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 appetitive and aversive 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 D2–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) and amnesiac 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.

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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 monoamnergic 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) (Livingston et al., 1984) and dundce (dnc) (1) (Dudai et al., 1976); MTM mutant ann(2)A (DeZazzo et al., 1999); hs-dCREB2-b transgenic fly (line 17–2) (Yin et al., 1994); UAS-shibire(1-1) 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., 2003); 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, or 1 M NaCl (Wako). Distilled water (DW) was used for the solution. The reinforcers used were 1 M sucrose (SUC) (Nacalai), 0.1% QH, or 1 M NaCl (Wako). 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. Immunolocalization of the larval and adult brains was performed as described previously (Kurusu et al., 2002). The antibodies used were as follows: Rat anti-CD8a (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-Alexa546 (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. 1A). This suppression was not specific to LIN as a significant RI decrement was also confirmed with another odorant, PA (Fig. 1B). Furthermore, suppression of larval RI required simultaneous exposure to the odor and QH as demonstrated in a temporal dissociation test (Fig. 1C), 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 possibility 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 conditions (∙ 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 rut1 and dnc1, 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 rut1 larvae and the quinine reactivity of dnc1 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.

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 training, we then compared larval gustatory re-
Sensory responses 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. 2F with Fig. 2F 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. 2B–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-shí1st (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 Furukubotokunaga, 2005). Most of the larval MB neurons are labeled with
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* in larval DA and OA/TA neurons using GAL4 drivers, *TH-GAL4* (Friaggi-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. 2A, 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* co-labeled larval *shi* neurons, 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* or *TDC2/shi* larvae were aversively trained with QH and tested at the permissive temperature (25°C), both genotypes of larvae showed normal memory scores (Fig. 4A). 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*, but not in *TDC2/shi* larvae (Fig. 4B). However, *TH/shi* larvae exhibited significant memory performance when trained at the permissive temperature and tested at the restrictive temperature. Memory performance of *TDC2/shi* was also significant in the 25–31°C shift experiment (supplemental Table 2, available at www.jneurosci.org as supplemental material) (Fig. 4C). *TDC2/shi* larvae showed lower performance in the 25–31°C shift compared with their performances in the other temperature shift experiments (not significant that the Kruskal–Wallis test, $p = 0.05$) (also see supplemental Fig. 4A, available at www.jneurosci.org as supplemental material). However, *TH/shi* 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. 4A, available at www.jneurosci.org as supplemental material), resulting a better performance when compared with the control *+/shi* larvae ($p < 0.05$ by the Kruskal–Wallis test followed by Dunn’s post hoc test).

However, when *TH/shi* or *TDC2/shi* larvae were appetitively trained with SUC at restrictive temperature and tested at...
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. 4B, 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 also 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 appetitive and appetitive memory tests. To examine whether the \(\Delta R\) 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 appetitive or appetitive US (\(\Delta R = -0.05 \pm 0.03\) with QH; \(\Delta R = +0.01 \pm 0.03\) 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 (\(\Delta R = -0.10 \pm 0.03\) with QH; \(\Delta R = +0.09 \pm 0.03\) 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 \(\Delta R\) value of TH/shi^{ts1} larvae in the 25–31°C shift experiment was not increased significantly from their \(\Delta R\) value in the 25–25°C control for either appetitive 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 conditions 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 by the Mann–Whitney U test), and indistinguishable from that of the control +/shi^{ts1} larvae.

Figure 4. Synaptic output of monoaminergic neurons is differentially required for acquisition of appetitive and appetitive memory. (A) 5 min of conditioning 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} larvae, as 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 but followed by Dunn's post hoc test, also confirmed by ANOVA). 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 (\(p > 0.5\) by the Kruskal–Wallis test but 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} larvae, as confirmed 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, also confirmed by ANOVA). 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} larvae) by the Kruskal–Wallis test, also confirmed by ANOVA).
odor, whereas they showed straightforward response to the odor at the other temperature conditions as with the other larvae including +/shi 

In other respects, olfactory responses of the TH/shi 

DA neurons exhibited interglomerulus innervation (supplemental Table 1, available at www.jneurosci.org as supplemental material). Both TH/shi 

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 (ARI) 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 does 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
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 reinforcement.

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.
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, D1–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-GALA/shi−1 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; Andreet 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.

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 appetitive and aversive 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; Voshall 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. 6A). 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. 5A–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. 6B). 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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