Differential Conditioning Produces Merged Long-term Memory in *Drosophila*

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Abstract

Multiple spaced trials of aversive differential conditioning can produce two independent long-term memories (LTMs) of opposite valence. One is an aversive memory for avoiding the conditioned stimulus (CS+), and the other is a safety memory for approaching the non-conditioned stimulus (CS–). Here, we show that a single trial of aversive differential conditioning yields one merged LTM (mLTM) for avoiding both CS+ and CS–. Such mLTM can be detected after sequential exposures to the shock-paired CS+ and unpaired CS–, and be retrieved by either CS+ or CS–. The formation of mLTM relies on triggering aversive-reinforcing dopaminergic neurons and subsequent new protein synthesis. Expressing mLTM involves αβ Kenyon cells and corresponding approach-directing mushroom body output neurons (MBONs), in which similar-amplitude long-term depression of responses to CS+ and CS– seems to signal the mLTM. Our results suggest that animals can develop distinct strategies for occasional and repeated threatening experiences.

Introduction

To survive in a complex environment, animals need to learn from threatening experiences to avoid potential dangers. From invertebrates to humans, aversive differential conditioning is widely used to study memories produced by threatening experiences (Carew et al., 1983; Corches et al., 2019; Gentile et al., 1986; Schneider et al., 1999; Tully and Quinn, 1985). After repetitive spaced trials of conditioning, animals form two complementary long-term memories (LTMs) of opposite valence (Jacob and Waddell, 2020; Pollak et al., 2008; Pollak et al., 2010), including the aversive memory to the conditioned stimulus (CS+) and the rewarding memory to the non-conditioned stimulus (CS–). Such complementary LTMs result in enhanced long-lasting discrimination between CS+ and CS– through guiding avoidance to CS+ and approach to CS–. However, it remains unclear whether and how occasional threatening experiences, such as single-trial conditionings, would induce long-lasting changes in future escape behavior.

From invertebrates to humans, experience-dependent long-lasting behavioral modifications mainly rely on the formation of LTMs (Kandel et al., 2014). In *Drosophila*, there are at least two categories of aversive olfactory LTMs that last for more than 7 days. One is the spaced training-induced LTM that can be observed after repetitive spaced training with inter-trial rests (multiple trials with a 15-minute rest interval between each), but not after either single-trial training or repetitive massed training without interval (Tully et al., 1994). Forming such aversive LTM requires new protein synthesis and the paired posterior lateral 1 (PPL1) cluster of dopaminergic neurons (DANs) to depress the connection between odor-activated Kenyon cells (KCs) in the mushroom body (MB) αβ lobe and downstream α2sc (MB-V2) MB output neurons (MBONs) (Aso et al., 2014a; Aso et al., 2014b; Cognigni et al., 2018; Modi et al., 2020; Pascual and Preat, 2001; Placais et al., 2012). The other is a recently reported context-dependent LTM that forms after single-trial training, which does not require protein synthesis-dependent consolidation (Zhao et al., 2019). The expression of context-dependent LTM relies on multisensory integration in the lateral horn and is not affected by blocking KCs. However, all these observations derived from the same design principle that evaluates memory performance through testing the discrimination between CS+ and CS– (Tully and Quinn, 1985). Thus, direct responses to CS+ and CS– have been largely overlooked.

In the current study, we introduced a third-odor test, in which flies were given a choice between either CS+ and a novel odor, or CS– and a novel odor. We therefore identified that the single-trial differential conditioning produces a merged LTM (mLTM) guiding avoidances of both CS+ and CS– for several days after training. The encoding and expression of such mLTM involve new protein synthesis, PPL1 DANs, αβ KCs, and α2sc MBONs. These findings suggest that animals utilize distinct escape strategies for facing occasional and repeated dangers.

Results

Single-trial Training Produces Aversive LTMs to Both CS+ and CS–

We first plotted the memory retention curve at various time points after training (**Figure 1A**). Consistent with previous reports (Davis and Zhong, 2017; Shuai et al., 2010; Tully et al., 1994), flies exposed to single-trial conditioning rapidly lost their ability to discriminate CS+ and CS– within 1 day. However, when trained flies were instead tested between either CS+ and a novel odor, or CS– and a novel odor, they gradually exhibited significant avoidances of both CS+ and CS– from 12 hours to more than 7 days after training (**Figure 1B**).

Based on these observations, we wondered whether such aversive LTMs require new protein synthesis to consolidate. Results show that such avoidances resulted from the formation of protein synthesis-dependent LTM^{CS+} and LTM^{CS-} because the administration of cycloheximide (CXM), a protein synthesis inhibitor, prevented the avoidances of both CS+ and CS– after 1 day (**Figure1C**). The time course of the consolidation of such LTMs could be revealed by cold-shock treatment, which is known

to abolish labile memories but leave consolidated LTM intact (Krashes and Waddell, 2008; Li et al., 2016; Tully et al., 1994). As shown in **Figure 1D**, it takes more than 3 hours for consolidation to be completed.

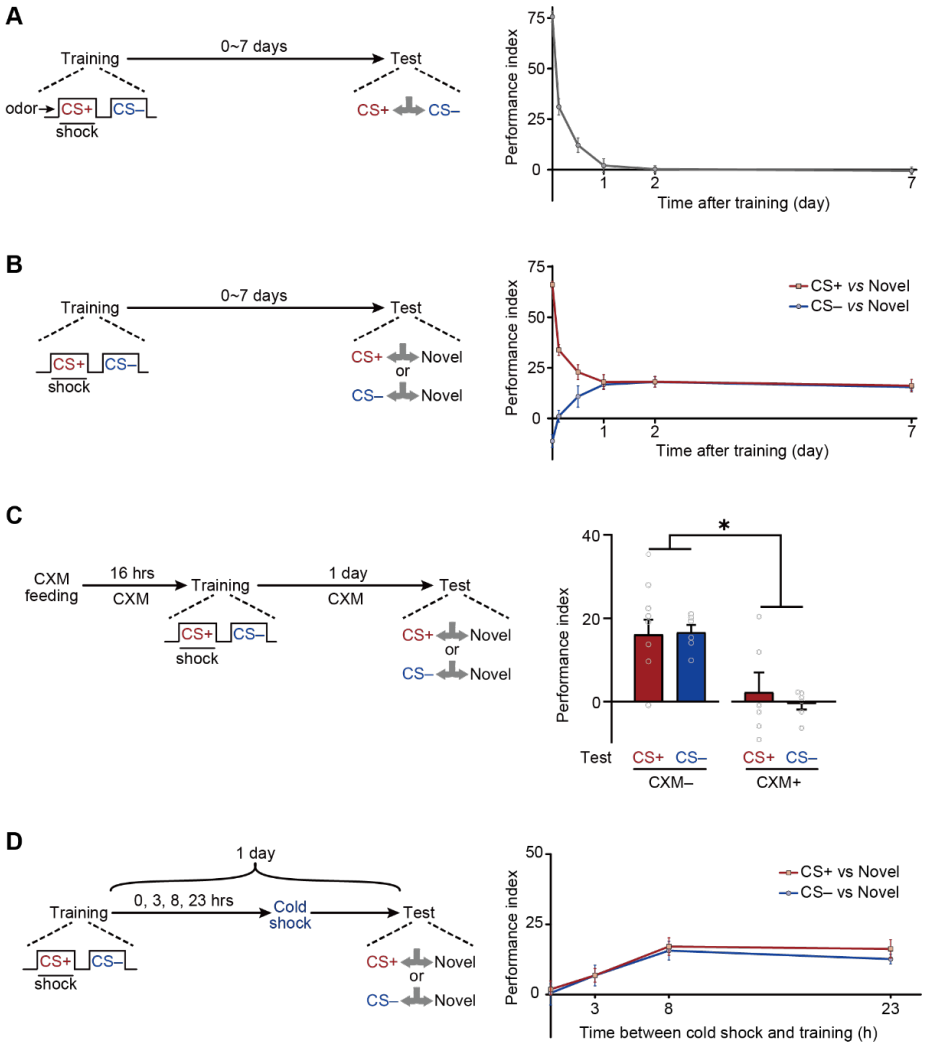

Figure 1. Long-term Avoidances of Both CS+ and CS– Induced by Aversive Differential Conditioning. (A) Single-trial differential conditioning induced discriminative memory that was forgotten within 1 day. **(B)** Conditioned flies exhibited long-lasting avoidances of both CS+ and CS–. **(C)** CXM treatment abolished 1-day avoidances of CS+ and CS–. **(D)** The 1-day avoidances were tested after cold-shock anesthesia at different time points following training. Aversive memory to CS+ and CS– requires a multi-hour consolidation process. All data shown are presented as mean ± SEM. *p < 0.05. NS, non-significant.

The Long-term Avoidances of CS+ and CS– Seem to Be Derived from the Same mLTM

The above results prompted us to investigate whether LTM^{CS+} and LTM^{CS-} are based on two parallel LTMs of the same valence, or the same LTM that can be retrieved by either odor. Two lines of evidence suggested that LTM^{CS+} and LTM^{CS-} are derived from the same memory component.

First, LTM^{CS+} and LTM^{CS-} were both extinguished by the re-exposure to either CS+ or CS– alone (**Figure 2A**). Trained flies were re-exposed to 3 cycles of either CS+ or CS– for 1 minute with 1-minute inter-trial intervals immediately before testing. Such treatment significantly reduced both LTM^{CS+} and LTM^{CS-}, as compared to those re-exposed to air, suggesting the LTM^{CS+} and LTM^{CS-} are both retrievable for either one of CS+ and CS–.

Second, LTM^{CS+} and LTM^{CS-} were both abolished when the temporal interval between CS+ and CS– during training was prolonged to 20 minutes (**Figure 2B**), suggesting that the formation of LTM^{CS+} and LTM^{CS-} depends on the contiguity of two shock-paired CS+ and CS– exposures, instead of temporally separated exposures. In addition, changing the sequence of exposure to CS+ and CS– did not affect either LTM^{CS+} or LTM^{CS-} (**Figure 2C**). Together, these results strongly suggest that, instead of two parallel LTMs, observed LTM^{CS+} and LTM^{CS-} are more likely to be derived from the same memory component. Since it was observable after two separated memory curves (CS+ and CS–) merged over time, we termed it as merged LTM (mLTM).

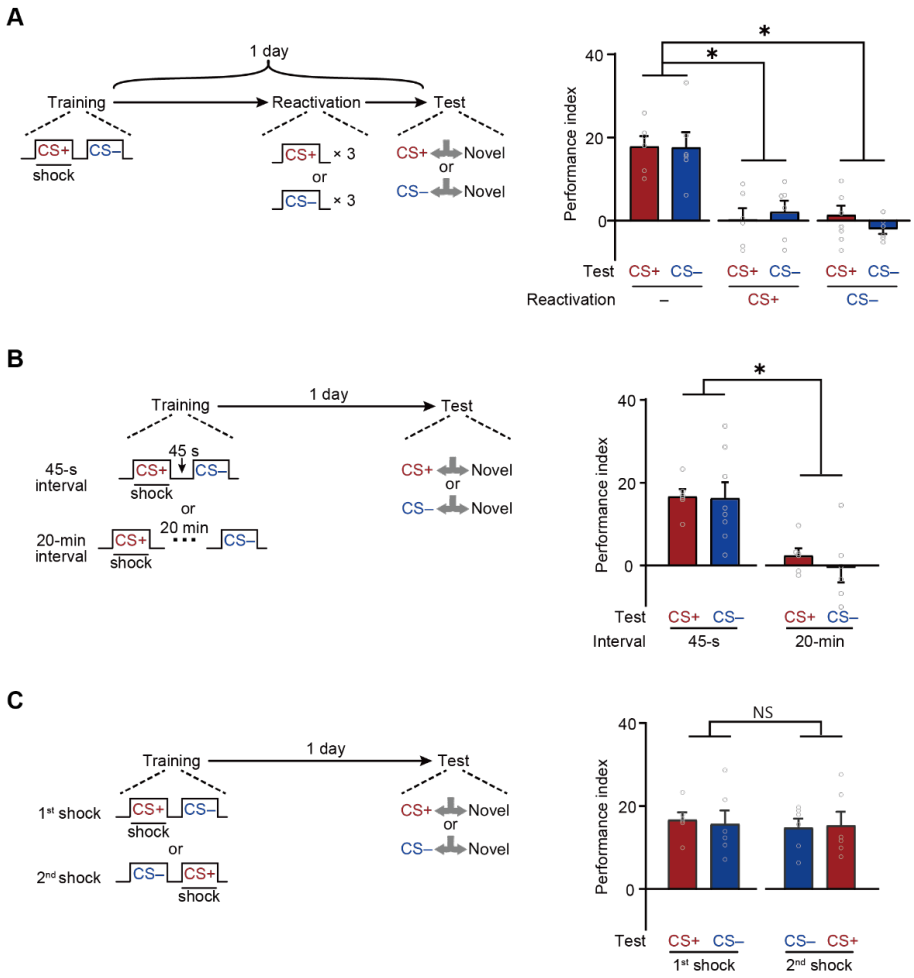

Figure 2. An mLTM Underlies the Long-term Avoidances of CS+ and CS–. (A) Three trials of re-exposure to either CS+ or CS– alone can impair both CS+ avoidance and CS– avoidance 1 day after training. **(B)** Prolonging the interval between CS+ and CS– to 20 minutes abolished 1-day avoidances. **(C)** Changing the sequence of CS+ and CS– during training did not affect 1-day avoidances. All data shown are presented as mean ± SEM. *p < 0.05.

The Encoding of mLTM Requires Aversive-reinforcing DANs

Then we further investigated the neural network mechanisms underlying such mLTM. In *Drosophila*, aversive olfactory memories are mainly encoded by the PPL1 cluster of DANs (Aso and Rubin, 2016; Claridge-Chang et al., 2009; Cognigni et al., 2018) through alterations of the connections between KCs and MBONs (Aso et al., 2014a; Aso et al., 2014b; Cognigni et al., 2018; Dubnau and Chiang, 2013; Modi et al., 2020; Pascual and Preat, 2001; Placais et al., 2012). We therefore tested whether PPL1 DANs involves in mLTM encoding by comparing 24-h memory in control flies with that in flies whose synaptic outputs from PPL1 DANs were blocked during training. For this purpose, we expressed the dominant-negative temperature-sensitive *UAS-Shibire*^{ts1} (*Shi*^{ts})-encoded dynamin (Kitamoto, 2001) driven by *TH-Gal4*, which labels main PPL1 DANs (**Figure S1A**). We specifically blocked output from TH DANs during training by raising the temperature of flies from 23°C to 32°C. Flies were then returned to 23°C and later tested for 1-day memory. The results showed that blocking the release of neurotransmitter from TH DANs during training impaired mLTM (**Figure 3A**), suggesting that the neuromodulation of PPL1 DANs is necessary for mLTM formation.

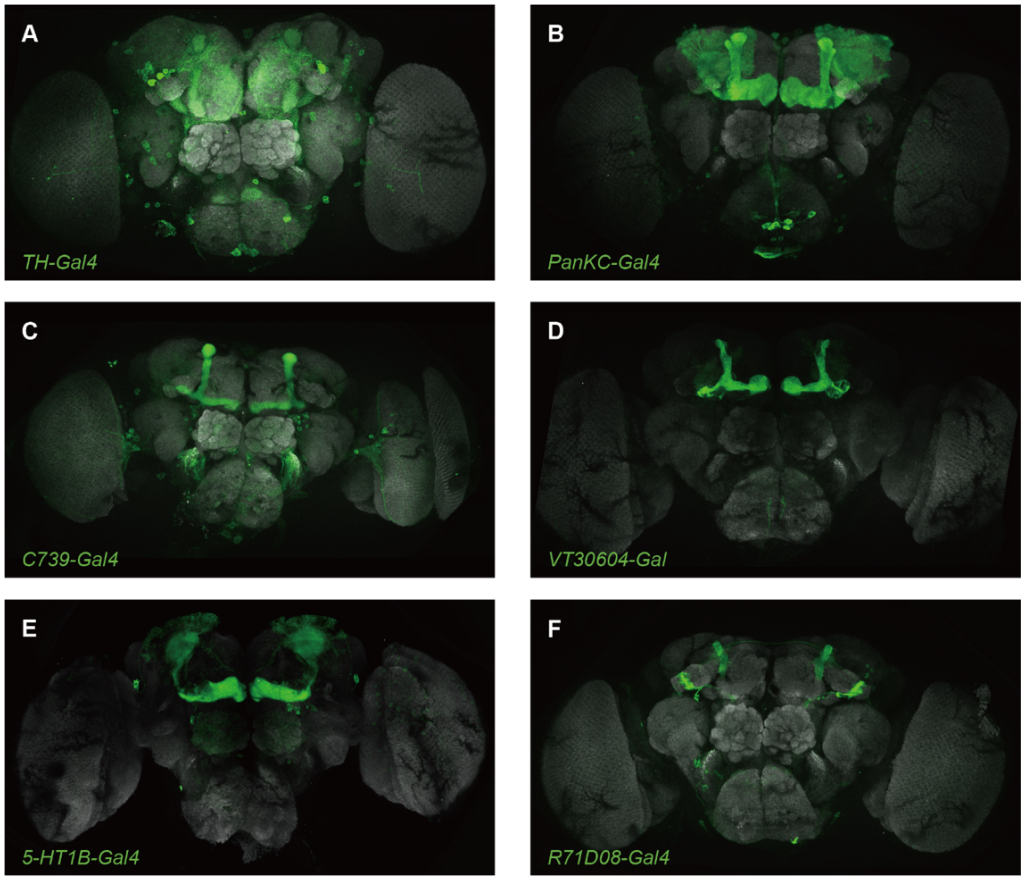

Figure S1. The Expression Patterns of All GAL4 Lines Were Used in This Study. Panels show GFP expression driven by the relevant GAL4 (green) and general neuropil stained with an antibody to the presynaptic marker nc82 (grey). **(A)** *TH-Gal4* broadly labels all PPL1 DANs. **(B)** *PanKC-Gal4* labels all KCs in MB. **(C)** *C739-Gal4* labels αβ KCs. **(D)** *VT20604-Gal4* labels α′β′ KCs. **(E)** *5-HT1B-Gal4* labels γ KCs. **(F)** *R71D08-Gal4* labels α2sc MBONs.

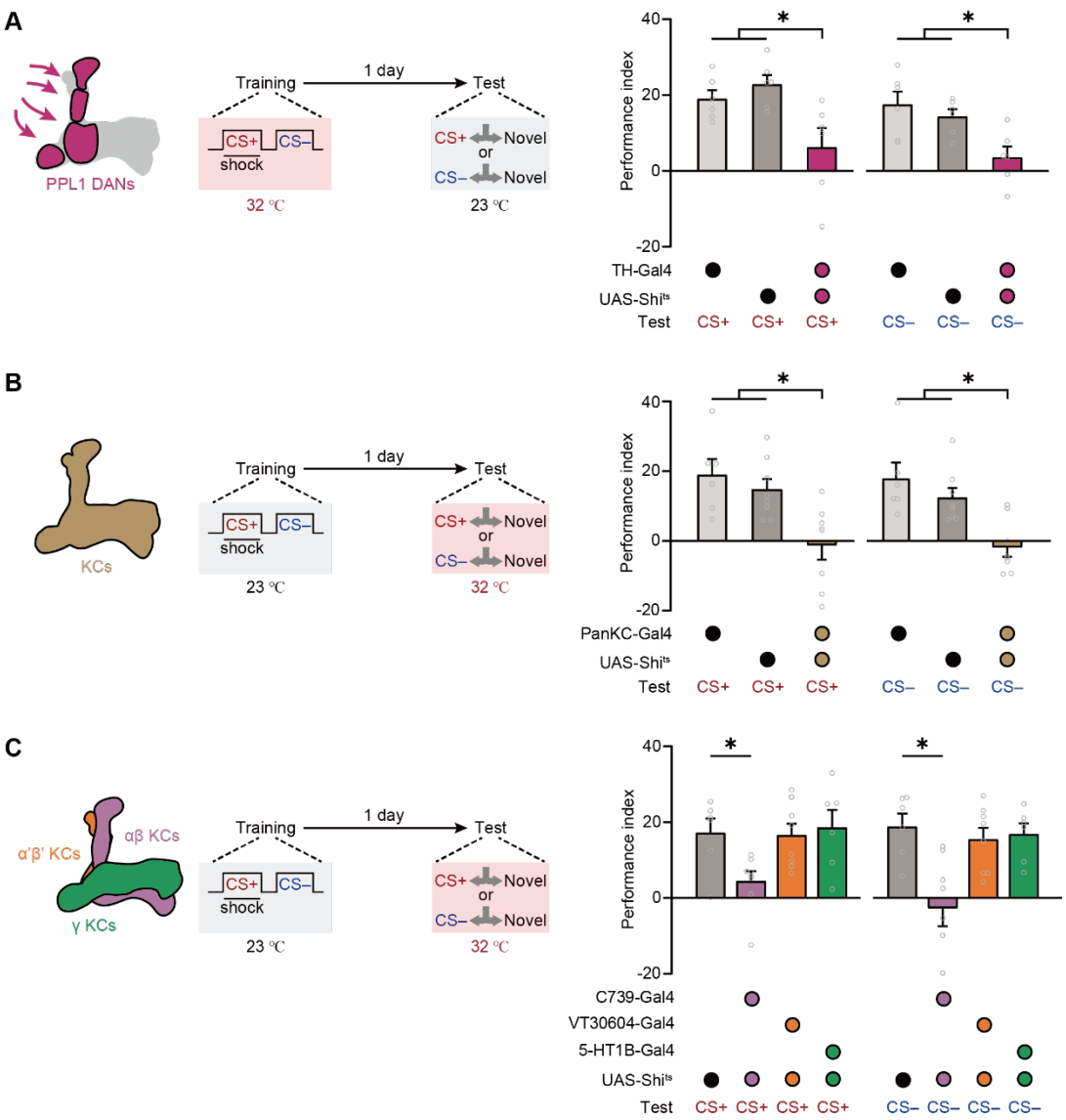

Figure 3. Expression of the mLTM Requires the αβ KCs. (A) Blocking PPL1 DANs during training using *TH-Gal4>UAS-Shi^{ts}* significantly impaired mLTM. **(B)** Blocking MB during testing using *PanKC-Gal4>UAS-Shi^{ts}* impaired the mLTM. **(C)** The mLTM was impaired by blocking αβ KCs (*C739-Gal4*), but not that of α'β' KCs (*VT30604-Gal4*) or γ KCs (*5-HT1B-Gal4*). All data shown are presented as mean ± SEM. *$p < 0.05$.

Retrieval of mLTM Requires Output of αβ KCs

We then examined the role of KCs in mLTM retrieval. We imposed the block by raising temperature to 32°C from 23°C, 15 minutes before the test. Inhibition of the synaptic output of all KCs (*PanKC-Gal4>UAS-Shi^{ts}*) through exposure to a restrictive temperature during the test abolished mLTM (**Figures S1B** and **3B**). We further tested the effects of blocking three distinct subgroups of KCs (**Figure 3C**), namely, those located within the MB αβ lobe (αβ KCs), which were labeled with *C739-Gal4*; those located within the α'β' lobe (α'β' KCs), which were labeled with *VT30604-Gal4*; and those located within the γ lobe (γ KCs), which were labeled with *5-HT1B-Gal4* (**Figures S1C** to **S1E**). The

results showed that mLTM did not express when the synaptic output of αβ KCs was blocked, whereas inhibition of α′β′ KCs and γ KCs had no significant effect (**Figure 3C**).

Long-lasting Depression of Responses in α2sc MBONs Underlies mLTM Expression

Then we further tried to identify which cluster of MBONs from αβ lobe of MB is required for mLTM. In agreement with prior reports that the V2 cluster of MBON (MB-V2, also known as α2sc MBONs) is important to aversive spaced training-induced LTM expression (Dubnau and Chiang, 2013; Modi et al., 2020; Pascual and Preat, 2001; Placais et al., 2012; Tully et al., 1994), we found that mLTM expression was significantly impaired by blocking α2sc MBONs during testing using *R71D08-Gal4* (**Figures 4A** and **S1F**). We then imaged odor-evoked calcium responses in the dendritic field of α2sc MBONs to CS+ and CS– through expressing jGCamP7f, a calcium-sensitive fluorescent protein (Dana et al., 2019), driven by *R71D08-Gal4*. To account for variance between different odors, the responses of trained flies were calibrated to the average responses of naïve flies to corresponding odors. Consistent with results of the behavioral assay, 1 day after training, odor-evoked calcium responses of α2sc MBONs to CS+ and CS– were both significantly reduced when compared to responses to the novel odor (**Figure 4B**). Thus, the aversion to CS+ and CS– are both linked to the neural plasticity conferred by the same MBON.

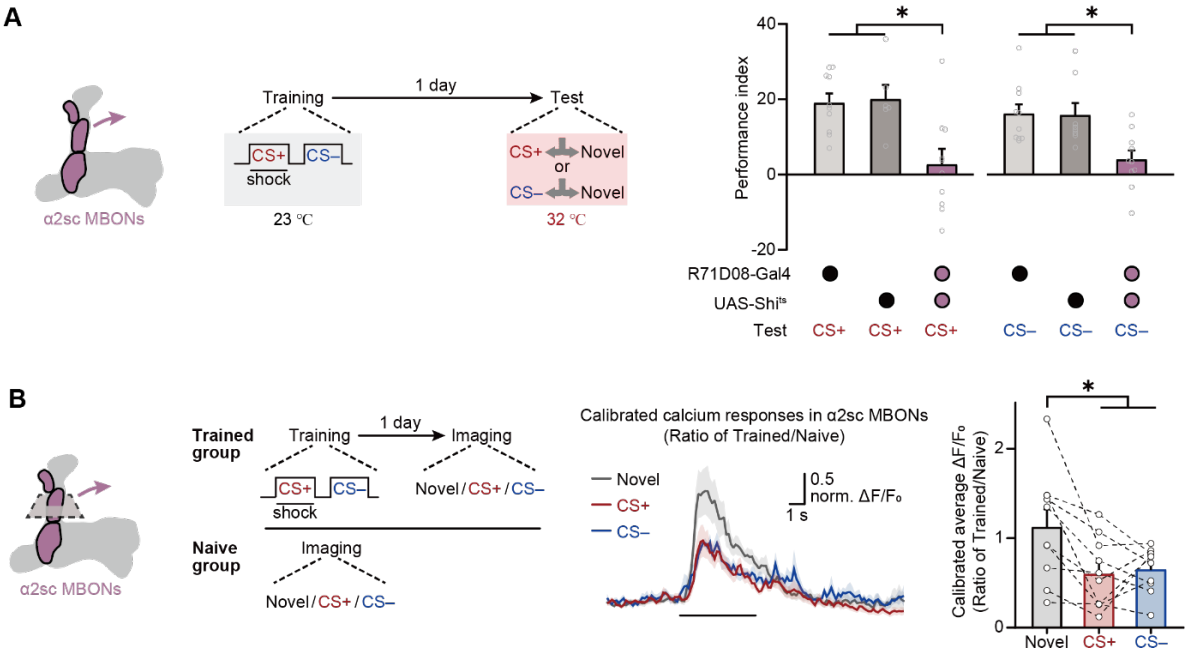

Figure 4. The mLTM Can Be Recorded as Depressed Odor-evoked Responses in α2sc MBONs. (A) Blocking α2sc MBONs *R71D08-Gal4>UAS-Shi^{ts}* impaired mLTM expression. **(B)** Left: training and imaging protocols. The imaging plane for α2sc MBONs is shown. The novel-odor-evoked response, CS+-evoked response, and CS–-evoked response were calibrated to the average responses of naïve flies to these odors. Right: the mLTM can be recorded as depressed odor-specific responses in α2sc MBONs. All data shown are presented as mean ± SEM. *$p < 0.05$.

Discussion

In the current study, the use of third-odor test leads to a conclusion that single-trial training produces an mLTM for guiding flies to avoid both CS+ and CS– for more than seven days. Three categories of evidence in support of this conclusion are outlined below.

First, throughout our study, the amplitudes of long-term avoidances of CS+ and CS– are always at a similar level under various conditions, including pharmacological treatment (**Figure 1C**), cold-shock treatment (**Figure 1D**), odor re-exposure (**Figure 2A**), paradigm alteration (**Figures 2B** and **2C**), and neural circuitry manipulations (**Figures 3** and **4**). Second, re-exposure to either one of CS+ and CS– alone can extinguish both CS+ avoidance and CS– avoidance (**Figure 2A**). Third, the long-term avoidances of CS+ and CS– can be recorded as the depression of odor-evoked responses in the same α2sc MBONs (**Figure 4**), meanwhile, CS+ avoidance and CS– avoidance both involve the same PPL1 DANs, αβ KCs, and α2sc MBONs (**Figures 3** and **4A**). Thus, CS+ avoidance and CS– avoidance derive from the same aversive mLTM, instead of two parallel LTMs of the same valence. The significance of these findings is further discussed below.

Combining with a recent report that uses a similar third-odor test to dissect LTMs induced by multi-trial spaced training (Jacob and Waddell, 2020), we are led to conclude that spaced multi-trial aversive differential conditioning produces two independent LTMs of opposite valence for avoiding CS+ and approaching CS–, whereas single-trial aversive differential conditioning yields one merged LTM that guides avoidances of both CS+ and CS–. Thus, animals can develop distinct escape strategies for different categories of dangers.

Moreover, the differences between single-trial training-induced mLTM and multi-trial training-induced complementary LTMs lead us to ask: how these differences are induced by those trials following the first one of repetitive spaced training? One possibility is that complementary LTMs are gradually transformed from the mLTM. Such transformation may be mediated by the slow oscillation of two pairs of DANs projecting to MB αβ lobe, which has been reported gating aversive LTM encoding (Placais et al., 2012). This oscillation may split the mLTM into two independent LTMs of opposite valence, and thus alter the long-term tendency of these odors, especially the CS–.

Finally, the result that prolonging the interval between CS+ and CS– during training fails to induce 1-day avoidances suggests a temporal proximity dependency of mLTM (**Figure 2B**). Such temporal property has been widely observed in higher cognitive functions, such as the chunking of sequential information processing (Craik and Lockhart, 1972; Dehaene et al., 2015), the contiguity effect of free recall (Folkerts et al., 2018; Kahana, 1996), and the memory allocation phenomenon observed in rodents (Cai et al., 2016; Rashid et al., 2016). This requirement of temporal proximity of mLTM suggests that a similar cognitive mechanism may be utilized in invertebrates as well.

Methods

Fly strains

All flies (*Drosophila melanogaster*) were raised on standard cornmeal medium at 23 °C and 60% relative humidity under a 12-hr light-dark cycle as described. The control strain was w^{1118}*(isoCJ1). UAS-Shibire*^{ts1}, *C739-Gal4*, and *TH-Gal4* were extant stocks in the laboratory. The following *Gal4* lines were obtained from the Bloomington *Drosophila* Stock Center (BDSC): *PanKC-Gal4* (#48571), *5HT1b-Gal4* (#27637), and *R71D08-Gal4* (#61645). *VT30604-Gal4* (#200228) was obtained from the Vienna Drosophila Resource Center (VDRC). *UAS-GCaMP7f* was a gift from Yi Sun, Westlake University.

Behavioral assays

All flies were raised at 23 °C and mixed-sex populations of 2–5-day-old flies were used in all experiments. The Pavlovian olfactory aversive conditioning procedure was performed as described previously in a behavioral room at 23 °C and 60% relative humidity.

During training, roughly 80 flies successfully received the following stimuli in a training tube, which contained a copper grid: air for 90 s, an odor paired with 12 pulses of 60 V electric shock (CS+) for 1 min, air for 45 s, a second odor without pairing the electric shock (CS–) for 60 s, and finally air for 45 s. 3-octanol (OCT, 10-15 μl in 10 ml mineral oil), 4-methylcyclohexanol (MCH, 10 μl in 10 ml mineral oil), and ethyl acetate (EA, 10 μl in 10 ml mineral oil) were used as standard odorants. This process describes a single training trial. Six sequential trials with 15-min intervals constitute spaced training.

For memory retrieval, trained flies were transferred into a T-maze, where they were allowed 2 min to choose between two odors (CS+ and novel odor, or CS– and novel odor). The testing odors were always OCT and MCH. Specifically, for the testing of CS+ performance, either OCT or MCH was alternately used as CS+, and EA was CS–; whereas for the testing of CS– performance, either OCT or MCH was alternately used as CS–, and EA was CS+.

Odor performance was quantified by a PI (performance index) calculated based on the fraction of flies in the two T-maze arms. A PI of 100 indicated that all flies avoid the odor exposed during training (either CS+ or CS–), while a PI of 0 indicated no odor preference, as reflected by 50:50 distributions between the arms. To balance naïve odor bias, two reciprocal groups were trained and tested simultaneously. One group was trained with OCT and EA during training, and the other was trained with MCH and EA during training. The complete PI was defined as the average PI of the two groups. For 3-min memory, flies were tested immediately after training. For longer memory retention, flies were placed in a fresh vial with the same contents as the vial they had been kept in before the training until the test.

For cold-shock anesthesia, flies were transferred into pre-chilled plastic vials and kept on ice for 2 min as described previously.

For cycloheximide (CXM) feeding, flies were provided food with (CXM+) or without (CXM–) 35 mM CXM (Sigma-Aldrich) dissolved in control solution, and 5% (wt/vol) glucose, for 16 hours before and after training until memory retention was tested.

For neural inactivation experiments using *UAS-Shi*^{ts}, crosses were reared at 23°C to avoid unintended inactivation. Flies were shifted to 32°C 15 minutes in advance for neural inhibition during training or test.

For odor re-exposure, flies were exposed to 3-cycles of conditioned odor (either CS+ or CS–) for 1 minute with 1-min interval.

Immunohistochemistry

Flies were quickly anesthetized on ice and whole brains were dissected in ice-cold PBS within 5 min, then stained as described. Brains were fixed in 4% paraformaldehyde in PBS for 30 min on ice. Brains were incubated for at least 72 h with the primary antibodies anti-GFP (chicken, 1:2000; Abcam, Cambridge, UK), anti-nc82 (mouse, 1:10; Developmental Studies Hybridoma Bank, Iowa City, IA, USA) and anti-DsRed (rabbit, 1:500; Takara Bio, Kyoto, JP). Brains were washed three times again in PBS with 0.2% Triton X-100 and transferred into secondary antibody solution (anti-chicken Alexa Fluor 488, 1:200; anti-mouse Alexa Fluor 647, 1:200; anti-rabbit Alexa Fluor 647, 1:200; Molecular Probes, Eugene, OR, USA) and incubated for 48 h at 4°C. Images were obtained using a Zeiss LSM710 confocal microscope (Carl Zeiss AG, Oberkochen, Germany).

In vivo two-photon calcium imaging

Two- to five-day-old female flies were used. After anesthetized in a plastic vial on ice for 15–20 s, flies were then gently inserted into a hole of a thin plastic rectangular plate and stabilized in the hole by glue. In a saline bath, the area surrounding the region of interest was surgically removed to expose the dorsal side of the brain. Fat and air sacs were gently removed to give a clear view of the brain. For calcium- response imaging, the 40× water immersion objective lens (NA=1.0; Zeiss) was lowered near the exposed brain.

Imaging was performed on a Zeiss LSM 7MP two-photon laser scanning microscope with an imaging wavelength at 910 nm (Carl Zeiss AG). The 512 × 512 pixel images were acquired at 2.6 Hz. In each trial, 10 seconds of baseline was recorded, followed by an odor-evoked response recording. Two to three minutes of rest were given in between trials when multiple trials were applied. GCaMP responses and the average value within 10 s before and during stimulus were quantified using custom software written in MatLab (MathWorks, MA, USA). For the brain region of interest during the experimental period, the average fluorescence value, F_{ave}, was then converted to $\Delta F/F_0$ using the formula $\Delta F/F_0 = (F_{ave} - F_0)/ F_0$, where F_0 is the baseline fluorescence value, measured as the average of 10 s before the stimulus. Flies were trained using CS+ (OCT, 10 μl in 10 ml mineral oil) and CS– (MCH10 μl in 10 ml mineral oil), and IA (10 μl in 10 ml mineral oil) was used as novel odor. To gain the calibrated

calcium responses of trained flies, the ratios of $\Delta F/F_0$ to different odors of trained and naïve flies were calculated.

Statistics

Statistics were performed with GraphPad Prism software (version 7; GraphPad Software, San Diego, CA, USA). All data satisfied the assumption of normal distribution (one-sample Kolmogorov–Smirnov test). Comparisons between two groups were performed using two-tailed t-tests. Comparisons of multiple groups were performed using one-way or two-way analysis of variance (ANOVA) tests followed by Bonferroni correction for multiple comparisons. P values less than 0.05 were considered statistically significant and are marked with an asterisk in figures; NS indicates non-significant differences (P > 0.05).

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Author contributions

B.Z. designed the study under the supervision of Y.Z and discussions from J.S. and Q.L.; B.Z., Q.L., and Y.Z. wrote the manuscript; B.Z. performed all behavioral assays; J.S. performed all imaging assays; B.Z. analyzed the data; J.S. and B.Z. prepared fly stock.

Declaration of interests

The authors declare no competing interests.

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