5*C*, available at www.jneurosci.org as supplemental material). Similarly, OA/TA neurons projected multiple parts of the adult MBs innervating the heel, parts of the γ-lobe, and the calyx (supplemental Fig. 6*A*,*B*, available at www.jneurosci.org as supplemental material) (Sinakevitch and Strausfeld, 2006). Dense innervation in the glomeruli of the AL was also evident (supplemental Fig. 6*C*, available at www.jneurosci.org as supplemental material).

In contrast to the complex and partially overlapping innervation patterns of the DA and OA/TA neurons in the adult brain, projection patterns of these monoaminergic neurons were much simpler in the larval brain, and exhibited distinctive and rather complementary innervation patterns in MB and AL. Thus, whereas DA neurons densely ramified on the heel and the vertical lobe of the larval MB (Fig. 5*A*), no innervation was detected in the larval calyx and AL (Fig. 5*B*,*C*). In contrast, OA/TA neurons exhibited little innervation on the vertical lobe (Fig. 5*D*). Finally, OA/TA neurons intensively innervated both the calyx and AL (Fig. 5*E*,*F*), in which a large number of OA puncta were observed along the projections of OA/TA neurons (supplemental Fig. 7, available at www.jneurosci.org as supplemental material). Most part of the medial lobe remained uninnervated by either DA or OA/TA neurons (data not shown).

Discussion

Aversive conditioning in *Drosophila* larvae

Because of its simple neural organization, larval learning in *Drosophila* has become an increasingly important model system (for review, see Gerber and Stocker, 2007). In the present study, we have characterized larval aversive memory induced by a paradigm we described previously with appetitive US (Honjo and Furukubo-Tokunaga, 2005). Control studies demonstrated that the induced difference in the larval olfactory response (ΔRI) is specific to the CS odor, and requires temporal association of aversive US and olfactory CS. Moreover, we have shown that larval aversive memory induced with LIN/QH or LIN/NaCl is short lived despite that the same training protocol induces more stable memory with LIN/SUC (Honjo and Furukubo-Tokunaga, 2005).

Gerber and Hendel (2006) described that larval aversive memory with gustatory US requires the presence of the aversive cue for its recall. However, we detected memory performance without aversive cue in the test plate (Fig. 1) and found no difference in memory performance by the inclusion of QH in the test plate (data not shown). The exact reason for this discrepancy is yet to be analyzed, but several differences are noteworthy. Whereas Gerber and Hendel (2006) train larvae reciprocally with CS (+) and CS (−) odors and examine differential odor preference, our protocol includes only a single CS odor and dose not involve odor discrimination task during memory retrieval. It is also noteworthy that the majority of larvae (88%) ingest QH in our protocol, whereas larvae rarely take up QH-containing gel in Hendel et al. (2005). Given that larvae have both external and internal gustatory neurons (Python and Stocker, 2002; Colomb et al., 2007), ingestion of US substance may induce additional stimulation of internal gustatory receptors. Alternatively, ingestion itself and/or maintenance of bitter substance in the gut could contribute to memory formation and retrieval in our protocol.

Differential stability of appetitive and aversive memory

In contrast to the medium-term stability of appetitive memory induced with LIN/SUC, larval aversive memory induced with LIN/QH or LIN/NaCl is short lived, although we use the same CS odor, the same US modality, and the identical training paradigm. However, our results do not exclude the possibility of inducing longer-lived memory with aversive training. Whereas Aceves-Piña and Quinn (1979) described that larval aversive memory induced with electric shock is lost in 30 min and independent of *amn*, Tully et al. (1994a) described that larval aversive memory induced with repetitive electric shocks requires *amn* and lasts through metamorphosis, suggesting that longer-lived memory including a CREB-dependent consolidated memory would be induced in larvae with aversive US depending on the training protocol. Moreover, it is also established that repetitive training with electric shock induces LTM in the adult fly (Tully et al., 1994b).

However, studies with appetitive US suggest that longer-lived memory can be induced with appetitive US in adult flies after much fewer trials (Tempel et al., 1983; Krashes and Waddell, 2008). Indeed, recent work (Krashes and Waddell, 2008) demonstrates that appetitive olfactory conditioning produces *radish* and protein synthesis-dependent long-term memory in the adult fly only by a single 2 min training session. While differences in the

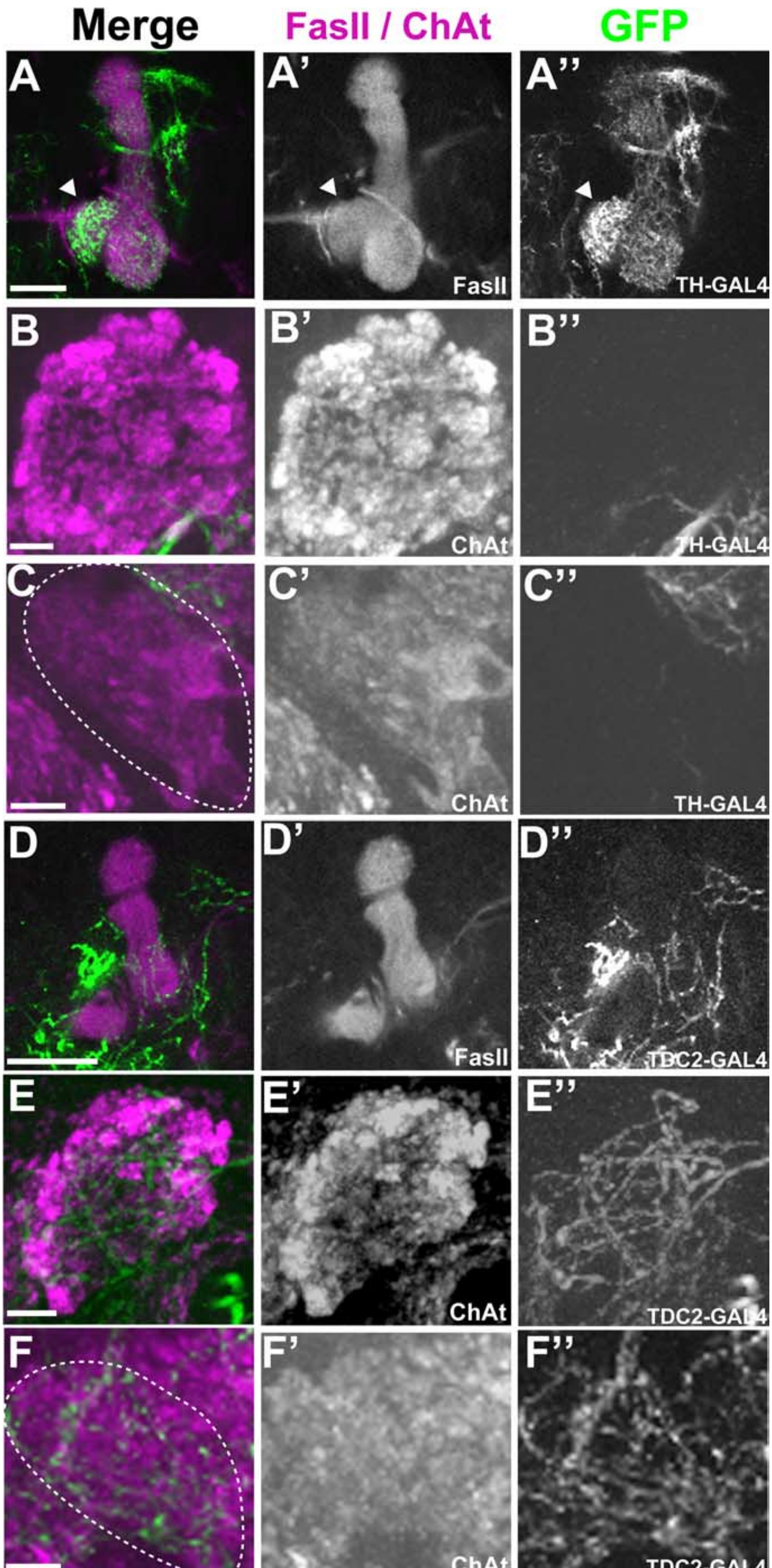

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Figure 5. Distinct projection patterns of the DA and OA/TA neurons in the larval brain. ***A–C***, Innervation patterns of DA neurons on MB and AL. In ***C*** and ***F***, the larval AL neuropil is demarcated with dotted circle. Note DA neurons densely innervate the MB heel (arrowhead) and the vertical lobe (***A–A″***), but not the calyx (***B-B″***) and the AL glomeruli (***C-C″***). ***D–F***, Innervation patterns of OA/TA neurons on MB and AL. OA/TA neurons intensively innervate the MB calyx (***E–E″***) and AL glomeruli (***F-F″***), but not the MB lobes (***D-D″***). The diffuse OA/TA neuron projections around the lobes (***D-D″***) have no direct contact on the lobes. Brains were immunolabeled for the indicated marker. Innervation patterns of DA and OA/TA neurons were visualized with *UAS-mCD8::GFP* using *TH-GAL4* (***A–C***) or *TDC2-GAL4* (***D–F***). Early third instar brain. Scale bars: (***A***, ***D***) 20 μm; (***B***, ***C***, ***E***, ***F***) 5 μm.

sensory modality of the US, the number of training cycles, and the physiological state of the animals (starved for reward conditioning) undermine direct comparison, combined with our results, these results suggest differential memory induction mechanisms in the brain that might be predisposed for more efficient induction of longer-lived memory with appetitive reinforcement.

Differential functions of monoaminergic neurons in memory induction

Mutational and pharmacological studies on insect associative learning emphasize the importance of monoaminergic systems in different types of reinforcements (Hammer and Menzel, 1998; Schwaerzel et al., 2003; Unoki et al., 2005, 2006; Kim et al., 2007; Vergoz et al., 2007). In the adult fly, OA is necessary for the formation of sugar reward memory, whereas synaptic output of DA neurons is required for acquisition, but not retrieval, of electric shock memory (Schwaerzel et al., 2003). In honeybees, the octopaminergic VUMmx1 neuron, which projects bilaterally to AL, MB, and lateral protocerebrum, mediates the reinforcing function of sugar US in reward conditioning of proboscis extension response (Hammer, 1993). Aversive learning in honeybees depends on DA, but not OA, receptors (Vergoz et al., 2007). Differential reinforcing properties of OA/TA and DA neurons was also examined in *Drosophila* larvae by expressing Channelrhodopsin-2 in the larval monoaminergic neurons (Schroll et al., 2006). Thus, they have shown that light-induced activation of larval OA/TA or DA neurons is sufficient to induce appetitive and aversive learning, respectively. The result of the present study that the synaptic activity of OA/TA or DA neurons is indeed differentially required for appetitive or aversive larval learning further supports the notion that different reinforcing mechanisms involving monoamine modulators are responsible for the induction of the two types of memory in the insect brain.

However, Kim et al. (2007) showed that, in addition to severe impairment of electric shock-mediated aversive learning, D_1–DA receptor mutations exhibit moderate impairment of sugar-mediated learning, suggesting that DA signaling also is required for appetitive learning in the adult fly. The discrepancy between this mutant study and the study with *TH-GAL4/shi*^{ts1} flies (Schwaerzel et al., 2003) might in part be explained by extensive physiological and/or developmental alterations caused by chronic loss of DA signaling by the genetic disruptions in the mutants, which could alter other brain functions affecting associative learning, such as arousal and/or attention states (Wu et al., 2000; Andretic et al., 2005; van Swinderen, 2007; Seugnet et al., 2008). Whether dDA1 is required for both appetitive and aversive memory is yet to be demonstrated in larvae.

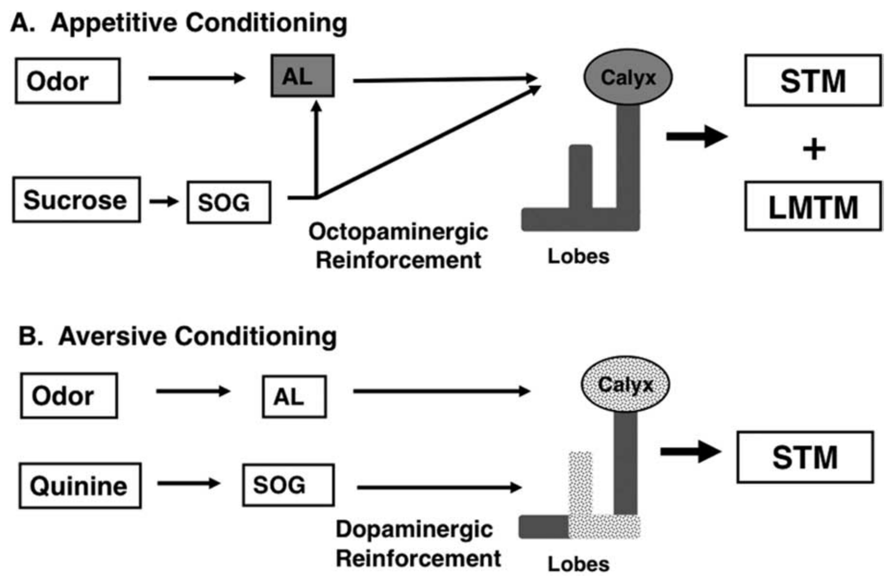

Figure 6. Convergence of the CS and US signals in the *Drosophila* larval brain. The organization of the larval olfactory system shows a parallelism to the adult system, but is straightforward, lacking cellular redundancy. Moreover, the US pathways are also simplified in the larval brain with less redundancy and complexity. The CS pathway; the odor CS information sensed by olfactory sensory neurons is transmitted to specific sets of the AL glomeruli. From there, projection neurons further convey the CS information to the calyx, the dendritic structure of MB. The US pathways; ***A***, The appetitive pathway; the sucrose reward stimuli sensed by sugar-responsive gustatory neurons is first transmitted to SOG, the primary gustatory center. Although the precise connectivity in SOG is unknown, the appetitive US information is transmitted to the modulatory OA/TA neurons, which project in duplicate onto the dendritic structures of AL and MB. The reward US information may thus be associated with the odor CS information both at the AL glomeruli and the MB calyx, leading to the formation of STM and a larval medium-term memory (LMTM). Similar connectivity is found in the adult OA/TA neurons (see supplemental Fig. 6, available at www.jneurosci.org as supplemental material). ***B***, The aversive pathway; the bitter sensing gustatory neurons project to SOG, from which the aversive US information is transmitted to the modulatory DA neurons. DA neurons project on the MB lobes, but neither on the calyx nor the AL glomeruli. Consequently, the convergence of the odor CS and the aversive US signals may be achieved only on MB via spatially separate inputs on the different parts of the Kenyon cells. Only STM is formed with the aversive reinforcement. Notably, this simple innervation pattern of DA neurons is modified in the adult brain; they also project to the AL glomeruli and the MB calyx along with extensive innervation to other parts of the brain (supplemental Fig. 5, available at www.jneurosci.org as supplemental material).

Differential US–CS convergence circuitries in appetitive and aversive memories

In associative learning, the strength of the association between CS and US is thought to determine learning efficacy, and memory is formed at the convergence site of the two stimuli in the brain by altering interacting synaptic strength. Given that both aversive and appetitive memory traces are stored in partially overlapping neural circuitries that are upstream of the MB output synapses, how do different types of reinforcement then lead to different efficacy in inducing longer-lived memory components even with a same CS odorant? It has been shown that olfactory learning is completely abolished by MB expression of a constitutive active Gα protein (Connolly et al., 1996). Kenyon cells express G-protein-coupled receptors for the monoamines that represent the US reinforcing property; both OA and DA receptors are preferentially expressed in MB neurons (Han et al., 1996, 1998). Whereas little is known about the intracellular biochemical events triggered by the activation of these receptors, different G-protein-coupled receptors for the monoamines might trigger distinctive molecular components, such as A-kinase anchoring proteins (Lu et al., 2007; Schwaerzel et al., 2007), that may differentiate the efficacy in inducing longer-lived memory within an overlapping set of neurons.

However, differential efficacy in longer-lived memory induction might, in part, be accounted for by the neural circuits that mediate reinforcing US signals (Fig. 6). The sugar reward information sensed by gustatory sensory neurons is first transmitted to SOG, and further conveyed to the higher memory centers in the larval brain (Colomb et al., 2007; Vosshall and Stocker, 2007). Intriguingly, the larval OA/TA neurons exhibit intensive innervation on the dendritic structures of AL and MB, suggesting that the sugar-US and the odor-CS signals converge on both sites to form memory traces (Fig. 6*A*). This duplicate convergence pattern of the reward-US and the odor-CS circuitries could, in part, account for the disposition for more efficient generation of stable memory components with appetitive US. With regards to this point, it is noteworthy that Thum et al. (2007) suggested that appetitive memory traces might be localized to both the Kenyon cells and the AL projection neurons in the adult fly. It is also noteworthy that local OA injection to either AL or MB, but not the lateral protocerebrum, produces associative reward learning in honeybees (Hammer and Menzel, 1998).

In contrast, the aversive QH information sensed by bitter sensing neurons is transmitted by DA neurons to only the MB lobe in the larval brain (Fig. 5*A–C*). Consequently, the odor CS and the aversive US are likely to converge on MB alone. Moreover, the US and CS signals were conveyed to the spatially distant subregions of the Kenyon cells (Fig. 6*B*). Considering these differential US–CS convergence patterns, we suggest that, although animals can learn to associate a given odors with various environmental cues by training, the efficacy of inducing longer-lived memory components might be predisposed by the innervation patterns of the endogenous reinforcing circuits in the brain that are genetically programmed through neural development.

Behavioral genetic studies with *Drosophila* have revealed a large number of genes and molecules involved in learning and memory, but the pivotal information as to the neurons and neural networks that mediate memory is still limited. The simple organization of the larval brain will help to identify the functional neural circuits in memory at a promising resolution.

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