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–) (**Figure 1-figure supplement 1**). 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**). Consistently, testing CS+ or CS– versus Air also revealed training-induced long-lasting avoidances of CS+ and CS– (**Figure 1-figure supplement 2**).

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.

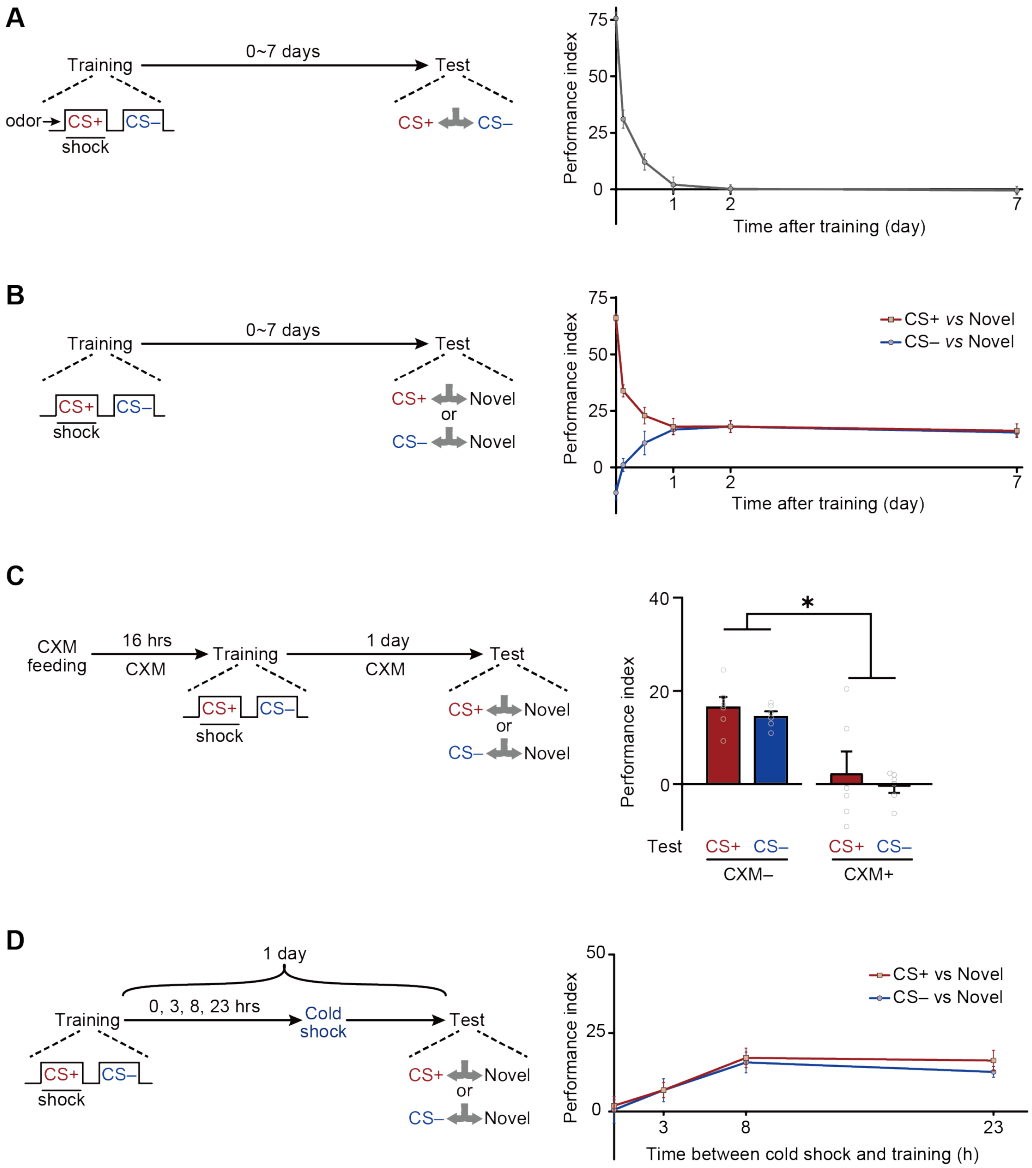


Figure 1. Long-term Avoidances of Both CS+ and CS– Induced by Aversive Differential Conditioning. (A) Single-trial differential conditioning induced discriminative memory that was forgotten within 1 day (n = 6-8). **(B)** Conditioned flies exhibited long-lasting avoidances of both CS+ and CS– (n = 8-10). **(C)** CXM treatment abolished 1-day avoidances of CS+ and CS– (n = 6). **(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 (n = 6-8). All data shown are presented as mean ± SEM. *p < 0.05.

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

97 from the same memory component.

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

104 Second, LTM^{CS+} and LTM^{CS-} were both abolished when the temporal interval
105 between CS+ and CS– during training was prolonged to more than 5 minutes (**Figure**
106 **2B**), suggesting that the formation of LTM^{CS+} and LTM^{CS-} depends on the contiguity of
107 two shock-paired CS+ and CS– exposures, instead of temporally separated exposures.
108 In addition, changing the sequence of exposure to CS+ and CS– did not affect either
109 LTM^{CS+} or LTM^{CS-} (**Figure 2C**), but training with CS+ or CS– alone failed to induce the
110 LTM^{CS+} or LTM^{CS-} (**Figure 2-figure supplement 1**).

111 Together, these results strongly suggest that, instead of two parallel LTMs,
112 observed LTM^{CS+} and LTM^{CS-} are more likely to be derived from the same memory
113 component. Since it was observable after two separated memory curves (CS+ and CS–)
114 merged over time, we termed it as merged LTM (mLTM).

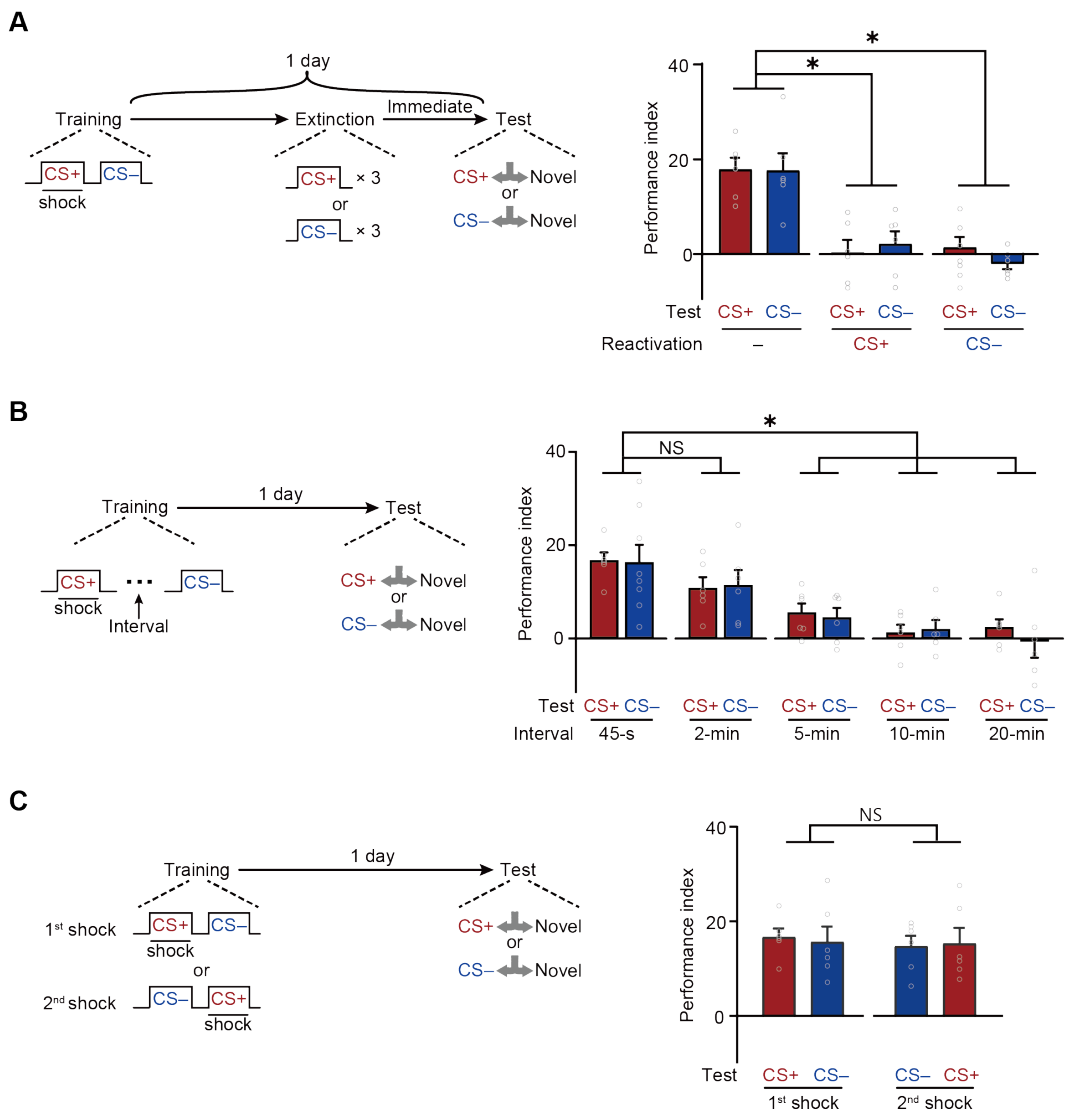


Figure 2. An mLTM Underlies the Long-term Avoidances of CS+ and CS–. (A) Three trials of re-exposure to either CS+ or CS– alone can impair both CS+ avoidance and CS– avoidance 1 day after training (n = 6). **(B)** Prolonging the inter-trial interval (ITI) between CS+ and CS– to more than 5 minutes significantly impaired 1-day avoidances (n = 6-8). **(C)** Changing the sequence of CS+ and CS– during training did not affect 1-day avoidances (n = 6). All data shown are presented as mean ± SEM. *p < 0.05. NS, non-significant.

The Encoding of mLTM Requires Aversive-reinforcing DANs

Then we further investigated the neural network mechanisms underlying such mLTM. In *Drosophila*, associative olfactory memories are mainly encoded by the 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.,

129 2014b; Cognigni et al., 2018; Dubnau and Chiang, 2013; Modi et al., 2020; Pascual and
130 Preat, 2001; Placais et al., 2012). We therefore tested whether DANs involves in mLTM
131 encoding by comparing 24-h memory in control flies with that in flies whose synaptic
132 outputs from different clusters of DANs were blocked during training. For this purpose,
133 we expressed the dominant-negative temperature-sensitive *UAS-Shibire*^{ts1} (*Shi*^{ts})-
134 encoded dynamin (Kitamoto, 2001) driven by *R58E02-Gal4*, which labels the
135 protocerebral anterior medial (PAM) cluster of DANs, and *R52H03-Gal4* and *TH-Gal4,*
136 which both labels PPL1 DANs (**Figure 3-figure supplement 1A, B, and C**). We specifically
137 blocked output from DANs during training by raising the temperature of flies from 23°C
138 to 32°C. Flies were then returned to 23°C and later tested for 1-day memory. The results
139 showed that blocking the release of neurotransmitter from PPL1 DANs, but not PAM
140 DANs, during training impaired mLTM (**Figure 3A**), suggesting that the
141 neuromodulation of PPL1 DANs is necessary for mLTM formation.

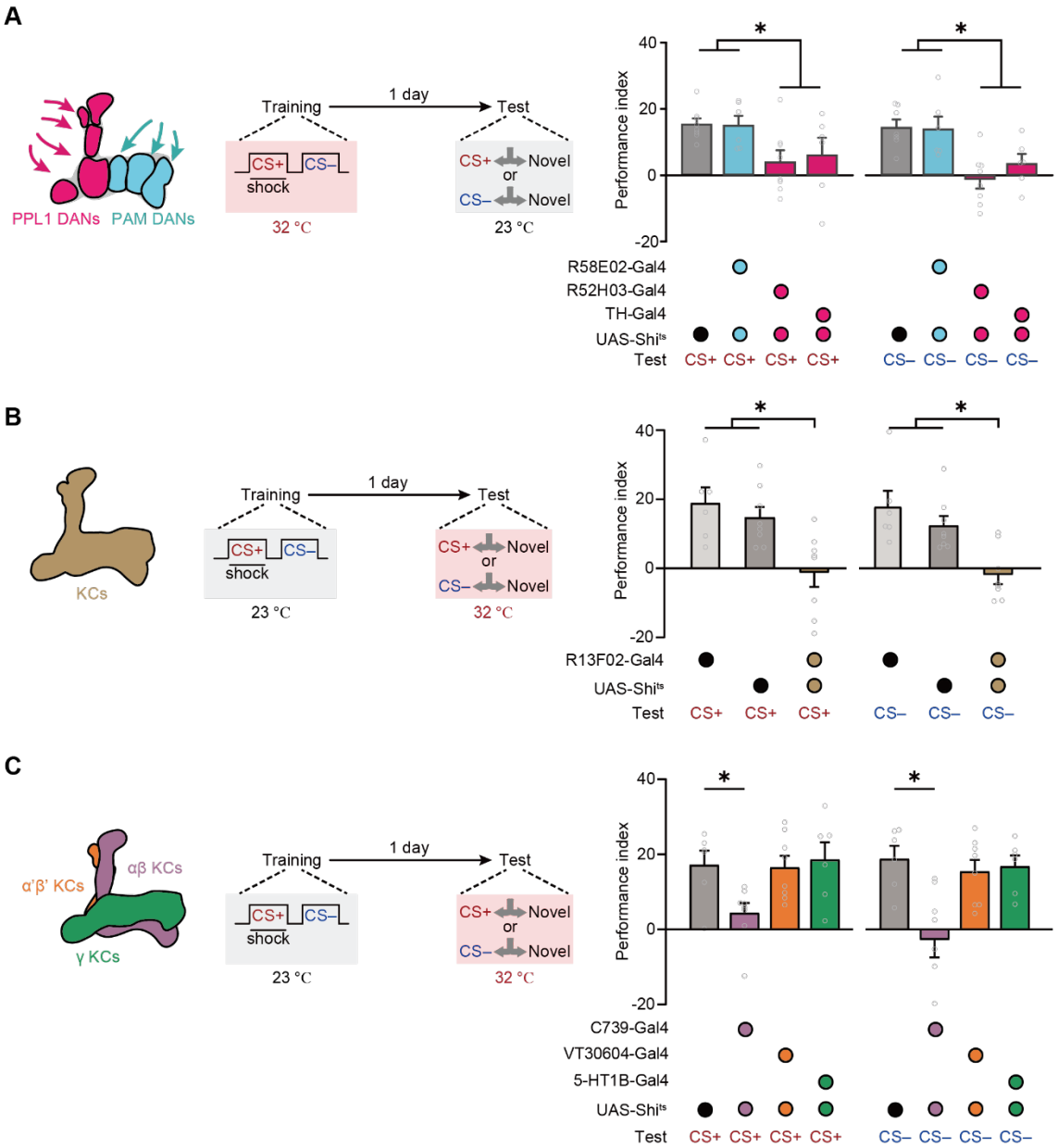


Figure 3. Expression of the mLTM Requires the αβ KCs. (A) Blocking PPL1 DANs (*R52H03-Gal4* or *TH-Gal4*) but not PAM DANs (*R58E02-Gal4*) during training significantly impaired mLTM (n = 6-8). **(B)** Blocking MB during testing using *R13F02-Gal4>UAS-Shi^{ts}* impaired the mLTM (n = 6-8). **(C)** The mLTM was impaired by blocking αβ KCs (*C739-Gal4*), but not that of α'β' KCs (*VT30604-Gal4*) or γ KCs (*5-HT1B-Gal4*) (n = 6-8). 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 (*R13F02-Gal4>UAS-Shi^{ts}*) through exposure to a restrictive

154 temperature during the test abolished mLTM (**Figure 3B and Figure 3-figure**
155 **supplement 1D**). We further tested the effects of blocking three distinct subgroups of
156 KCs (**Figure 3C**), namely, those located within the MB αβ lobe (αβ KCs), which were
157 labeled with *C739-Gal4*; those located within the α′β′ lobe (α′β′ KCs), which were
158 labeled with *VT30604-Gal4*; and those located within the γ lobe (γ KCs), which were
159 labeled with *5-HT1B-Gal4* (**Figure 3-figure supplement 1E to G**). The results showed
160 that mLTM did not express when the synaptic output of αβ KCs was blocked, whereas
161 inhibition of α′β′ KCs and γ KCs had no significant effect (**Figure 3C**).

162

163 ## The α2sc Cluster of MBONs Are Required for mLTM Expression

164 Then we further tried to identify which cluster of MBONs from αβ lobe of MB is
165 required for mLTM. Prior studies on the structure of MB network have uncovered that
166 the PPL1 DANs mainly integrate to the α2 and α3 compartments of the vertical α lobe
167 of MB, and corresponding MBONs (α2sc and α3 clusters) have been reported to be
168 important to aversive spaced training-induced LTM expression (Aso et al., 2014a; Aso
169 et al., 2014b; Bouzaiane et al., 2015; Dubnau and Chiang, 2013; Jacob and Waddell,
170 2020; Modi et al., 2020; Pascual and Preat, 2001; Placais et al., 2012; Schwaerzel et al.,
171 2003). We therefore attempted to block α2sc MBONs (*R71D08-Gal4*) and α3 MBONs
172 (*G0239-Gal4*) during testing, respectively (**Figures 4-figure supplement 1A, B**). Results
173 showed that mLTM expression was significantly impaired by blocking α2sc MBONs, but
174 not α3 MBONs (**Figure 4A, B**). This conclusion was further strengthened by the result
175 that inhibiting the neural activity of α2sc MBONs using heat-inducible expression of
176 the potassium channel Kir2.1 decreased the mLTM retrieval (**Figures 4-figure**
177 **supplement 2**). Thus, these results suggest that retrieving mLTM specifically relies on
178 the output of α2sc MBONs.

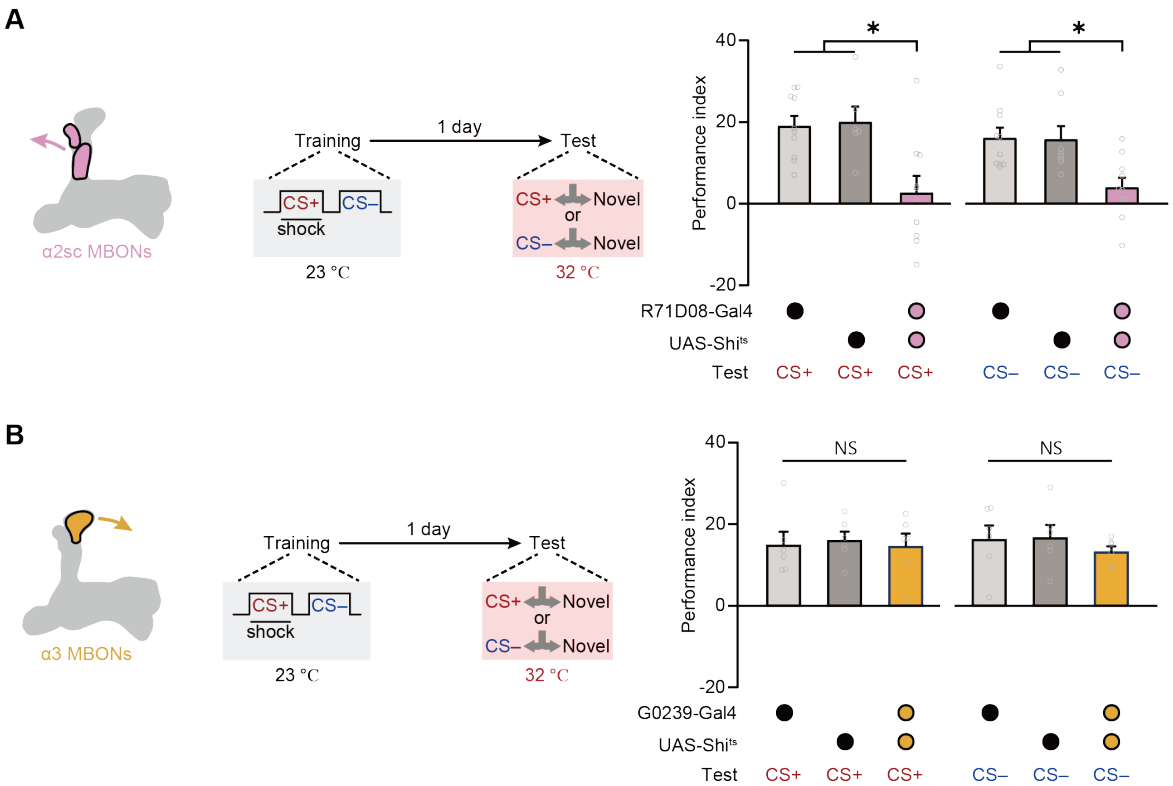
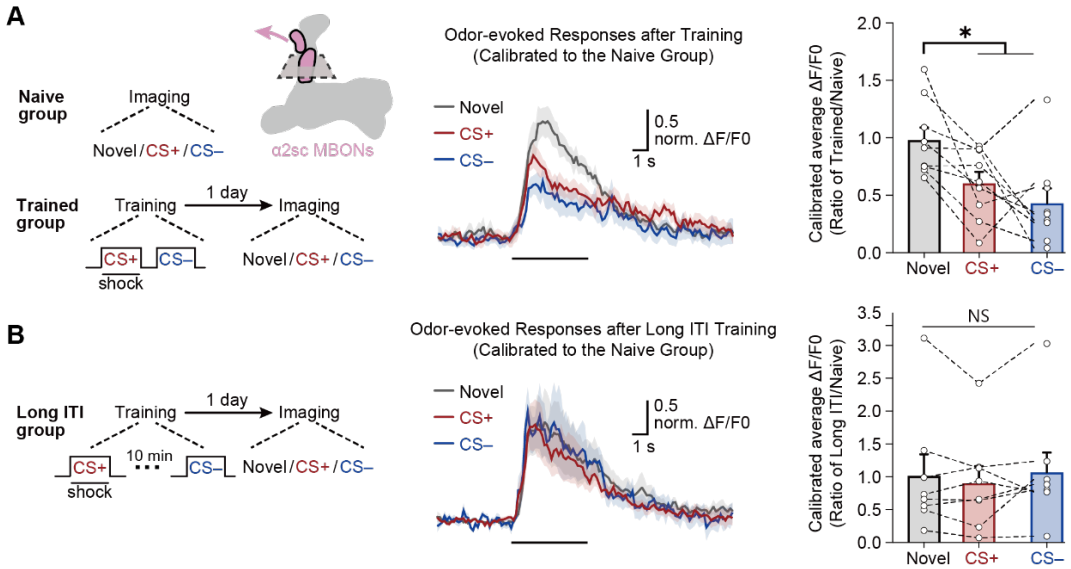


Figure 4. Expression of the mLTM Requires α2sc MBONs. (A) Blocking α2sc MBONs using *R71D08-Gal4>UAS-Shi^{ts}* impaired mLTM expression (n = 6-10). **(B)** Blocking α3 MBONs using *G0239-Gal4>UAS-Shi^{ts}* did not affect mLTM expression (n = 6). All data shown are presented as mean ± SEM. *p < 0.05. NS, non-significant.

Differential Conditioning Induces Long-lasting Depression of CS+ and CS– Odor-evoked Responses in α2sc

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 5A, B**). Moreover, such depression of CS+ and CS– odor-evoked responses can be abolished by prolonging the temporal interval between CS+ and CS– to 10 minutes during training (**Figure 5C**), which is consistent with the result of behavioral assay (**Figure 2B**). Interestingly, when we recorded the calcium responses immediately after training, the depressed responses to CS+ and CS– were observed, suggesting the

199 mLTM should be encoded during training (**Figure 5-figure supplement 1**). Together, our
200 data showed that the aversion to CS+ and CS– are both linked to the neural plasticity
201 conferred by the same MBON.



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203 **Figure 5. The mLTM Can Be Recorded as Depressed Odor-evoked Responses in α2sc**
204 **MBONs. (A)** Left: training and imaging protocols. The imaging plane for α2sc MBONs
205 is shown. The novel odor-evoked response, CS+ odor-evoked response, and CS– odor-
206 evoked response were calibrated to the average responses of corresponding odors in
207 naïve flies. Right: the mLTM can be recorded as depressed odor-specific responses in
208 α2sc MBONs (n = 9). **(B)** Prolonging the ITI between CS+ and CS– during training
209 abolished the depressed odor-specific responses (n = 8). All data shown are presented
210 as mean ± SEM. *p < 0.05. NS, non-significant.

211

212 <u>Discussion</u>

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

217 First, throughout our study, the amplitudes of long-term avoidances of CS+ and
218 CS– are always at a similar level under various conditions, including pharmacological
219 treatment (**Figure 1C**), cold-shock treatment (**Figure 1D**), odor re-exposure (**Figure 2A**),
220 paradigm alteration (**Figures 2B** and **2C**), and neural circuitry manipulations (**Figures 3**
221 and **4**). Second, re-exposure to either one of CS+ and CS– alone can extinguish both
222 CS+ avoidance and CS– avoidance (**Figure 2A**). Third, the long-term avoidances of CS+

223 and CS– can be recorded as the depression of odor-evoked responses in the same α2sc
224 MBONs (**Figure 4**), meanwhile, CS+ avoidance and CS– avoidance both involve the
225 same PPL1 DANs, αβ KCs, and α2sc MBONs (**Figures 3** and **4A**). Thus, CS+ avoidance
226 and CS– avoidance derive from the same aversive mLTM, instead of two parallel LTMs
227 of the same valence. The significance of these findings is further discussed below.

228 Combining with a recent report that uses a similar third-odor test to dissect LTMs
229 induced by multi-trial spaced training (Jacob and Waddell, 2020), we are led to
230 conclude that spaced multi-trial aversive differential conditioning produces two
231 independent LTMs of opposite valence for avoiding CS+ and approaching CS–, whereas
232 single-trial aversive differential conditioning yields one merged LTM that guides
233 avoidances of both CS+ and CS–. Thus, animals can develop distinct escape strategies
234 for different categories of dangers. When the same dangerous situation has been
235 experienced repeatedly, animals would remember the detailed information to guide
236 behavior in the next similar situation. However, when the dangerous event has only
237 been experienced occasionally, animals would choose to avoid all potentially
238 dangerous cues as a more reserved survival strategy.

239 Moreover, the differences between single-trial training-induced mLTM and multi-
240 trial training-induced complementary LTMs lead us to ask: how these differences are
241 induced by different training sessions? Jacob and Waddell (2020) reported that multi-
242 trial spaced training induces depressed responses to CS+ in α2sc MBONs and α3
243 MBONs are required for aversive LTM to CS+, whereas the modulated responses to CS–
244 in β′2mp MBONs and γ3, γ3β′1 MBONs appears to be responsible for the safety-
245 memory to CS–. In contrast, here we found that single-trial training is sufficient to
246 induce the depressed responses to both CS+ and CS– in α2sc MBONs. Therefore, our
247 results suggest a lower threshold and specificity of the plasticity between KCs-α2sc
248 MBONs, compared to KCs-α3 MBONs, KCs-β′2mp MBONs, and KCs-γ3, γ3β′1 MBONs
249 connections. Consequently, changing these synaptic connections requires involving
250 more training sessions.

251

252 ## Methods

253 ### Fly strains

254 All flies (*Drosophila melanogaster*) were raised on standard cornmeal medium at
255 23 °C and 60% relative humidity under a 12-hr light-dark cycle as described (Shuai et
256 al., 2010). The control strain was *w*^{1118}*(isoCJ1). UAS-Shibire*^{ts1}, and *UAS-Kir2.1;tub-*

257 *gal80^{ts}* were extant stocks in the laboratory. Gal4 lines used have been described
258 previously: *TH-Gal4* (Friggi-Grelin et al., 2003), *C739-Gal4* (McGuire et al., 2001),
259 *R58E02-Gal4*, *R52H03-Gal4*, *R13F02-Gal4*, and *R71D08-Gal4* (Jenett et al., 2012),
260 *5HT1b-Gal4* (Yuan et al., 2005), *G0239-Gal4* (Pai et al., 2013), and *VT30604-Gal4*
261 (#200228) (Wu et al., 2013). *UAS-GCaMP7f* was a gift from Yi Sun, Westlake University.

262 ## Behavioral assays

263 All flies were raised at 23 °C and mixed-sex populations of 2–5-day-old flies were
264 used in all experiments. The Pavlovian olfactory aversive conditioning procedure was
265 performed as described previously in a behavioral room at 23 °C and 60% relative
266 humidity described (Shuai et al., 2010).

267 During training, roughly 80 flies successfully received the following stimuli in a
268 training tube, which contained a copper grid: air for 90 s, an odor paired with 12 pulses
269 of 60 V electric shock (CS+) for 1 min, air for 45 s, a second odor without pairing the
270 electric shock (CS–) for 60 s, and finally air for 45 s. 3-octanol (OCT, 10-15 μl in 10 ml
271 mineral oil), 4-methylcyclohexanol (MCH, 10 μl in 10 ml mineral oil), and ethyl acetate
272 (EA, 10 μl in 10 ml mineral oil) were used as standard odorants. This process describes
273 a single training trial. Six sequential trials with 15-min intervals constitute spaced
274 training. For memory retrieval, trained flies were transferred into a T-maze, where they
275 were allowed 2 min to choose between two odors (CS+ and novel odor, or CS– and
276 novel odor).

277 Odor performance was quantified by a PI (performance index) calculated based
278 on the fraction of flies in the two T-maze arms. A PI of 100 indicated that all flies avoid
279 the odor exposed during training (either CS+ or CS–), while a PI of 0 indicated no odor
280 preference, as reflected by 50:50 distributions between the arms. To balance naïve odor
281 bias, two reciprocal groups were trained and tested simultaneously and the testing
282 odors were always OCT and MCH, one serves as the CS+ or CS– and the other serves
283 as the novel odor alternatively. One complete PI was generated from the average PI of
284 a couple of rotate training and testing. Specifically, for the testing of CS+ performance,
285 either OCT or MCH was alternately used as CS+, and EA was CS–; whereas for the
286 testing of CS– performance, either OCT or MCH was alternately used as CS–, and EA
287 was CS+. For 3-min memory, flies were tested immediately after training. For longer
288 memory retention, flies were placed in a fresh vial with the same contents as the vial
289 they had been kept in before the training until the test.

290 For odor avoidance testing, a similar PI was calculate based on the fraction of flies

291 in the two T-maze arms. A PI of 100 indicated that all flies choose the Air arm, while a
292 PI of 0 indicated no preference.

293 For cold-shock anesthesia, flies were transferred into pre-chilled plastic vials and
294 kept on ice for 2 min as described previously described (Shuai et al., 2010).

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

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

301 For odor re-exposure, flies were exposed to 3-cycles of conditioned odor (either
302 CS+ or CS–) for 1 minute with 1-min interval as described previously (Wang et al., 2019).
303 All mentioned re-exposure treatment were performed immediately before testing.

304 ## Immunohistochemistry

305 Flies were quickly anesthetized on ice and whole brains were dissected in ice-cold
306 PBS within 5 min, then stained as described (Shuai et al., 2010). Brains were fixed in 4%
307 paraformaldehyde in PBS for 30 min on ice. Brains were incubated for at least 72 h with
308 the primary antibodies anti-GFP (chicken, 1:2000; Abcam, Cambridge, UK), anti-nc82
309 (mouse, 1:10; Developmental Studies Hybridoma Bank, Iowa City, IA, USA) and anti-
310 DsRed (rabbit, 1:500; Takara Bio, Kyoto, JP). Brains were washed three times again in
311 PBS with 0.2% Triton X-100 and transferred into secondary antibody solution (anti-
312 chicken Alexa Fluor 488, 1:200; anti-mouse Alexa Fluor 647, 1:200; anti-rabbit Alexa
313 Fluor 647, 1:200; Molecular Probes, Eugene, OR, USA) and incubated for 48 h at 4°C.
314 Images were obtained using a Zeiss LSM710 confocal microscope (Carl Zeiss AG,
315 Oberkochen, Germany).

316 ## In vivo two-photon calcium imaging

317 Two- to five-day-old female flies were used. After anesthetized in a plastic vial on
318 ice for 15–20 s, flies were then gently inserted into a hole of a thin plastic rectangular
319 plate and stabilized in the hole by glue. In a saline bath, the area surrounding the region
320 of interest was surgically removed to expose the dorsal side of the brain. Fat and air
321 sacs were gently removed to give a clear view of the brain. For calcium- response
322 imaging, the 40× water immersion objective lens (NA=1.0; Zeiss) was lowered near the
323 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. Specifically, all odor-evoked responses after training were divided to the mean responses to the same odors of same number naïve flies. Thus, a calibrated $\Delta F/F_0$ of 1.0 indicated no difference in the odor-evoked responses to the correspondence odor between trained and naïve group.

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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357 Declaration of interests

358 The authors declare no competing interests.

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