Distinct molecular underpinnings of *Drosophila* olfactory trace conditioning

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Trace conditioning is valued as a simple experimental model to assess how the brain associates events that are discrete in time. Here, we adapted an olfactory trace conditioning procedure in *Drosophila melanogaster* by training fruit flies to avoid an odor that is followed by foot shock many seconds later. The molecular underpinnings of the learning are distinct from the well-characterized simultaneous conditioning, where odor and punishment temporally overlap. First, Rutabaga adenylyl cyclase (Rut-AC), a putative molecular coincidence detector vital for simultaneous conditioning, is dispensable in trace conditioning. Second, dominant-negative Rac expression, thought to sustain early labile memory, significantly enhances learning of trace conditioning, but leaves simultaneous conditioning unaffected. We further show that targeting Rac inhibition to the mushroom body (MB) but not the antennal lobe (AL) suffices to achieve the enhancement effect. Moreover, the absence of trace conditioning learning in *D1 dopamine receptor* mutants is rescued by restoration of expression specifically in the adult MB. These results suggest the MB as a crucial neuroanatomical locus for trace conditioning, which may harbor a Rac activity-sensitive olfactory "sensory buffer" that later converges with the punishment signal carried by dopamine signaling. The distinct molecular signature of trace conditioning revealed here shall contribute to the understanding of how the brain overcomes a temporal gap in potentially related events.

learning and memory | olfaction | cAMP | Rho GTPase

In trace conditioning, the conditional stimulus (CS) and the unconditional stimulus (US) are separated in time by a stimulus-free interval (1). This so-called "trace interval" can last for a fraction of a second in eyeblink conditioning but many seconds in fear conditioning, which poses a challenging question: how does the brain overcome this temporal gap to form the association between the CS and US (2)? Intriguingly, trace conditioning in mammals engages neural substrates fundamentally different from delay conditioning, where the CS precedes but also temporally overlaps with the US (3). Early evidence comes from lesion studies with experimental animals showing that acquisition of trace conditioning requires intact hippocampal formation (4, 5) and medial prefrontal cortex (6), whereas delay conditioning can occur even with the entire forebrain removed (7, 8). Later studies involving human subjects further validate the involvement of different brain circuits in these two conditioning variants and even suggest, more surprisingly, that conscious awareness might be a prerequisite for trace but not delay conditioning (9, 10). It is then hypothesized that the participation of hippocampus and neocortex, as well as the associated higher cognitive function, is necessary in trace conditioning to maintain a representation of the CS or CS/US contingency so as to bridge the temporal gap (11, 12). However, little is known about what form this representation takes and how it eventually converges with the US.

Pavlovian conditioning has also been extensively studied in invertebrate animals (13). In *Drosophila*, one of the best-studied paradigms is olfactory differential aversive conditioning (14), wherein fruit flies smell two odors [normally 3-octanol (OCT) and 4-methycyclohexanol (MCH)], one (CS^+) associated with negative reinforcement but the other (CS^-) not. A simultaneous conditioning procedure is frequently used (Fig. 1*A*) in which the 1-min CS^+ odor exposure cooccurs with the US punishment, composed of twelve 1.5-s pulses of 60 V electric shock distributed in a 1-min period (15). Studies over the past three decades have substantiated the mushroom body (MB) as a major site where learning-related plasticity takes place (16). This brain locus is the third-order olfactory area in insects where the CS and US combine (17). Information about the CS reaches the MB via the projection neurons of the antennal lobe (AL), the insect equivalent of the olfactory bulb (18). The reinforcement signal from the US is conveyed via the dopamine neurons, which also form synapses with the MB (19). In a simplified molecular model, the CS^+ and US converge on Rutabaga adenylyl cyclase (Rut-AC), which in turn triggers the cAMP/PKA signaling cascade that drives synaptic plasticity and learned behavior (20–22). The neural circuits processing the CS and US information are now being studied at single-cell resolution (e.g., 19, 23, 24) and an increasing repertoire of learning/memory-related molecules are being identified (e.g., 25, 26). The abundant knowledge of this conditioned behavior makes the fruit fly an attractive model to study trace conditioning (14, 27, 28).

Here, we adapted our olfactory aversive trace conditioning procedure via insertion of an odor-free interval between the offset of CS^+ and the onset of US (Fig. 1*A*). Single-trial training is sufficient to elicit considerable learning performance. The molecular underpinnings of the trace learning were found to be dramatically different from simultaneous conditioning, with respect to the requirement of Rut-AC and the involvement of Rac-mediated forgetting.

Results

***rut* Mutants Perform Normally in Trace Conditioning.** Learning performance generated after single-trial training with different conditioning procedures is shown in Fig. 1*B*. Consistent with previous reports (14, 27), trace conditioning elicits considerable conditioned aversion of the CS^+ in wild-type flies. Learning becomes less efficient as the trace interval increases, but is still evident for intervals up to 60 s. Two *rut* mutants were tested along with the wild-type flies; rut^{1047} bears a P{Gal4} insertion (25), whereas rut^{1} carries a point mutation and is functionally null (29). Surprisingly, both *rut* mutants have completely normal performance in trace conditioning despite their severe learning defects in simultaneous conditioning (Fig. 1*B*). Likewise, rut^{1}/rut^{1047} heterozygote shows a learning defect in simultaneous but not trace conditioning (Fig. 1*C*). Both rut^{1} and rut^{1047} have been

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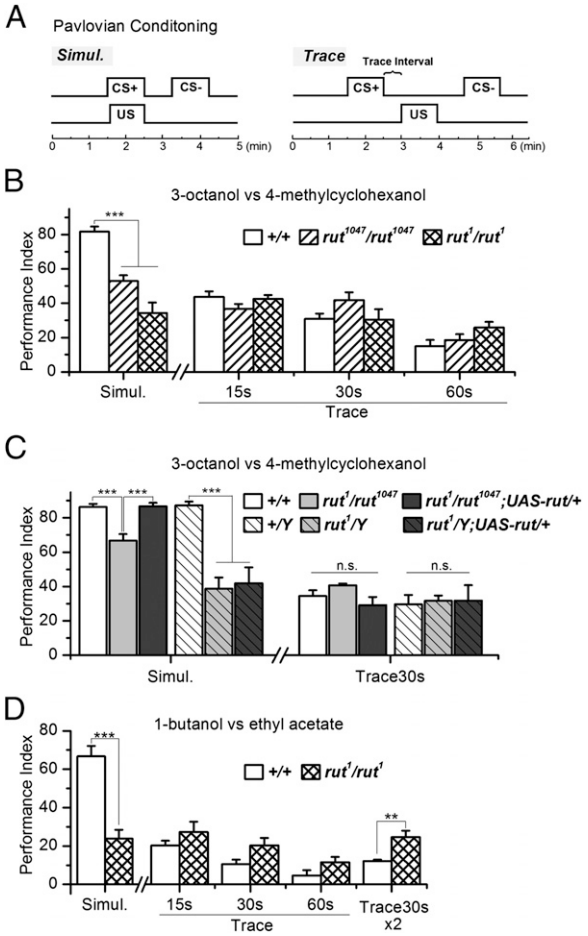

Fig. 1. Trace conditioning of olfactory aversion in wild type and *rut* mutants. (*A*) Trace conditioning (Trace) differed from standard simultaneous conditioning (Simul.) only in an interstimulus trace interval between the CS^+ odor and the US foot shock. (*B*) Learning performance after Simul. or Trace with various trace intervals (15, 30, and 60 s). Odors used were indicated. *rut* mutants showed learning defects in Simul. (ANOVA, $P < 0.001$), but normal performance in Trace (ANOVA, $P > 0.1$ for all intervals); $n = 6$. Error bars indicate SEM. (*C*) rut^1/rut^{1047} transheterozygote also showed selective defect in Simul. learning. The defect was rescued by restoration of *rut* expression in rut^1/rut^{1047}; *UAS-rut*/+, but no increase in Trace performance was observed. Female and male flies were segregated after testing to obtain designated genotypes. $n = 4$ for Simul.; 6 for Trace30s. Error bars indicate SEM. $^{***}P < 0.001$; NS, nonsignificance. (*D*) In the training with hydrophilic odors, rut^1 mutant also showed lower learning performance in Simul. (ANOVA, $P < 0.001$), but normal or even higher performance in Trace (ANOVA, $P = 0.27$, 0.06, 0.12 for 15, 30, and 60 s, respectively; $P = 0.006$ for Trace30s × 2 with intertrial interval of 5 min). $n = 5$. Error bars indicate SEM.

equilibrated to the wild-type genetic background, so it is unlikely that the trace conditioning defect is masked by a second-site mutation. This assertion is further strengthened by the fact that restoration of *rut* expression by a upstream activation sequence (*UAS*)-*rut* transgene (30) rescues the learning defect in simultaneous conditioning, but does not further increase trace conditioning performance (compare the performance of rut^1/rut^{1047} and rut^1/rut^{1047}; *UAS-rut*/+ in Fig. 1*C*). Thus, both wild-type and *rut*-deficient mutants acquire learning in the trace conditioning paradigm.

In the above experiments, we followed the conventional protocol (15) and used the hydrophobic odors, OCT and MCH. Galili et al. recently pointed out that MCH and possibly other hydrophobic odors are difficult to remove from the training apparatus by air flushing (28). To ensure the trace conditioning is not an artifact of residual odor, we monitored odor dissipation in the training tube via a photoionization detector (PID). Odor concentration follows a rapid decay after the termination of odor delivery, but the PID detected a trace amount of slowly decaying odor that took over 1 min to return to baseline (Fig. S1*A*). However, such a low level of residual odor at the time window relevant to the trace conditioning paradigm (≥30 s) is not sufficient to induce measurable learning performance in flies (Fig. S1*B*). Odors adhering to the fly cuticle or retained in the sensillum lymph are unlikely to be a problem as it is known that olfactory sensory neuron activity rapidly returns to baseline following odor offset (31). In addition, we validated the above *rut* results by training flies with a pair of hydrophilic odors, 1-butanol (BU) and ethyl acetate (EA). These two odors show much faster dissipation, decaying to baseline within 15 s (Fig. S1*A*), and the former is used by Galili et al. in their recent trace conditioning study (28). We observed a similar phenotype. rut^1 mutant shows defect in simultaneous conditioning, but normal or even slightly higher learning performance in trace conditioning (Fig. 1*D*). This result, combined with those described above, demonstrates that Rut-AC is dispensable in trace conditioning, as opposed to its essential role in simultaneous conditioning (20, 30, 32).

Inhibition of Rac-Mediated Forgetting Enhances Trace Conditioning. Another distinction between the two paradigms comes from the assessment of a molecular pathway mediating forgetting of early labile memory (25). Rac is a member of Rho family small G proteins, which play critical roles in neuronal actin cytoskeleton remodeling (33). We recently reported that inhibition of Rac activity lengthens early memory retention after simultaneous conditioning, but leaves initial learning unaffected (25). Intriguingly, when a temporal gap separates the CS^+ and the US in trace conditioning, the same manipulation exerts a profound effect on learning.

We used the Gal4/$Gal80^{ts}$ system (32) to drive adult onset expression of a dominant-negative form of *Drosophila* Rac1, Drac1(N17) (34). As described previously (25), flies were raised in 18 °C; a 3-d heat-shock treatment at 30 °C was used to inactivate the ubiquitously expressed *tubulin-*$Gal80^{ts}$ ($Gal80^{ts}$) and switch on Gal4-dependent transgene expression. Remarkably, inhibition of Rac activity throughout the adult brain (*elav-Gal4*/+; $Gal80^{ts}$/+; *UAS-Drac1(N17)*/+) enhances the learning of trace conditioning compared with the two parental controls (Fig. 2*A*). Significant enhancement is obvious for trace conditioning at trace intervals of 15, 30, and 60 s. At an extremely long interval of 300 s, some residual learning at a score of ∼10 is still evident, which presumably arises from attraction to CS^- via backward conditioning (27); however, no statistically significant differences were observed among groups, validating the enhancement is specific to trace conditioning. Heat-shock induction of transgene expression is a prerequisite of the enhancement, because no differences among genotypes were observed for uninduced groups kept at 18 °C (Fig. 2*A*).

Drac1(N17)-expressing flies show normal task-relevant sensorimotor responses and acquisition of simultaneous conditioning (25); thus the observed trace conditioning enhancement is unlikely attributable to a superior ability in pairing foot-shock punishment with an ambient odor trace in the training tube. We performed three more control experiments below.

First, we trained flies with simultaneous conditioning but lowered odor concentration by further dilutions of 10^1-, 10^2-, and 10^4-fold (Fig. S2*A*). Drac1(N17)-expressing flies show performance largely comparable to the controls. A marginal yet statistically significant difference was observed compared with the *elav-Gal4*/+; $Gal80^{ts}$/+ control in the 10^1-fold dilution, but not in the 10^2- or 10^4-fold dilution or in any comparison with the *UAS-Drac1(N17)*/+ control. Therefore, the detection or reinforcement

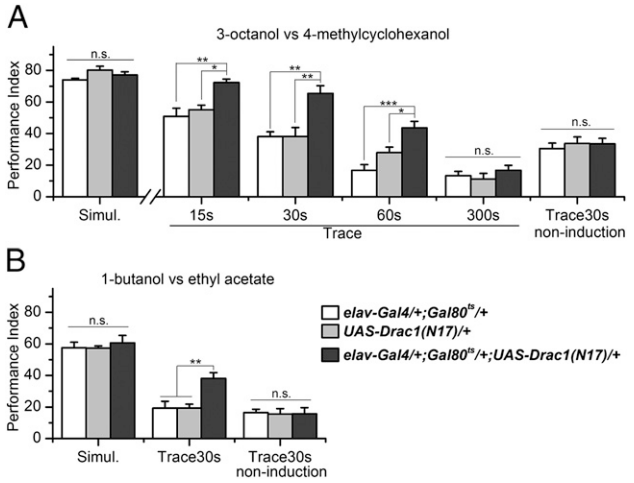

Fig. 2. Drac1(N17) expression enhances trace conditioning. (*A*) Heat shock at 30 °C for 3 d was used to induce transgene expression. The learning performance of Drac1(N17)-expressing flies (*elav-Gal4/+; Gal80^{ts}/+; UAS-Drac1(N17)/+*) was significantly higher than two similarly heat-shock–treated parental controls in Trace at trace intervals of 15, 30, and 60 s (ANOVA, $P < 0.05$), but not at the interval of 300 s (ANOVA, $P > 0.15$). Fly groups without heat-shock induction showed similar performance in Trace30s learning (ANOVA, $P > 0.95$). The Simul. data are from Shuai et al. (25) and are presented for ease of comparison. $n = 6$. Error bars indicate SEM. (*B*) Confirmation experiments that used hydrophilic odors for training (ANOVA, $P < 0.01$ for Trace30s; $P > 0.95$ for Simul. and Trace30s noninduction). $n = 5$ or 8. Error bars indicate SEM.

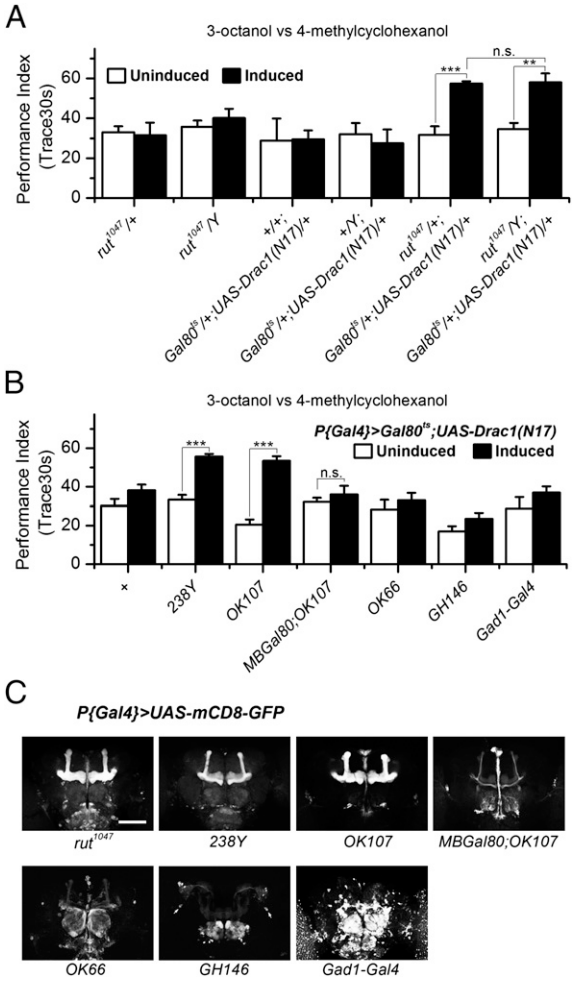

Fig. 3. Drac1(N17) expression in the mushroom body is sufficient for the enhancement. (*A*) Trace enhancement was observed when induced expression of Drac1(N17) was driven by *rut*^{1047} Gal4 (ANOVA, $P < 0.001$ for *rut*^{1047}/+, 0.01 for *rut*^{1047}/*Y*, compared with the uninduced group). No statistically significant difference was detected when the enhancement in the *rut*^{1047}/*Y* hemizygous background was compared with that in the *rut*^{1047}/+ heterozygous background (ANOVA, $P = 0.9$). Female and male flies were segregated after testing to obtain designated genotypes. $n = 4$–12. Error bars indicate SEM. (*B*) *Gal80^{ts}; UAS-Drac1(N17)* flies were crossed to wild-type flies (+) and the indicated Gal4 drivers. Trace enhancement after heat-shock induction was detected only when Drac1(N17) was expressed by the two MB Gal4s, *OK107* and *238Y* (ANOVA, $P < 0.001$). The enhancement was blocked when *OK107* was combined with *MBGal80* (ANOVA, $P = 0.45$). $n \geq 6$. Error bars indicate SEM. (*C*) Gal4 expression patterns visualized by mCD8-GFP. *rut*^{1047} has primary expression in the MB; *238Y* and *OK107* presumably label all of the MB neurons; *MBGal80* largely suppresses *OK107* expression in the MB; *OK66*, local neurons of the AL, but faint expression in the MB is also visible; *GH146*, projection neurons of the AL and APL neurons, cell bodies of APL neurons are marked with arrowhead; *Gad1-Gal4*, GABAergic neurons. (Scale bar, 100 μm.)

of a weak odor is not significantly influenced by Drac1(N17) expression.

Second, we trained flies to avoid a specific concentration of one odor, i.e., to discriminate between regular concentration and 10-fold further dilution of MCH. PID measurements show that the very low level of residual odor after a 30-s air flushing does not provide information about the initial odor concentration (Fig. S1*B*). Therefore, we reasoned that trace conditioning of intensity discrimination should not be confounded by lingering odors. Accordingly, Drac1(N17)-expressing flies significantly outperformed controls when intensity discrimination was trained in a trace conditioning procedure, but not in a simultaneous conditioning procedure (Fig. S2*B*).

Third, we trained flies with a hydrophilic odor pair, BU and EA, which are cleared more rapidly from the training tube (Fig. S1*A*). Again, Drac1(N17) expression selectively enhances trace conditioning (Fig. 2*B*). The enhancement depends on heat-shock induction (Fig. 2*B*) and is not explained by differences in olfactory acuity of the hydrophilic odors (Table S1). Because we got consistent results with the hydrophobic and hydrophilic odor pairs, in later experiments we only used the hydrophobic odor pair (OCT and MCH), which generates higher learning scores.

Mushroom Body Is a Crucial Site for Trace Conditioning. We found that the trace conditioning enhancement could be reproduced when Drac1(N17) expression was driven by the *rut*^{1047} Gal4 together with *Gal80^{ts}* (Fig. 3*A*). Notably, enhancement reaches approximately the same level in the *rut*^{1047}/*Y* hemizygote and in the *rut*^{1047}/+ heterozygote. Thus, *rut* deficiency does not compromise the superior ability of transgenic mutants in trace conditioning learning, further supporting that Rut-AC is not involved. *rut*^{1047} Gal4 preferentially labels the MB (Fig. 3*C*). The result therefore also hints that Drac1(N17) expression in the MB is sufficient for the enhancement.

We further confirmed this idea by using two pan-MB drivers, *238Y* and *OK107* (Fig. 3*C*) to induce Drac1(N17) expression specifically in the MB. Significant enhancement was observed compared with their respective uninduced control groups (Fig. 3*B*). Consistently, the enhancement associated with *OK107* is blocked (Fig. 3*B*) by the introduction of the *MBGal80* transgene (35), which specifically suppresses Gal4 activity in the MB (Fig. 3*C*). We also targeted Drac1(N17) expression to the AL with *OK66* and *GH146*, which label local neurons (36) and projection neurons (37) of the AL, respectively (Fig. 3*C*). However, no differences were observed between induced and uninduced groups (Fig. 3*B*). It is worthwhile to note that *GH146* also labels

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the anterior paired lateral (APL) neurons, a pair of GABAergic neurons that innervate the MB and suppress olfactory associative learning (38). The absence of enhancement (Fig. 3*B*) in *GH146* and additionally in GABAergic *Gad1-Gal4* (39), however, does not support a role for Drac1(N17) in the APL neurons. The mapping results thus suggest the MB as a predominant neuroanatomical locus in mediating the enhancement effect of Drac1(N17).

Dopamine Receptor in the Mushroom Body Supports Trace Conditioning. Dopaminergic neurons are believed to convey the reinforcement signal from foot shock (19, 24). Accordingly, mutants of the *D1 dopamine receptor* (*dDA1*), $dumb^1$ and $dumb^2$, show no learning of simultaneous conditioning (40). We report here that trace conditioning learning is abolished in these mutants as well (Fig. 4*A*), which suggests that trace conditioning also relies on dopamine signaling to transmit the US punishment information. Importantly, the piggyBac inserted in the first intron of the *dDA1* gene in the $dumb^2$ mutant contains UAS, which can produce functional dDA1 receptor in the presence of a Gal4 driver (40). Taking advantage of this property, we found that restoration of dDA1 expression in the MB with the *OK107* driver fully rescued the deficit of $dumb^2$ in trace conditioning (Fig. 4*B*). Moreover, the rescue effect was evident when expression was restored specifically in the adult MB (Fig. 4*C*). The localization of dDA1 function implies that in trace conditioning, the US signal is relayed to the MB, where it presumably converges with information about the now absent CS^+.

Discussion

It has been postulated that trace and delay conditioning are two fundamentally different types of learning (11). Evidence is accumulating in mammals concerning the involvement of different brain systems (3). Here, we characterized trace conditioning in the fruit fly and used mutant analyses to show that it is distinct from the well-characterized simultaneous conditioning at the molecular level. These data complement the mammalian circuit-level studies and, more importantly, open up a molecular understanding of the internal trace that the brain uses to bridge the temporal gap.

Trace Conditioning of Olfactory Aversion in *Drosophila*. Odor footshock pairing elicits robust learning in fruit flies (14). The current study adapted this assay to study trace conditioning simply by modifying the timing relationship between the CS^+ odor and the US punishment. To mimic the widely used simultaneous conditioning paradigm (15), CS^- presentation is kept at 45 s after the punishment. Single-trial training is sufficient to elicit considerable learning performance; the learning index for OCT and MCH is ~35 for trace conditioning at a trace interval of 30 s. Although a portion of the score (~10) might be attributed to attraction to the CS^- via backward conditioning (27), the behavioral results clearly indicate a marked ability of fruit flies to associate events that are temporally discrete (14, 27, 28).

Residual odor is a great concern in olfactory trace conditioning, particularly in light of the hydrophobic nature of OCT and MCH (28). We conducted careful control experiments to show that air flushing for 30 s during the trace interval is sufficient to reduce residual odors in the training tube below the threshold for fruit fly learning. In addition, the results were replicated with a pair of hydrophilic odors (BU and EA) with much faster dissipation kinetics (28). Consistent phenotypes were observed for the rut^1 mutant and Drac1(N17)-expressing flies using hydrophobic and hydrophilic odors. These experiments therefore addressed the concerns raised by the slower kinetics of hydrophobic odors. Galili et al. (28) excluded MCH from their recent trace conditioning study due to its slower dissipation. The apparent discrepancy in these results may arise from the fact that Galili et al. used a 10-times higher concentration of MCH and a trace interval of only 5 s, which together do not allow for sufficient odor decay.

Distinctive Mechanisms Support Trace and Simultaneous Conditioning. One remarkable finding of the current study is that flies devoid of Rut-AC perform normally in trace conditioning. This result is interesting in view of the belief that dually regulated adenylyl cyclase plays a central role in invertebrate associative learning (16, 41). The function of Rut-AC is best described as a molecular coincidence detector that is synergistically activated by the CS-evoked calcium entry and the US-evoked G protein-coupled receptor activation (20–22). It has been hypothesized that the stimulus-free gap in trace conditioning can be bridged by the temporal integration property of Rut-AC (21, 42). However, our results disagree with this hypothesis. The normal or even higher performance of *rut*-deficient mutants suggests that CS–US association in trace conditioning may recruit separate molecular machineries or occur in a distinct group of neurons (26, 43). Also pertinent to our study is that cAMP levels in the prefrontal cortex negatively influence working memory performance (44). Therefore, whereas cAMP signaling is essential for some learning tasks, it is dispensable or even detrimental for others (45).

Another intriguing finding is that induced expression of dominant-negative Rac enhances the learning of trace but not simultaneous conditioning. Notably, no learning enhancement was observed in a number of simultaneous conditioning variants with altered training parameters, including lowered odor concentration and conditioned intensity discrimination in the current work, as well as reduced shock pulses and lowered shock voltage in our previous report (25). Thus, the differential effects are not explained by a ceiling effect or other ancillary factors. Trace conditioning testing was performed almost immediately

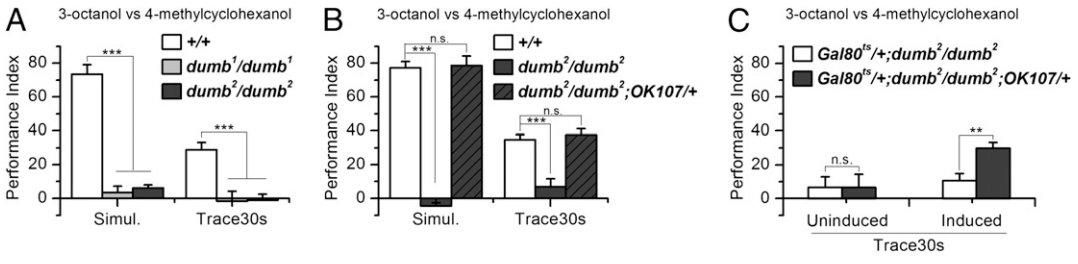

Fig. 4. Genetic lesions of *D1 dopamine receptor* abolish trace conditioning. (*A*) $dumb^1$ and $dumb^2$ mutants showed no learning in either Simul. or Trace (ANOVA, $P < 0.001$ compared with wild type). $n = 6$. Error bars indicate SEM. (*B*) Restoration of dDA1 expression in the MB ($dumb^2/dumb^2$; *OK107*/+) fully rescued the learning phenotype of $dumb^2$ mutant in both paradigms (ANOVA, $P > 0.95$ compared with wild type). $n = 4$ for Simul.; 6 for Trace30s. Error bars indicate SEM. (*C*) Adult rescue of $dumb^2$ mutant. $Gal80^{ts}$; $dumb^2/dumb^2$; *Ok107*/+ were compared with $Gal80^{ts}$; $dumb^2/dumb^2$ control that lacks the Gal4 driver. Learning performance in Trace was restored for flies subjected to 30 °C heat shock for 3 d (ANOVA, $P < 0.01$ for induced groups), but not for those kept at 18 °C (ANOVA, $P > 0.95$ for uninduced groups). $n = 6$ for uninduced; 5 for induced. Error bars indicate SEM.

(within 3 min) after the training, rendering a better retention of the acquired associative memory also unlikely. Trace conditioning becomes less efficient as trace interval increases, indicating that an inner trace of the odor gradually degrades with time. We therefore speculate that inhibition of Rac activity might preserve this transient "sensory buffer" so as to facilitate trace conditioning. In the learning of simultaneous conditioning, the co-occurrence of odor and shock makes it possible to process the CS and US information automatically, e.g., via simple convergence on coincidence detection molecules like Rut-AC; hence the requirement of an olfactory sensory buffer is superfluous, which explains the lack of enhancement from Rac inhibition. The above speculation is particularly attractive considering a recently established role of Rac in the forgetting of a cold-shock sensitive early associative memory (25). It appears that the perdurance of two short-lived memory forms, one registered after a passive olfactory experience and lasting tens of seconds and the other registered after an associative reinforcement and lasting several hours, are both sensitive to Rac signaling manipulation.

Mushroom Body May Hold a Sensory Buffer of the Odor. Drac1(N17) takes effect in the MB, the center for olfactory learning and sensory integration in insects (46). The localization of the Drac1 (N17) effect, combined with the full rescue of the *dDA1* mutant phenotype in the MB, implies a possible trace conditioning model in which the MB bridges the temporal gap by holding a short-term sensory buffer of the odor, which later converges with the reinforcement signal carried by dopamine signaling. In accordance with this model, two recent studies in fruit fly (28) and honey bee (47) found no correlation between trace conditioning behavior and the postodor calcium response patterns in olfactory sensory neurons and projection neurons of the AL. Both studies pointed out the likelihood that the sensory buffer relevant to trace conditioning is in neurons downstream of the AL, most likely in the MB. Nonetheless, the AL may still retain odor information in biochemical signals other than calcium or in short-term synaptic plasticity (48, 49). The rapidly evolving molecular imaging techniques in fruit flies (50) may help to delineate the nature of the putative sensory buffer and how it interacts later with a biologically significant stimulus.

Another remaining puzzle is that both simultaneous and trace conditioning, although recruiting different molecular mechanisms, rely on the MB as a mutual crucial site. This seems at variance with the view from mammalian studies, where trace conditioning recruits neural circuits distinct from delay conditioning. Species or paradigm differences might explain the discrepancy, but it awaits to be fully addressed by future studies exploring whether brain regions outside the MB are additionally engaged in trace conditioning in fruit flies and, more importantly, whether various MB subdivisions (51) contribute differentially to these two conditioning variants.

Materials and Methods

Fly Stocks. *rut*^{1} and *rut*^{1}*; UAS-rut* were gifts from Josh Dubnau (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY); *Gad1-Gal4* from Liqun Luo (Stanford University, Stanford, CA); and *dumb*^{1} and *dumb*^{2} from Kyung-An Han (University of Texas at El Paso, El Paso, TX). All of the other strains were extant stock in the laboratory and were described in a previous paper (25). We were not able to obtain flies homozygous for both *dumb*^{2} and *OK107*. In the *dumb*^{2} rescue experiment, *dumb*^{2}*/dumb*^{2}*; OK107/+* were crossed to *dumb*^{2}*/dumb*^{2} or *Gal80*^{ts}*/Gal80*^{ts}*; dumb*^{2}*/dumb*^{2}. Progeny genotypes were easily segregated after behavioral experiments on the basis of the bright red eye color of flies bearing OK107 driver.

Behavioral Assays. Pavlovian conditioning of odor avoidance response was performed in a controlled environment room of 25 °C and 70% relative humidity as described (15).

During training, around 100 flies were loaded into a training tube covered with copper grid. Odors (Sigma-Aldrich/Fluka) were dissolved in heavy mineral oil (Fisher Scientific) and brought to the training tube by a moisturized air current bubbling through the odor vials at 750 mL/min. Normally, the dilution in (vol/vol) was: 1.5×10^{-3} for OCT, 1×10^{-3} for MCH, 2×10^{-3} for BU, and 2×10^{-3} for EA; further dilutions from these starting concentrations were indicated in some experiments. The odor source of BU and EA was replenished after every 8 min of odor presentation considering their faster run down. With this procedure, no apparent effects on behavioral scores were observed. In simultaneous conditioning, the two odors were presented to flies sequentially; each lasted for 60 s and was followed by flushing of fresh air for 45 s. The US (twelve 1.5-s pulses of 60 V electric foot shock at 5-s interpulse intervals) was present during the delivery of the first odor (CS^+) but not the second (CS^-). Trace conditioning differed from simultaneous conditioning only in the temporal relationship between CS^+ and US, i.e., CS^+ preceded US but a no-odor interval separated the offset of CS^+ and onset of US. CS^- delivery was maintained at 45 s following US. Odorless clean air bubbling through heavy mineral oil was delivered at 750 mL/min whenever there was no odor delivery. It is estimated that the training tube (inner volume of ~15 mL) was refreshed every 1.2 s.

To assay for learning performance, flies were allowed to choose between CS^+ and CS^- in a T maze for 120 s immediately after the training. Performance index (PI) was calculated (15) as the fraction of flies avoiding CS^+ minus the fraction of flies avoiding CS^-. To eliminate odor bias, each PI was averaged over two reciprocally trained groups, e.g., one associating shock with OCT, the other with MCH. PI was normalized to a range of 0–100, with 0 indicating no learning and 100 indicating perfect learning.

Statistics. The data are shown as means ± SEM and analyzed by ANOVA followed by Bonferroni's post hoc test in Origin 8.0 software. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; NS, nonsignificance ($P > 0.05$).

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