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Research

The GABAergic anterior paired lateral neurons facilitate olfactory reversal learning in *Drosophila*

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Reversal learning has been widely used to probe the implementation of cognitive flexibility in the brain. Previous studies in monkeys identified an essential role of the orbitofrontal cortex (OFC) in reversal learning. However, the underlying circuits and molecular mechanisms are poorly understood. Here, we use the T-maze to investigate the neural mechanism of olfactory reversal learning in *Drosophila*. By adding a reversal training cycle to the classical learning protocol, we show that wild-type flies are able to reverse their choice according to the alteration of conditioned stimulus (CS)-unconditioned stimulus (US) contingency. The reversal protocol induced a specific suppression of the initial memory, an effect distinct from memory decay or extinction. GABA down-regulation in the anterior paired lateral (APL) neurons, which innervate the mushroom bodies (MBs), eliminates this suppression effect and impairs normal reversal. These findings reveal that inhibitory regulation from the GABAergic APL neurons facilitates olfactory reversal learning by suppressing initial memory in *Drosophila*.

[Supplemental material is available for this article.]

Cognitive flexibility is one of the most salient characteristics of biological intelligence. Even for insects, the ability to respond flexibly to an ever-changing environment is vital for survival. The reversal learning paradigm is often used to study the neural basis of cognitive flexibility. A typical reversal learning process involves establishing and then altering an association between a conditioned stimulus (CS) and an unconditioned stimulus (US, either reward or punishment) (Kringelbach 2005; Murray et al. 2007). One of the most significant findings regarding reversal learning was the identification of the importance of the orbitofrontal cortex (OFC) for reversal acquisition in monkeys (Iversen and Mishkin 1970). Furthermore, the role of OFC in reversal learning seems to be conserved across species: rats, cats, mice, monkeys, and humans with OFC damage acquire reversals more slowly (Murray et al. 2007), which suggests the existence of a general underlying mechanism. In addition, work in honeybees has shown that the mushroom bodies (MBs), the brain structures strongly associated with olfactory learning and memory, are required for the normal acquisition of reversal learning (Devaud et al. 2007).

In *Drosophila*, olfactory reversal learning is usually assayed using the T-maze platform initially designed to study olfactory classical learning (Quinn et al. 1974; Tully and Quinn 1985; Tully et al. 1990; Dubnau et al. 2001; Wu et al. 2007; Shuai et al. 2010). Basically, a reversal training cycle (cycle 2) with reversed CS-US contingency is added after the normal classical training cycle (cycle 1) and before the testing period. The flies selectively avoid the odor more recently paired with shock, regardless of what they learned during cycle 1 (Tully and Quinn 1985). Furthermore, the cycle 2 training interacts nonadditively with the cycle 1 memory (Tully et al. 1990), which indicates an amplification effect of the cycle 2 training. Another important finding is that down-regulation of the small G protein Rac in MBs impairs the reversal learning capacity but leaves the classical learning ability intact (Shuai et al. 2010). This result strongly implies that different mechanisms underlie classical and reversal learning in *Drosophila* and that the reversal protocol could be used to examine the neural mechanism specifically underlying reversal learning.

In this report, we attempt to determine in which part of the brain reversal learning occurs and how it is modulated. We first investigated the effectiveness of the one-trial instant reversal learning protocol, which was used as a primary paradigm here. Combining this paradigm with genetic tools for reversible blockade of different brain areas, we found that the MBs are the brain structures responsible for reversal acquisition. In addition, we found that a reduction of GABA synthesis in the anterior paired lateral (APL) neurons, a pair of inhibitory neurons innervating the MBs, severely impairs reversal learning. With a set of novel protocols that enables us to separately evaluate the memory strengths of the two training cycles in reversal learning, we found that the initial memory from the cycle 1 training is suppressed by cycle 2 training in wild-type flies. However, for those flies with reduced GABA synthesis in the APL neurons, the initial learning could not be properly inhibited. Therefore, we conclude that the APL neurons, through appropriate GABA release, inhibit the initial learning and promote successful reversal.

Results

Wild-type flies are able to reverse their established CS-US associations

We began with the basic protocols of classical learning and reversal learning in two commonly used wild-type fly lines: Wild-type Berlin (WTB) and Canton-S. We assayed classical learning according to a standard protocol (Tully and Quinn 1985). Canton-S flies learned slightly better in the classical learning protocol (PI [performance index] = 0.77 ± 0.02) than WTB flies (PI = 0.61 ± 0.03) (Fig. 1B, gray bar). For reversal learning, flies were retrained

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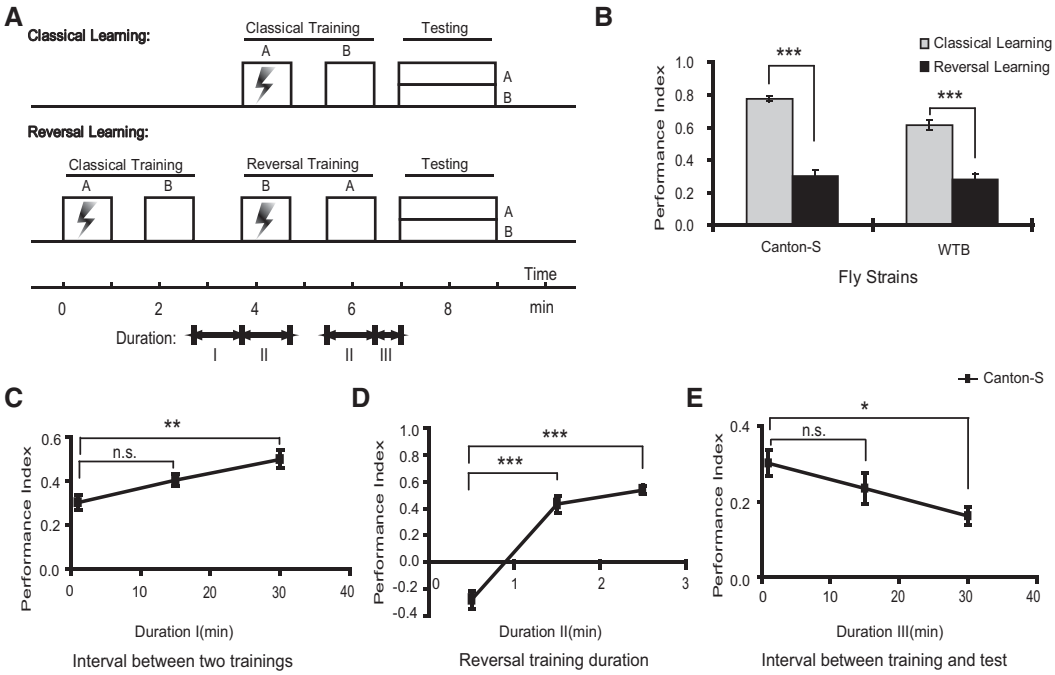

Figure 1. Wild-type flies are able to reverse their established CS-US association, and the reversal learning PI is affected by varying durations of training and interval. (*A*) A standard classical learning protocol was used (Tully and Quinn 1985). During the classical training period, the flies were presented with odor A associated with electric shock and then with odor B without shock; afterward, they would make a choice when the two odors were presented simultaneously in the testing period. The reversal learning protocol consisted of two consecutive training cycles, a classical training cycle (cycle 1) and a reversal training cycle (cycle 2), with the odor-shock pairing reversed. The interval between the two training cycles is labeled Duration I, the length of cycle 2 is Duration II, and the interval between cycle 2 and testing (delay time) is Duration III. (*B*) The performance of Canton-S and WTB flies in classical learning (gray bar) and reversal learning (black bar). For the basic reversal learning protocol used here, Durations I and II were 1 min, and Duration III was 30 sec. The calculation of PIs was in accord with the second cycle. Wild-type flies generated a stable reversal learning PI at around 0.30 (0.30 ± 0.04 for Canton-S and 0.28 ± 0.03 for WTB flies), which suggests that they avoided the odor paired with shock in cycle 2 more than in cycle 1. (*C–E*) Variations of the basic reversal learning protocol. (*C*) The reversal learning PI improved if Duration I was prolonged. (*D*) The reversal learning PI also increased with the lengthening of Duration II. (*E*) The learning PI decreased along with Duration III. $n = 11$ for classical learning in Canton-S, $n = 8$ for the other groups in *B*. $n = 10$ for the group with 30 min in Duration I, 1 min in Duration II, and 30 sec in Duration III; $n = 6$ for the other groups in *C–E*. Data are means ± SEM.

by a second cycle (cycle 2) with the odor-shock pairing (CS-US contingency) reversed (Fig. 1A). In other words, the flies were first presented with odor A (MCH) paired with shock and odor B (OCT) without shock in cycle 1 (A+B–), and then they were presented with odor B paired with shock and odor A without shock in cycle 2 (B+A–). To eliminate odor bias, each experimental trial (in this protocol as well as in all other protocols mentioned in this report) contains two subtrials with odor A and odor B switched and their PIs averaged, similar to that in the classical learning protocol (Tully and Quinn 1985). The reversal learning PIs were 0.28 ± 0.03 for WTB flies and 0.30 ± 0.04 for Canton-S flies (Fig. 1B, black bar). These results showed that after a single reversal training cycle, wild-type flies will reverse their choice between the two odors relative to the first training cycle (cycle 1); i.e., flies selectively avoid the odor most recently paired with an aversive stimulus. Therefore, flies are capable of performing olfactory reversal learning tasks, as previously documented (Tully and Quinn 1985; Tully et al. 1990).

We then examined how training protocol manipulation affects the final learning PIs. First, we changed the length of the interval between cycle 1 and cycle 2, depicted as Duration I in Fig. 1A. When we changed the duration from 1 min to 15 min and 30 min, the learning PIs increased from 0.30 ± 0.04 to 0.41 ± 0.03 and 0.50 ± 0.04, respectively (Fig. 1C). Therefore, the reversal learning score improves when the interval between the first and the second training is lengthened, as reported previously (Tully et al. 1990; Reaume et al. 2010). This improvement is presumably because of the decay of memory from the initial learning in cycle 1. We next tested how reversal PI depends on the duration of stimulus delivery in cycle 2 (Duration II in Fig. 1A). When Duration II was shortened to 30 sec, the flies were unable to reverse their choice, as reflected by the negative reversal learning PI (−0.28 ± 0.07) (Fig. 1D). When Duration II was lengthened to 1.5 min, the learning PI increased to 0.43 ± 0.06, which was significantly higher than the PI (0.30 ± 0.04) in the basic protocol where Duration II was 1 min. Due to the increased exposure of the reversal training, the learning PI could be further increased to 0.54 ± 0.03 if Duration II was prolonged to 2.5 min (Fig. 1D). These results demonstrate how the reversal learning PI is directly influenced by reversal training strength, which is represented by Duration II. Finally, we varied the delay period between cycle 2 and testing (Duration III in Fig. 1A). The PI decreased slightly to 0.23 ± 0.04 when Duration III was extended from 30 sec to 15 min. If Duration III was further increased to 30 min, the reversal learning PI was decreased to 0.16 ± 0.02 (Fig. 1E), showing a decay of the memory from the reversal learning. These results show that the reversal learning PI increases with increased training length or increased duration between cycle 1 and cycle 2, but it decreases with increased duration between cycle 2 and the testing period.

Reversal learning is different from memory decay, memory extinction, or two-odor learning

If the memory strength of cycle 1 is equal to that of cycle 2 during testing, they should cancel each other out and yield a final PI near zero. However, under the reversal learning protocol, flies always

avoided the odor most recently paired with shocks, which generated a positive reversal PI that was much higher than zero (Fig. 1B) and clearly reflects the "reversal" effect. Nevertheless, there remains the possibility that this effect could be accounted for by the process of memory decay or extinction that might have a larger impact on the more distant memory of cycle 1 relative to cycle 2. We, therefore, examined whether the difference could be attributed to the effects of memory decay or extinction. To measure the amount of memory decay between cycle 1 training and testing, we used a classical decay protocol (Fig. 2A) in which cycle 2 was replaced with a delay period. The PI of classical decay (Fig. 2B, gray bar) was similar to that of classical learning (Fig. 2B, white bar), which indicates that no measurable memory decay of cycle 1 training occurs during the reversal process. We also considered the memory extinction component. As depicted in Figure 2A, we performed the classical extinction protocol, in which only odor A was presented in cycle 2 (A+B– in cycle 1 and A– only in cycle 2). The results of this experiment are shown in Figure 2B, and the classical extinction PI was not significantly different from the classical learning PI, which demonstrates that the memory extinction of the cycle 1 training does not have any measurable impact. We, therefore, conclude that the effect of memory decay or extinction from the cycle 1 training does not account for the final positive reversal learning PI. The stable reversal PI can be attributed to reversal training overpowering the initial training episode. Thus, our protocol reveals that the process of reversal learning is distinct from memory decay or extinction.

A previous study reported that, in the course of classical learning, CS+ odor presentation alone is sufficient to produce an optimal learning result (Yu et al. 2006). We designed a different protocol set and confirmed that classical learning is actually a "one-odor learning" paradigm in which the CS– odor is dispensable (Supplemental Fig. S1). To investigate whether the CS– odor is also dispensable in the reversal learning paradigm, we performed the experiments depicted in Figure 2C. A reversal learning protocol was performed together with a timeline-aligned CS– odor absent protocol (A+B+) to obtain a comparable result. The CS– odor absent protocol yielded a PI of 0.08 ± 0.08 (Fig. 2D, black bar), which indicates that the flies could not consistently choose between the two equally punished odors. Furthermore, our tests showed that the reversal learning PI was significantly higher than the CS– odor absent PI (Fig. 2D). Therefore, unlike in classical learning, the presence of CS– odor is effective and indispensable in reversal learning. This result further underlines the distinction between reversal learning and classical associative learning, and it excludes the possibility that reversal learning is merely an additive "two-odor learning" (Yin et al. 2009). Collectively, these results demonstrate that our protocol revealed a unique process in reversal learning that is distinct from memory decay, memory extinction, or two-odor learning.

The MBs are required for olfactory reversal learning

There is a great deal of evidence supporting a central role for MBs in olfactory associative learning and memory in *Drosophila* (Heisenberg 2003; Davis 2005; McGuire et al. 2005; Akalal et al. 2006; Berry et al. 2008; van Swinderen 2009). After a normal classical training cycle, the olfactory memory forms in multiple nodes of the olfactory nervous system, including the MBs (Davis 2011). As reversal learning is composed of two consecutive, albeit contradictory, associative training cycles, it is partially dependent on

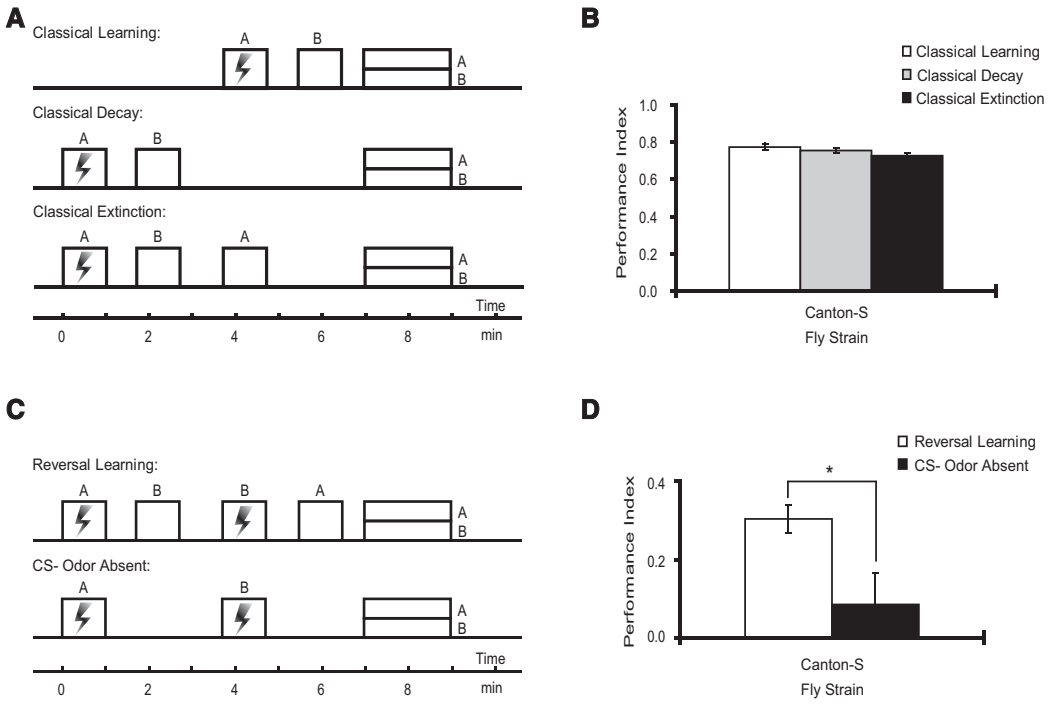

Figure 2. Reversal learning is different from memory decay, memory extinction, and two-odor learning. (*A*) Two new protocols were used to investigate the effects of memory decay and extinction from cycle 1 training in reversal learning. The "Classical Decay" protocol replaced cycle 2 with a delay period. The "Classical Extinction" protocol contains only odor A delivery in cycle 2. (*B*) There were no significant differences between the PIs of the three protocols. (*C*) The reversal learning protocol was compared with the "CS-Odor Absent" protocol, in which the odors not associated with shock were removed from the basic protocol. (*D*) The PIs of the corresponding protocols depicted in panel *C* are shown. The "CS-Odor Absent" PI was significantly different from the basic reversal learning PI. $n = 6–8$ for each group. Data are means ± SEM. (*) $P < 0.05$ (Student's *t*-tests).

associative learning. In addition, the MBs are important for many other cognitive functions that are probably related to behavioral flexibility, such as the regulation of habit formation (Brembs 2009) and decision-making (Zhang et al. 2007). A study in honeybees supports the notion that MBs are required for reversal learning (Devaud et al. 2007). Hence, we hypothesized that the MBs are important for olfactory reversal learning in *Drosophila*. To investigate the relationship between MBs and reversal learning, we combined genetic tools available in *Drosophila* for reversible blockade of neural transmission (UAS-*shibire*^{ts1}) with MB-specific *Gal4* lines (MB247-*Gal4*, c739-*Gal4*, and c305a-*Gal4*) to block MB neural output during reversal learning. At the restrictive temperature (>29°C), *shibire*^{ts1} blocks synaptic vesicle release by inhibiting vesicle recycling, thereby transiently inactivating neurotransmission (Kitamoto 2001). We first assayed classical learning in every fly line to confirm that the temperature switching system worked properly (Supplemental Fig. S2A,B). We then performed reversal learning protocols. As shown in Fig. 3A, when we performed the entire reversal learning protocol at restrictive temperature, the PIs of MB247-*Gal4*/UAS-*shibire*^{ts1}, c739-*Gal4*/UAS-*shibire*^{ts1}, and c305a-*Gal4*/UAS-*shibire*^{ts1} flies were severely impacted. These results clearly demonstrate the importance of synaptic transmission from the MBs for reversal learning. In control experiments, we tested the reversal learning of flies carrying a UAS-*shibire*^{ts1} transgene driven by the dorsal paired medial (DPM)-specific *Gal4* line c316-*Gal4* (Waddell et al. 2000). The c316-*Gal4*/UAS-*shibire*^{ts1} flies exhibited similar reversal learning PIs at both restrictive and permissive temperatures (Fig. 3A), which suggests that the DPM neurons may not be involved in reversal learning.

Previous studies suggested that the α/β lobes of MBs form a later memory trace than the α′/β′ lobes and that output from the α/β lobes is only required during memory retrieval in the testing period (Dubnau et al. 2001; McGuire et al. 2001; Schwaerzel et al. 2002; Krashes et al. 2007). We, therefore, investigated whether α/β lobe output is required during reversal acquisition using the protocol depicted in Figure 3B. Briefly, cycle 2 was performed at the restrictive temperature, whereas cycle 1 and testing were performed at the permissive temperature. The PIs obtained from these experiments were compared to those from the corresponding control protocol performed entirely at the permissive temperature. To allow for a sufficient recovery of the reversible temperature-sensitive *shibire*^{ts1} transgene, the interval between the two training cycles and the delay period after cycle 2 were lengthened to 30 min. The reversal learning PIs of MB247-*Gal4*/UAS-*shibire*^{ts1} and c739-*Gal4*/UAS-*shibire*^{ts1} flies were not affected when their cycle 2 training was performed at the restrictive temperature. In contrast, the c305a-*Gal4*/UAS-*shibire*^{ts1} flies exhibited severely impaired reversal learning performance under the same condition (Fig. 3B). Moreover, the c316-*Gal4*/UAS-*shibire*^{ts1} flies showed no differences between protocols. Several careful investigations of the expression patterns of MB-specific *Gal4* lines showed that the MB247 line had strong expression in the α/β lobes and γ lobes but very weak expression in the α′/β′ lobes and that the c739 line is exclusively restricted to the α/β lobes (Armstrong et al. 1998; Zars

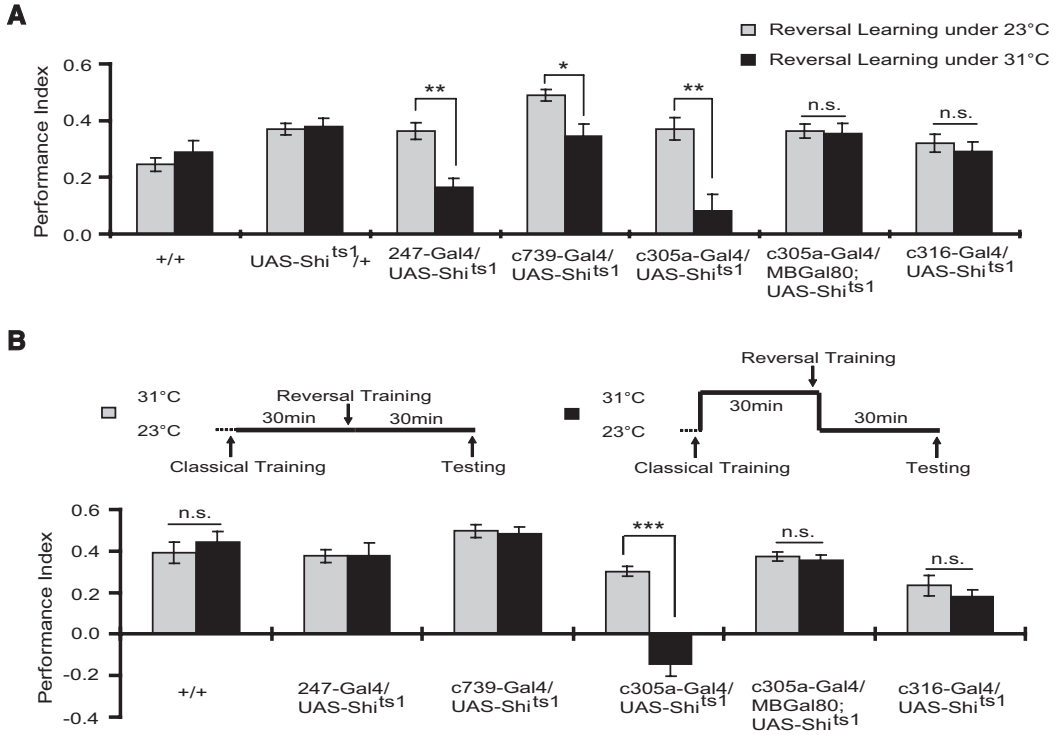

Figure 3. Olfactory reversal learning is acquired in MBs. (*A*) Reversal learning was performed at the permissive (23°C) and restrictive (31°C) temperature, and the respective PIs were compared. Flies carrying UAS-*shibire*^{ts1} together with the 247-*Gal4*, c739-*Gal4*, or c305a-*Gal4* driver showed significantly lower reversal learning PIs at the restrictive temperature. In contrast, the c305a-*Gal4*/MB*Gal80*; UAS-*shibire*^{ts1} and c316-*Gal4*/UAS-*shibire*^{ts1} flies did not exhibit any obvious impairment at the restrictive temperature. (*B*) The reversal learning protocol was elongated so that the intervals were 30 min each. The control protocol (gray bar) was at the permissive temperature, whereas the test protocol (black bar) required that flies be acclimated to the restrictive temperature for 30 min before they were reversal-trained. They were subsequently moved back to the permissive temperature and tested at the permissive temperature after 30 min. Only cycle 2 was performed under the restrictive temperature. Compared with the PIs obtained from the control protocol, only the c305a-*Gal4*/UAS-*shibire*^{ts1} flies displayed severely disrupted reversal learning performance. $n = 6–8$ for each group. Data are means ± SEM. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$; (n.s.) not significant (Student's *t*-tests).

et al. 2000; Krashes et al. 2007; Tanaka et al. 2008). In addition, the c305a line is relatively α′/β′ lobe-specific and does not express in α/β or γ lobes (Krashes et al. 2007). The c305a line also expresses in the antennal lobes, but its involvement in associative learning was mainly attributed to α′/β′ lobes (Krashes et al. 2007). To further clarify the role of α′/β′ lobes in reversal learning, we incorporated the MB{*Gal80*} transgene to repress the c305a-*Gal4* expression in the MB. When combined with c305a, the MB{*Gal80*} transgene specifically abolished *Gal4* activity in α′/β′ neurons while leaving the expression in the antennal lobe (AL) and other regions either slightly reduced or unchanged (Krashes et al. 2007). As shown in Figure 3A, the c305a-*Gal4*/MB*Gal80*;UAS-*shibire*^{ts1} flies exhibited similar reversal learning PIs at both restrictive and permissive temperatures. Furthermore, the reversal learning PI of the c305a-*Gal4*/MB*Gal80*;UAS-*shibire*^{ts1} flies was not affected when only the cycle 2 training was performed at the restrictive temperature (Fig. 3B). Thus, the effect of c305a-*Gal4* could be safely attributed to the α′/β′ lobes. Together, the experimental results displayed in Fig. 3 imply that the acquisition of olfactory reversal learning maps to the neural circuitry that includes the α′/β′ MB neurons.

Reduction of GABA synthesis in the APL neurons impairs olfactory reversal learning

To further explore the neural mechanisms underlying reversal learning, we attempted to screen various mutant fly lines with learning and memory defects. However, as mentioned above, the olfactory reversal learning is grounded in classical learning, which makes it difficult to determine any reversal-specific mechanism once classical learning ability is impaired. This led us to consider the manipulation of the neurotransmitter GABA, because a series of important studies revealed that the down-regulation of MB GABA improves classical learning (Liu et al. 2007; Liu and Davis 2009; Liu et al. 2009). RNA interference (RNAi) was used to knock down the expression of a crucial enzyme for GABA synthesis, the glutamatic acid decarboxylase (GAD), in APL neurons, and it enhanced classical learning performance (Liu et al. 2009). Currently, there is no available APL neuron-specific *Gal4* line, and the most widely used among the *Gal4* lines containing APL is GH146-*Gal4*. Although GH146-*Gal4* also drives expression in projection neurons (PNs), these neurons are mostly cholinergic and not affected by GAD knockdown (Tanaka et al. 2008; Liu and Davis 2009). We, therefore, focused on this line for the reversal learning assays. The flies carrying the GH146-*Gal4* driver and the UAS-*Gad*-RNAi transgene displayed a higher classical learning PI than control flies carrying only the GH146-*Gal4* driver or *Gad*-RNAi transgene (Fig. 4B), as previously reported (Liu et al. 2009). However, their reversal learning PI was significantly lower than that of control flies (Fig. 4B). To confirm this result, we tested three variations of the basic reversal learning protocol as shown in Fig. 1C–E and observed consistent impairment of reversal learning in all cases (Fig. 4C–E).

Furthermore, we tested other APL-relevant *Gal4* lines. The NP225-*Gal4* line has a similar expression pattern with the GH146-*Gal4* line, except that it does not include the APL neurons (Tanaka et al. 2008; Liu et al. 2009). We found that the reversal learning PI of NP225-*Gal4*/*Gad*-RNAi flies was indistinguishable from that of control flies (Fig. 4F), further ruling out the possible role of PNs. In addition, we tested the NP2631 and NP5288 *Gal4* lines, which also drive expression in APL neurons (Tanaka et al. 2008; Pitman et al. 2011). Similar to GH146-*Gal4*/*Gad*-RNAi flies, the NP2631/*Gad*-RNAi and NP5288/*Gad*-RNAi flies both exhibited disrupted reversal learning PI (Fig. 4F). Taken together, these results demonstrate that reducing GABA synthesis in the APL neurons impairs olfactory reversal learning in *Drosophila* and indicate that the APL neurons play an important role in reversal learning.

The APL neurons facilitate reversal learning through inhibiting the initial memory

Since the GABAergic APL neurons arborize only within the MB neuropile (Tanaka et al. 2008; Liu and Davis 2009; Pitman et al. 2011), we speculated that, during reversal learning, the APL neurons might inhibit the cycle 1 memory inside MBs (Liu and Davis 2009), thereby facilitating successful reversal acquisition. To test our hypothesis, we adopted another set of protocols. Since the flies were tested for the two trained odors in the basic reversal learning protocol, we could only evaluate the memory strength of the two training cycles relative to each other and were unable to determine the "absolute values" of memory strength. Therefore, we introduced a third odor (odor C) into the testing period. As shown in Figure 5A, the protocol set included four separate subprotocols that were similar to the reversal learning protocol but with some small variations. Protocols 1 and 3 (both without shock) served as blank controls for Protocols 2 and 4, respectively. To eliminate odor bias, we subtracted the scores of control groups (Protocol 1 or 3) from the scores of their corresponding conditioned groups (Protocol 2 or 4) and averaged this result with another result calculated from a similar experiment but with odor A and odor B switched to yield the final PI (Supplemental Table S1). This value obtained from Protocols 1 and 2 was indicated as "PI2-PI1", and the value from Protocol 3 and 4 was marked as "PI4-PI3" (Fig. 5B). In Protocols 1 and 2, odor C replaced odor B in the testing period, whereas in Protocols 3 and 4, odor C replaced odor A during the testing period. Through these protocols, we intended to separately measure the memory strengths of odor A and odor B relative to a third odor C. PI2-PI1 measures the memory strength of odor A relative to odor C, which represents the memory strength of cycle 1 during testing; PI4-PI3 is the corresponding measurement for odor B, which represents the memory strength of cycle 2. A separate measurement of these two odors' memory strengths showed that the cycle 1 memory is suppressed after reversal training in wild-type flies, as PI2-PI1 was significantly lower than PI4-PI3 (Fig. 5B). Furthermore, we tested the performance of GH146-*Gal4*/*Gad*-RNAi flies and their control lines using this set of protocols. As demonstrated in Figure 5B, GH146-*Gal4*/*Gad*-RNAi flies failed to inhibit their initial memory from cycle 1 training, as their PI2-PI1 and PI4-PI3 values were not significantly different. However, control flies carrying only the GH146-*Gal4* driver or *Gad*-RNAi transgene performed similarly to the wild-type flies; they were able to suppress the cycle 1 memory, as indicated by significantly different PI2-PI1 and PI4-PI3 (Fig. 5B). Therefore, reduction of GABA synthesis in the APL neurons impairs the flies' ability to properly inhibit the initial memory from cycle 1 training in reversal learning.

The impairment of reversal learning through GABA down-regulation in APL is a physiological effect

The lack of temporal control of the *Gal4*-UAS system over transgene expression could potentially cause structural brain defects during development. To rule out this possibility, we used the TARGET system (McGuire et al. 2003) to introduce temporal control over the tissue-specific expression of transgenic *Gad*-RNAi. Flies were engineered to carry a ubiquitously expressed temperature sensitive *Gal80*^{ts} (tub-*Gal80*^{ts}) gene together with the GH146-*Gal4* driver and the *Gad*-RNAi transgene. The flies were all raised at 18°C; at this temperature, *Gal80* suppresses the *Gal4* driver. If the flies were further trained and tested at 18°C, both the classical learning and reversal learning PIs of GH146-*Gal4*/*Gad*-RNAi flies were indistinguishable from the control (Fig. 6A). However, if the flies were moved after eclosion to 32°C for 2 d, thereby releasing the inhibition of *Gal80*^{ts} on GH146-*Gal4*,

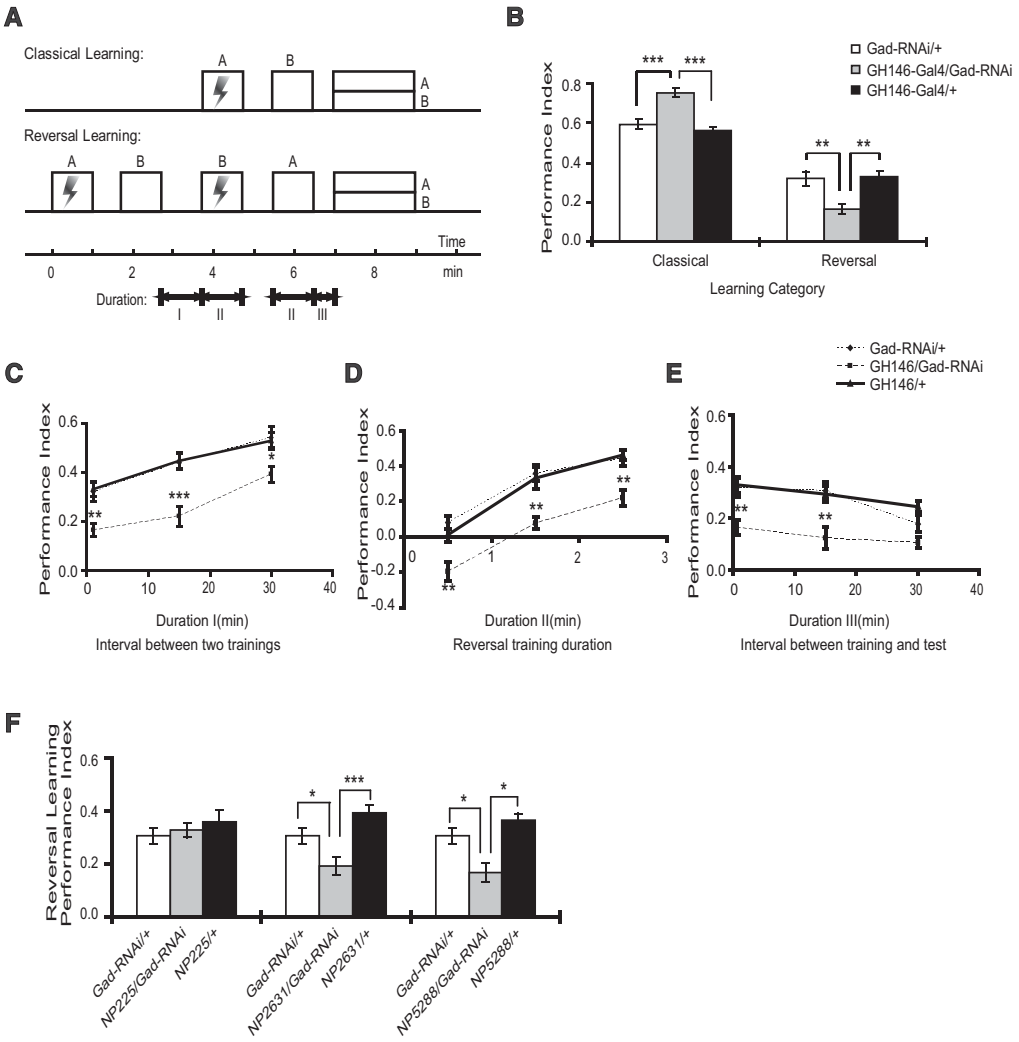

Figure 4. Reduction of GABA synthesis in APL neurons impairs reversal learning. (*A*) The classical and reversal learning protocols used. (*B*) Flies carrying the GH146-*Gal4* driver and the *Gad*-RNAi transgene exhibited an enhanced PI for classical learning, whereas their reversal learning PI was significantly lower than that of control flies carrying only the GH146-*Gal4* driver or the *Gad*-RNAi transgene. (*C–E*) Variations of the reversal learning protocol were performed. The GH146-*Gal4*/*Gad*-RNAi flies showed obviously impaired reversal learning ability compared with their control groups (GH146-*Gal4*/+ and *Gad*-RNAi/+ flies). (*F*) NP2631-*Gal4*/*Gad*-RNAi and NP5288-*Gal4*/*Gad*-RNAi flies also showed significantly lower reversal learning PI comparing with their control groups. However, the NP225-*Gal4*/*Gad*-RNAi flies did not show decreased performance compared with their control counterpart. $n = 6–8$ for each group. Data are means ± SEM. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$; (Student's *t*-tests in *B* and *F*, ANOVA in *C–E*).

GABA down-regulation had an effect. The GH146-*Gal4*; *Gal80*^{ts}/*Gad*-RNAi flies showed improved performance in classical learning and impaired reversal learning (Fig. 6B). Together, these results confirm that the disruption of reversal learning through reducing GABA synthesis in APL neurons is a physiological rather than developmental effect.

Discussion

The effectiveness of one-trial instant reversal

The one-trial instant reversal paradigm was chosen as the primary reversal protocol for several reasons in addition to its being concise and simple to execute. First, if cycle 2 immediately follows cycle 1, the memory decay factor of cycle 1 is minimized, and the contribution of the reversal factor is highlighted. In fact, an elegant early analysis of the interaction between cycle 1 memory and cycle 2 training already disclosed that, although the reversal learning PI increases along with the delay of the cycle 2 training, the nonadditive effect of the two cycles is most salient when the delay is shorter than 30 min (Tully et al. 1990). Therefore, it would be more suitable to adopt the short delay protocol when examining the reversal effect.

Second, we demonstrated the necessity of our protocol (A+B– B+A–) for the investigation of reversal learning. Since CS+ odor presentation alone is sufficient to produce an optimal learning result in classical learning (Yu et al. 2006), CS– odor is dispensable in classical learning. Therefore, it brought the concern about whether CS– odor is also dispensable in reversal learning. A timeline-aligned direct comparison between the basic reversal learning protocol (A+B– B+A–) and the CS– odor absent protocol (A+B+) indicated that the reversal protocol exerted a stronger inverting power than CS– absent protocol (Fig. 2D). Therefore, CS– odor is indispensable in reversal learning protocol. Additional experiments showed that the flies developed distinguishable memory strengths for the two trained odors before testing, as significantly different PIs were observed when the two odor

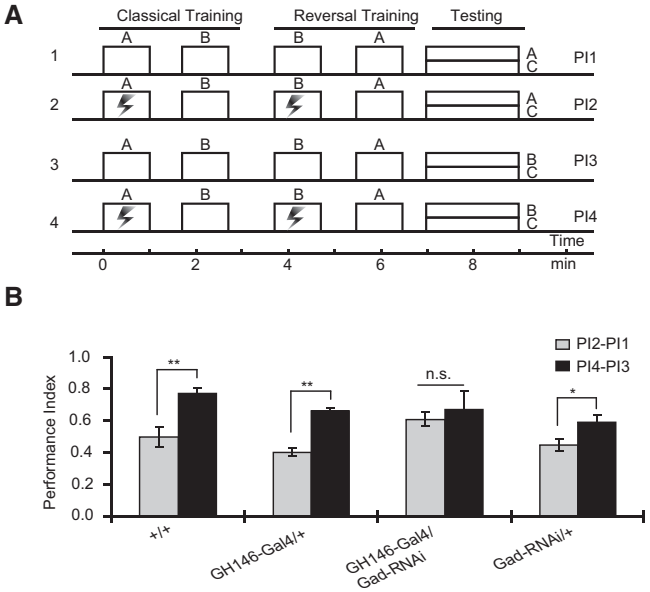

Figure 5. The APL neurons facilitate reversal learning through inhibiting the initial learning. (*A*) A third odor (C) was introduced in the testing period to serve as a reference odor; otherwise, the protocols were the same as those used for reversal learning. Protocols 1 and 3 (without electric shock) were reference trials for Protocols 2 and 4, respectively. In Protocols 1 and 2, odor C replaced odor B during testing, whereas in Protocols 3 and 4, odor C replaced odor A. (*B*) Flies were trained on the protocols indicated in panel *A*. Gray bars represent the values of PI2 minus PI1 (PI2-PI1), and black bars represent those of PI4 minus PI3 (PI4-PI3). Flies with reduced GABA synthesis that carry both the GH146 driver and the *Gad*-RNAi transgene showed indistinguishable values of PI2-PI1 and PI4-PI3. In contrast, wild-type flies and those carrying only the GH146 driver or the *Gad*-RNAi transgene exhibited a significant difference between PI2-PI1 and PI4-PI3. $n = 6–7$ for each group. Data are means ± SEM. (*) $P < 0.05$; (**) $P < 0.01$; (n.s.) not significant (Student's *t*-tests).

memories were separately evaluated (Fig. 5). Moreover, a detailed investigation of a "two-event choice" protocol (Yin et al. 2009), which actually is also A+B+, revealed that the flies are unable to make a consistent choice when the time delay between the two conditioning sessions is shorter than 2 min (Yin et al. 2009). This confirmed the effectiveness of our protocol and further differentiated the reversal learning from a decision-making process, which was probed through the "two-event choice" protocol (Yin et al. 2009).

Third, the serial reversal paradigm that has been used in other model systems demonstrated that increased reversal experience expedited the reversal acquisition (Iversen and Mishkin 1970; Schoenbaum et al. 2003; Mota and Giurfa 2010). We have also assayed sequential reversal learning but so far have not observed improved reversal PIs with increasing reversal cycle numbers in *Drosophila* (Supplemental Fig. S3). The difference could be due to a lower efficiency of Pavlovian training on the T-maze platform compared with operant training.

The importance of inhibitory regulation in reversal learning

The importance of MBs for olfactory learning and memory in *Drosophila* has been extensively addressed (Heisenberg 2003; Davis 2005; McGuire et al. 2005; Akalal et al. 2006; Berry et al. 2008; van Swinderen 2009). Genetic suppression of Rac expression in MBs disturbed reversal learning (Shuai et al. 2010). In honeybees, MBs are required for reversal learning (Devaud et al. 2007). Our experimental results further demonstrated that *Drosophila* olfactory reversal learning also requires the MBs (Fig. 3). It is not surprising that the MBs have such a vital role since our reversal learning protocol is based on classical associative learning. For the same reason, it is difficult to distill reversal-specific mechanisms in MBs. Therefore, we emphasize the role of GABA regulation in reversal learning.

Previous studies have shown that down-regulation of GABA through knock- down of the $GABA_A$ receptor, resistance to dieldrin (RDL), or through reducing GABA synthesis in the APL neurons improves the associative learning ability of flies (Liu et al. 2007; Liu and Davis 2009; Liu et al. 2009). The role of the GABAergic APL neurons in *Drosophila* learning and memory has recently garnered attention. The APL neurons are required to sustain labile memory, and gap junctions between the APL and the DPM neurons play a critical role in olfactory memory (Pitman et al. 2011; Wu et al. 2011). Moreover, in locusts, the giant GABAergic neurons (GGN), which are anatomical equivalents to the *Drosophila* APL neurons, were found to form a normalizing negative-feedback loop within the MBs (Papadopoulou et al. 2011). Taken together with our finding that reducing GABA synthesis in APL neurons disrupted the reversal learning ability in flies (Figs. 4, 5), we propose that the APL neurons mediate the olfactory reversal learning, which might be acquired inside MBs. Specifically, we speculate that when the flies face reversal training that contradicts previous learning, the APL neurons inhibit the initial memory and facilitate the reversal acquisition by releasing an appropriate amount of GABA. Since the APL neurons innervate the MBs broadly, it is difficult to imagine how the release of GABA would occur specifically on the MB neurons that represent the initially learned odor. Perhaps there exists some retrograde information from the MB neurons, representing the initially learned odor, that is sent to the presynaptic dendrites of the APL neurons, thus regulating the GABA release accordingly.

Our attempts to manipulate RDL expression and up-regulate GABA synthesis in APL neurons failed to yield meaningful results; we did not observe any significant change of reversal learning in those experiments (Supplemental Figs. S4, S5). The reason might be that the efficiencies of those manipulations were not sufficient to generate detectable differences in reversal learning. Nevertheless, enhanced GABAergic innervation has been shown to improve reversal learning in mice (Morellini et al. 2010), which supports our speculation regarding GABA modulation in reversal learning.

The relationship between memory extinction and reversal learning

Memory extinction occurs when the CS+ that was previously associated with US is presented without US pairing (Pavlov 1927; Myers and Davis 2007). We have shown that in our reversal protocol, the memory extinction component could not fully account for the reversal learning PI (Supplemental Fig. 2A). In honeybees, the blockade of MBs led to reversal defects without affecting extinction (Devaud et al. 2007), which suggests an obvious divergence between extinction and reversal. Despite these apparent differences, there appears to be some common underlying mechanisms. Overlapping neural systems mediating extinction and reversal were reported in humans (Schiller and Delgado 2010). In *Drosophila*, odorant memory extinction is supposed to be an intracellular process and antagonizes the previous memories at the molecular level, affecting cAMP signaling (Schwaerzel et al. 2002). Our experimental results demonstrated that reversal is also cAMP-dependent (Supplemental Fig. S6), which indicates that there is a similar foundation for extinction and reversal at the molecular level. Based on those evidences, the relationship between

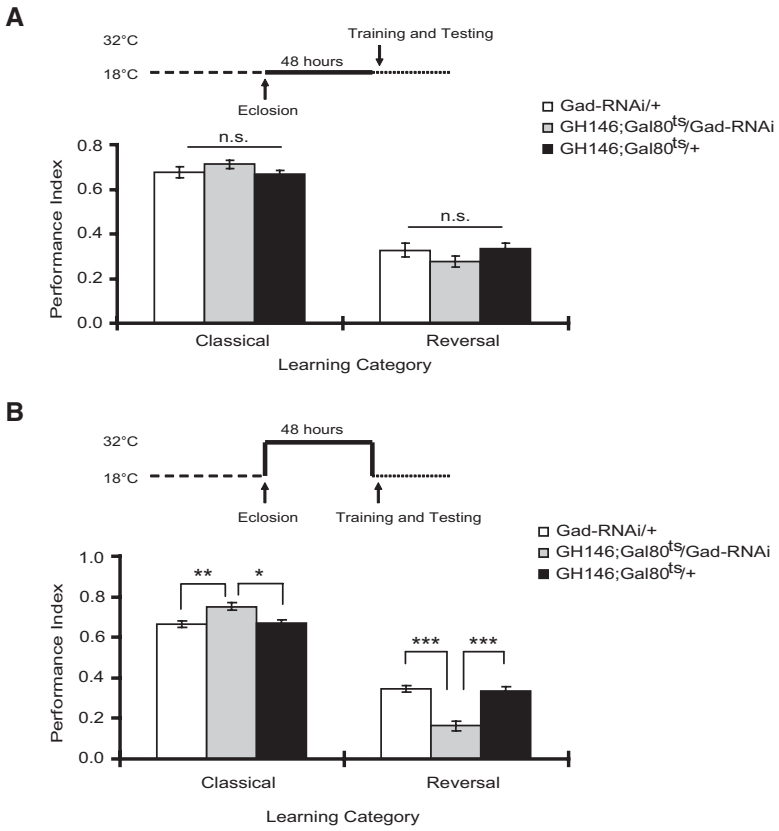

Figure 6. Reversal learning is physiologically disrupted when GABA is reduced in APL. (*A*) Flies were raised, trained, and tested at 18°C. The experimental flies carried the GH146-*Gal4* driver, the *Gad*-RNAi transgene, and a tubulin *Gal80^{ts}* repressor. Control flies carried only the GH146-*Gal4* driver or *Gad*-RNAi transgene combined with tubulin *Gal80^{ts}*. In both classical and reversal learning, PIs of the experimental group were not significantly different from those of the controls. (*B*) Flies were raised at 18°C until eclosion, then shifted to 32°C for 2 d prior to training and testing. In classical learning, the experimental group performed significantly better than the control groups, whereas their reversal learning was severely impaired relative to the control. $n = 6–12$ for each group. Data are means ± SEM. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$; (n.s.) not significant (Student's *t*- tests).

memory extinction and reversal learning seems to be complicated and might involve different but not completely separate neuronal mechanisms.

Materials and Methods

Fly stocks and culture

The flies were cultured using standard food (Guo et al. 1996) in a 12-h light/dark cycle at 25°C and 60%–70% relative humidity.

The fly strains ***w***(CS10), *Rdl*i8-10J, *Gad*-RNAi, c772-*Gal4*, and c739-*Gal4* were generous gifts from Dr. Ronald Davis. The c316-*Gal4* line and MB*Gal80*; UAS-*shibire*^{ts1} fly lines were kindly provided by Dr. Scott Waddell. The *rut^{2080}*, *rut^{2080}*; UAS-*rut*+ flies were kindly provided by Dr. Li Liu. Dr. Dave Featherstone kindly offered us the UAS-*Gad1* flies. The c305a-*Gal4* fly was ordered from the Bloomington *Drosophila* Stock Center. The NP225-*Gal4*, NP2631-*Gal4*, and NP5288-*Gal4* lines were ordered from the *Drosophila* Genetic Resource Center (DGRC). The rest of the fly lines were extant in our lab.

Behavioral assays

Classical learning

Classical learning refers to Pavlovian olfactory aversive conditioning and was performed according to a standard protocol (Tully and Quinn 1985). Briefly, a group of ~120 flies was first trained by being exposed to an odor paired with shock (odor A, CS+) and sequentially to another odor (odor B, CS–) without shock (US). The odors were 3-octanol [OCT] (1.5×10^{-3}) and 4-methylcyclohexanol [MCH] (1×10^{-3}) (Fluka) diluted in heavy mineral oil (Fisher). The US was composed of 12 1.25-sec pulses of 75-V electric foot shock. To test the learning effect, trained flies were pushed down to a choice chamber where they could choose between CS+ and CS– within a 120-sec testing period. A performance index was calculated from the number of flies distributed in the two T-maze arms. The PI was equal to the number of flies avoiding the CS+ odor minus the number of flies avoiding the CS– odor divided by the total number of flies. To eliminate odor bias, each experimental trail ($n = 1$) included two reciprocal groups, with the two odors switched. The final PI was the average of the two groups.

Reversal learning

After the training cycle of classical learning, a second training cycle was added with the CS-US contingency reversed, i.e., the odor previously paired with shock in the first cycle was not paired with shock in the second cycle and vice versa. There was a 60-sec interval between the two training cycles. After both cycles, the flies chose between both odors, as in classical learning. The PI was calculated as stated above, except that the odor paired with shock in the second cycle was taken as the CS+. Again, each experimental trial ($n = 1$) consisted of two reciprocal groups.

Third–odor tests

The various protocols containing the third odor (odor C) used benzaldehyde [BEN] (1×10^{-3}) (Fluka) diluted in heavy mineral oil. Odor C was only presented during the testing period. When the PI was computed, the odor paired with shock in the second cycle was taken as the CS+, the unpaired odor in the second training cycle was considered to be the CS–, and odor C played the same role as the absent odor in the testing. Another group of flies exposed to exactly the same odor sequence without any US delivery (untrained) was also tested with the same testing odor pair as above. This group served as a control for naive odor bias. The score of the untrained group was subtracted from that of the trained group to yield a half PI. This value was averaged with another half PI, in which odor A and odor B were interchanged, to produce a final PI.

Data analysis

Data are shown as means ± SEM. Comparisons were performed by Student's *t*-tests (for two groups) or one-way ANOVA with post-hoc Fisher LSD test (for multiple groups). (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$; n.s. indicates no significant difference ($P > 0.05$). Unless stated otherwise, all experiments are $n \geq 6$.

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