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Forgetting Is Regulated through Rac Activity in *Drosophila*

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SUMMARY

Initially acquired memory dissipates rapidly if not consolidated. Such memory decay is thought to result either from the inherently labile nature of newly acquired memories or from interference by subsequently attained information. Here we report that a small G protein Rac-dependent forgetting mechanism contributes to both passive memory decay and interference-induced forgetting in *Drosophila*. Inhibition of Rac activity leads to slower decay of early memory, extending it from a few hours to more than one day, and to blockade of interference-induced forgetting. Conversely, elevated Rac activity in mushroom body neurons accelerates memory decay. This forgetting mechanism does not affect memory acquisition and is independent of Rutabaga adenylyl cyclase-mediated memory formation mechanisms. Endogenous Rac activation is evoked on different time scales during gradual memory loss in passive decay and during acute memory removal in reversal learning. We suggest that Rac's role in actin cytoskeleton remodeling may contribute to memory erasure.

INTRODUCTION

Initially acquired memory is vulnerable to forgetting. Traditionally, two psychological concepts, usually placed in opposition, have been raised to account for forgetting: decay and interference (Jonides et al., 2008; Wixted, 2004). The former holds that memory simply evaporates with time, whereas the later claims that forgetting principally arises from loading of irrelevant information. With the nature of the underlying process remaining unspecified, the decay and interference explanations of forgetting are under continuous debate (for recent debate, see Altmann, 2009; Lewandowsky et al., 2009). In recent years, molecular genetic approaches have led to the identification of a cohort of key memory molecules, inspiring theoretical explanations of numerous basic memory phenomena, such as coincidence detection (Bourne and Nicoll, 1993), consolidation (Kandel, 2001), memory allocation (Han et al., 2007), and spacing effect (Pagani et al., 2009). However, efforts to understand the molecular basis of early memory forgetting have long been overlooked, presumably due to the pervasive notion that early labile memory is dependent upon phosphorylation of pre-existing molecules by a variety of kinases (Kandel, 2001; Micheau and Riedel, 1999) and that such modification will be reversed passively by basal activities of cellular phosphatases (Genoux et al., 2002; Mansuy, 2003). Thus a dedicated mechanism for removing early memory may not exist.

However from a theoretical point of view it has long been speculated that there are adaptive benefits of a forgetting strategy that can respond to the environmental information (Anderson and Schooler, 1991; Bjork, 1989; Kraemer and Golding, 1997). For instance, when the biological significance of the acquired memory is decreased after an extended period of "disuse," or when the existing memory is inconsistent with current circumstances and thus might harm an individual's survival, the forgetting process may function to remove the unnecessary or inappropriate memory. On the basis of this notion, we launched an effort to identify *Drosophila* mutants of enhanced early memory with the expectation that such enhancement might result from a defect in forgetting. In analyzing these mutants (unpublished data), the effects of Rac-signaling relevant genes attracted our attention and prompted our study of Rac's role in forgetting.

Pavlovian olfactory aversive conditioning has been extensively characterized in *Drosophila* (Tully and Quinn, 1985). Single-session training yields a memory retention curve consisting of rapid forgetting of the labile early memory, including mainly short-term memory (STM) and mid-term memory (MTM), and a gradual appearance of a longer-lasting component, anesthesia-resistant memory (ARM). The early memory disappears within a few hours, leaving ARM the only memory component lasting over 1 day (DeZazzo and Tully, 1995). In addition to ARM, there exists another consolidated memory form, protein-synthesis-dependent long-term memory (LTM), which is elicited only with repetitive spaced training and lasts for at least a week (Tully et al., 1994). The present study focuses on one-session training-induced labile early memory and reveals that this component can be prolonged to more than 1 day by interfering with the functions of Rac.

Rac belongs to the Rho family GTPases. This family of small G proteins act as key regulators of cytoskeleton dynamics as well as other cellular processes by switching between GTP-bound active forms and GDP-bound inactive forms (Etienne-Manneville and Hall, 2002). They have been extensively studied in neuronal

development and activity-dependent structural plasticity where cytoskeleton remodeling is acutely required (Luo, 2000; Van Aelst and Cline, 2004). Their physiological roles in mature nervous systems, however, are much less well-defined. A major obstacle in approaching this question is attributed to the deleterious effects caused by perturbing their activities throughout development (Johndrow et al., 2004; Wang and Zheng, 2007). However conditional expression of dominant mutants can circumvent the developmental defects and thus serves as the preferred experimental strategy. With the genetic tools accessible to *Drosophila*, we demonstrate that Rac activity is critically involved in active regulation of early memory forgetting.

RESULTS

Two dominant Rac mutant proteins with amino acid substitution have been successfully used to characterize physiological functions of Rac in *Drosophila* (Luo et al., 1994). The dominant-negative N17 mutant (T17N) inhibits endogenous Rac activity by competing for an upstream activator, whereas the constitutively active V12 mutant (G12V) renders Rac persistently active as a consequence of its abolished intrinsic GTPase activity. Tissue-specific expression of transgenes encoding dominant mutants of *Drosophila* Rac1 (Drac1) was obtained through the Gal4/UAS binary system (Brand and Perrimon, 1993) whereas the temporal control of adult-onset expression was achieved by integration with *tubulin-Gal80*^{ts} (*Gal80*^{ts}), which encodes a ubiquitously expressed temperature-sensitive Gal80 protein that suppresses Gal4-induced expression at the permissive temperature (18°C) but not at the restrictive temperature (30°C) (McGuire et al., 2003). The specificity of expression was verified by coupling with a GFP reporter, which produced a pattern (Figure S1A available online) consistent with that reported previously (McGuire et al., 2003).

Inhibition of Rac Activity Slows down Memory Decay

To probe the effects of Rac inhibition, dominant-negative Drac1(N17) was first expressed by a pan-neuronal *elav-Gal4* driver (Lin and Goodman, 1994) in combination with *Gal80*^{ts}. Crosses were reared at the permissive temperature (18°C). two- to four-day-old progeny were collected and exposed to 30°C for 3 days to induce the expression of Drac1(N17), which was verified by immunoblotting (Figure S1D). To evaluate behavioral effects, these Drac1(N17)-expressing adults were subjected to Pavlovian olfactory aversive conditioning (see Experimental Procedures) at 25°C along with similarly treated parental controls.

We compared retention curves at various time points after one-session training (Figure 1A). Drac1(N17)-expressing flies (*elav-Gal4/+; Gal80*^{ts}*/+; UAS-Drac1(N17)/+*) exhibited normal memory in the first 30 min after training (at 3, 15, and 30 min) but showed significantly slower memory decay at later time points from 2 hr up to 24 hr.

The normal performance in the first 30 min implies that the observed slower memory decay is not likely a result of strengthened acquisition of the initial memory. To further distinguish between a role of Rac in memory decay and in initial acquisition, we performed three additional experiments.

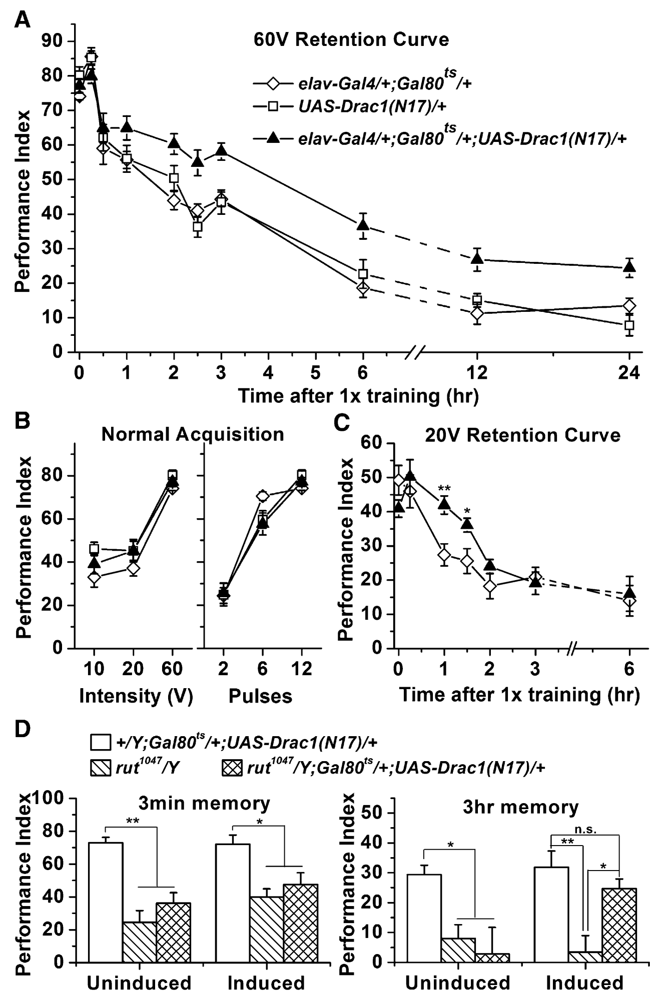

Figure 1. Normal Acquisition but Slower Memory Decay in Drac1(N17)-Expressing Flies

For induction of Drac1(N17) expression, flies received heat shock at 30°C for 3 days before Pavlovian conditioning.

(A) Retention curves were generated by testing conditioned odor avoidance at various time points after one-session training. Drac1(N17)-expressing flies (*elav-Gal4/+; Gal80*^{ts}*/+; UAS-Drac1(N17)/+*) displayed normal memory performance shortly after training (ANOVA, $p > 0.2$ for time points up to 1 hr) but slower memory decay thereafter (ANOVA, p = 0.006, 0.02, 0.002, 0.009, 0.002, 0.02 compared to *elav-Gal4/+; Gal80*^{ts}/+, 0.12, 0.002, 0.002, 0.046, 0.02, 0.0002 compared to *UAS-Drac1(N17)/+* for 2 hr, 2.5 hr, 3 hr, 6 hr, 12 hr, 24 hr, respectively). n = 6–16, means ± SEM.

(B) Immediate memory performance after one-session training with varied electric shock intensities (left) or number of electric shock pulses (right). n = 6–7, means ± SEM.

(C) Retention curves after weak training with 20 V electric shock (ANOVA, p = 0.008 for 1 hr, 0.02 for 1.5 hr). n = 5–10, means ± SEM.

(D) Induced expression of Drac1(N17) failed to reverse the immediate (3 min) memory defect of *rut*^{1047} mutant but significantly improved its 3 hr memory retention. Statistical significance (*$p < 0.05$; **$p < 0.01$) or nonsignificance (n.s.) is indicated. n = 6–12, means ± SEM.

See also Figure S1 and Table S1.

First, acquisition curves were examined for each genotype (Figure 1B) by plotting immediate (3 min) memory as a function of training intensity (the intensity of electric shock, 10 V, 20 V,

and 60 V; the number of shock pulses, 2, 6, and 12; see Experimental Procedures). Consistent with the idea of normal acquisition, no statistically significant differences were found between Drac1(N17)-expressing flies and controls. Second, to exclude the possibility that the slower memory decay was a consequence of ceiling effects in initial acquisition, we examined retention curves after 20 V training (Figure 1C) wherein the initial memory was acquired at a lower level (Performance Index [PI] = ∼45 versus ∼75 in regular 60 V training). Retention curves after this weak training showed much faster decay kinetics that were understandably different from those found for regular training. For Drac1(N17)-expressing flies, slower memory decay was still observed at the time points of 1 hr and 1.5 hr, but not after 2 hr. The quality of the initial memory acquired from the weaker training paradigm might contribute to the inability to observe differences at later time points. For this reason, we conducted the third experiment, in which we examined Drac1(N17)-dependent memory enhancement in a learning mutant that showed a lower acquisition level with regular training intensity. *rutabaga* (*rut*) was chosen for this purpose because *rut*-encoded adenylyl cyclase is considered as the coincidence detector in associative learning (Davis, 2005). First, we isolated the P{Gal4} line rut^{1047} as a mutant of *rut* (see Figures S1E–S1H for details). Induced expression of Drac1(N17) by rut^{1047}, which labels all the mushroom body (MB) lobes and several other brain regions (Figures S1G and S1H), did not affect initial acquisition defect associated with *rut* mutation (Figure 1D; PI = 48 ± 7 for induced group of rut^{1047}/Y; $Gal80^{ts}$/+; *UAS-Drac1(N17)*/+, as compared to 72 ± 6 for +/Y; $Gal80^{ts}$/+; *UAS-Drac1(N17)*/+ control). However, 3 hr memory was strongly enhanced in the *rut* mutant with induced expression of Drac1(N17) (Figure 1D; PI = 25 ± 3 for induced group of rut^{1047}/Y; $Gal80^{ts}$/+; *UAS-Drac1(N17)*/+, as compared to 3 ± 6 for rut^{1047}/Y alone). This result not only confirms that Rac does not affect initial acquisition but also suggests that mechanisms underlying memory decay are at least in part independent of the Rut-mediated mechanisms for memory formation.

Task-relevant sensorimotor responses were not significantly altered by Drac1(N17) expression (Table S1). Moreover, as genetic background controls, *elav-Gal4*/+; $Gal80^{ts}$/+; *UAS-Drac1(N17)*/+ flies without heat shock induction showed normal learning and memory performance (Figure S1I).

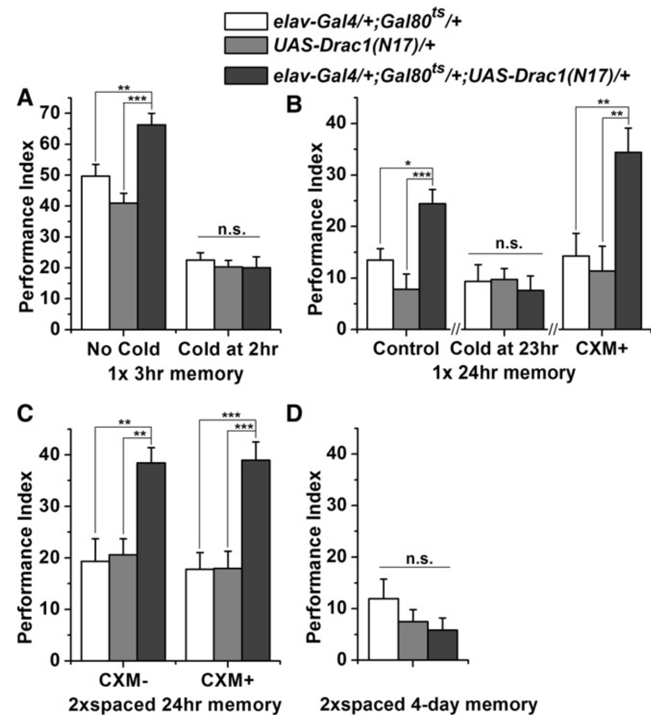

Figure 2. Feature Analysis of the Prolonged Memory in Drac1(N17)-Expressing Flies

(A) Three hour memory after one-session training. The differences between Drac1(N17)-expressing flies and controls were eliminated by cold-amnesia treatment at 2 hr (Cold at 2 hr, ANOVA, p > 0.95). n = 10, means ± SEM.

(B) Twenty-four hour memory after one-session training. The elevated performance of Drac1(N17)-expressing flies was blocked by a cold-amnesia treatment at 23 hr (Cold at 23 hr, ANOVA, p > 0.95) but not by feeding flies with a protein synthesis inhibitor, cycloheximide (CXM+, ANOVA, p < 0.01). n = 6–16, means ± SEM.

(C) Twenty-four hour memory after two-session spaced training. Drac1(N17)-expressing flies showed memory retention remarkably higher than controls, irrespective of CXM feeding or not (ANOVA, p < 0.001 and 0.01 for the CXM+ and CXM− groups, respectively). n = 17 or 9, means ± SEM.

(D) Four day memory after two-session spaced training. The performance of Drac1(N17)-expressing flies was not significantly different from controls (ANOVA, p > 0.4). n = 8, means ± SEM.

See also Figure S2.

The Prolonged Memory Is Distinct from Known Consolidated Memory Components

Next, we attempted to determine features of the prolonged memory in Drac1(N17)-expressing flies, with a particular interest in examining whether it resembled the two well-characterized consolidated memory forms that can last more than 1 day: ARM and LTM (DeZazzo and Tully, 1995). We first applied a cold shock amnesia treatment (Quinn and Dudai, 1976), which should disrupt anesthesia-sensitive labile early memory forms but not ARM. Such treatment applied at 2 hr (Figure 2A) or even at 23 hr (Figure 2B) after one-session training abolished the elevated memory performance in Drac1(N17)-expressing flies, indicating that the prolonged memory is very different from ARM. We then fed flies with the protein synthesis inhibitor cycloheximide (CXM) (Tully et al., 1994). CXM feeding, although impeding LTM formation in control flies (Figure S2), had no discernable effects on blocking the enhanced 24 hr memory performance observed in Drac1(N17)-expressing flies after one-session training (Figure 2B), or two-session spaced training (Figure 2C). Thus, the prolonged memory is independent of protein synthesis and thereby does not resemble LTM. This conclusion is further supported by the observation that the prolongation effect lasted less than 4 days (Figure 2D).

Taken together, the prolonged memory observed in Drac1(N17)-expressing flies stands apart from the known consolidated memory forms in *Drosophila*. Although lasting over 1 day, it retains the fragile and protein-synthesis-independent features of early memory. Thus, one likely possibility is that Rac inhibition preserves memory by hampering an endogenous process required for memory decay or forgetting.

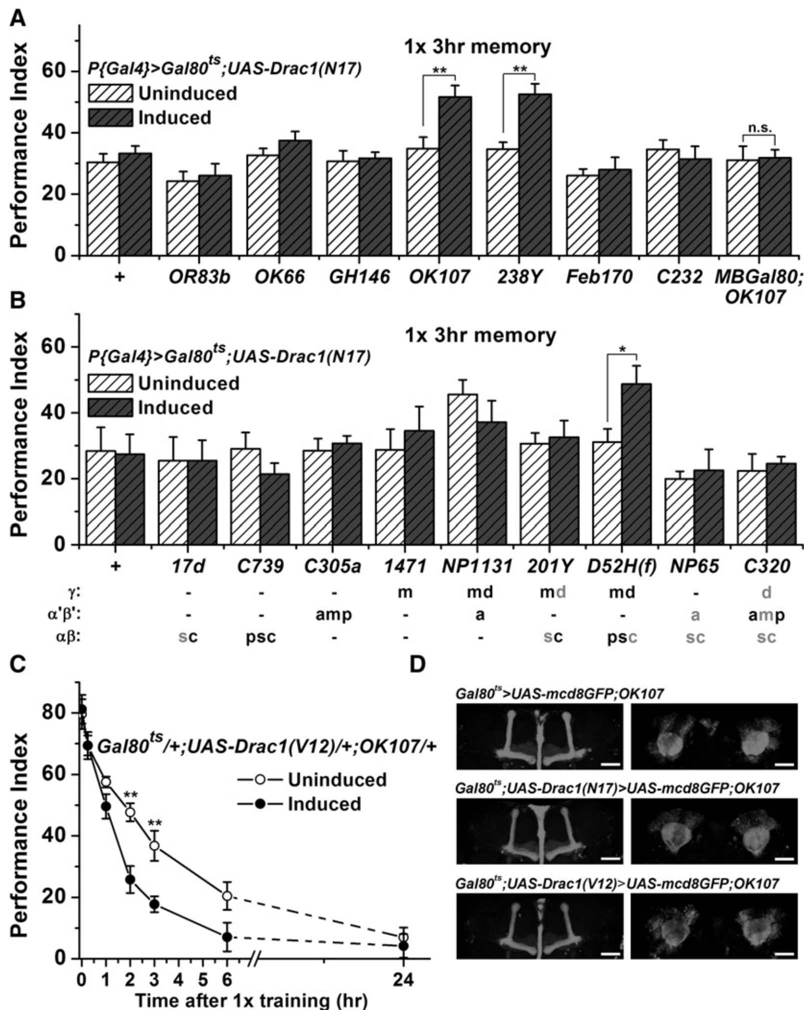

Figure 3. Bidirectional Regulation of Memory Decay by Rac in the Mushroom Body

(A) *Gal80^{ts}; UAS-Drac1(N17)* flies were crossed to wild-type flies (+) and the indicated Gal4 drivers. Three hour memory enhancement after heat shock induction was detected only when Drac1(N17) was expressed by the two strong MB Gal4s, *OK107* and *238Y* (ANOVA, $p < 0.01$ for both Gal4s). No effect on 3 hr memory was found when Drac1(N17) was expressed using *OK107* combined with *MBGal80*, which specifically inhibits Gal4 activity in the MB. n = 6–10, means ± SEM.

(B) *Gal80^{ts}; UAS-Drac1(N17)* flies were crossed with several subtype-specific MB-Gal4s (Aso et al., 2009). Effects of heat shock induction on 3 hr memory were examined as above. For the X chromosome-located *D52H*, only female results are shown. Statistically significant differences between the induced and uninduced groups were only found with *D52H(f)* (ANOVA, $p < 0.05$). n = 5–10, means ± SEM.

Further subdivisions of lobes: c, core; s, surface; p, posterior; a, anterior; m, middle; d, dorsal. Gray indicates relatively weak expression.

(C) The induced group of *Gal80^{ts}/+; UAS-Drac1(V12)/+; OK107/+* showed accelerated memory decay compared to the corresponding uninduced control (ANOVA, p = 0.82, 0.99, 0.1, 0.002, 0.002, 0.07, 0.6 for 3 min, 15 min, 1 hr, 2 hr, 3 hr, 6 hr, 24 hr, respectively). n = 6–12, means ± SEM.

(D) Gross morphologies of the MB (left, lobe; right, calyx) were normal after induced expression of Drac1(N17) or Drac1(V12). Three to six adult brains were examined for each genotype. Scale bar is 50 μm.

See also Figure S3 and Table S1.

Involvement of the Mushroom Body in Slowing down Memory Decay

Immunohistochemical analysis with an antibody against human Rac1 detected widely distributed immunosignals in the adult brain (Figures S3A–S3E). This widespread pattern likely reflects principally the expression of *Drac1*, as the immunosignals were decreased in a hypomorphic mutant of *Drac1* (Figure S3F) but not in null mutants of *Drac2* and *Mtl* (data not shown).

To determine where in the brain Rac functions were required for regulation of memory decay, several Gal4 lines (Figure S3G), in combination with *Gal80^{ts}*, were utilized to drive acute local expression of Drac1(N17). Enhanced 3 hr memory performance (Figure 3A) was observed only when Drac1(N17) expression was driven by *OK107* and *238Y*, two Gal4 drivers preferentially expressed in all MB neurons (Aso et al., 2009). Enhancement was not evident (Figure 3A) when expression was targeted to olfactory sensory neurons (*OR83b*), local and projection neurons of the antennal lobe (*OK66* and *GH146*), or ellipsoid body of the central complex (*Feb170* and *C232*). This MB dependence is further supported by the observation that the enhancement disappeared (Figure 3A) when *OK107*-driven expression of Drac1(N17) in the MB was suppressed by *MBGal80* (Krashes et al., 2007). Thus, the suppressive effect of Drac1(N17) on memory decay likely occurs in the MB, which is consistent with a central role of the MB in Pavlovian olfactory memory (Davis, 2005; Heisenberg, 2003; Margulies et al., 2005).

The MB intrinsic neurons can be further classified into three major subtypes with axonal projections in different lobes: following their birth order, the γ, $\alpha'\beta'$, and $\alpha\beta$ neurons (Crittenden et al., 1998; Lee et al., 1999). Recent studies have increasingly emphasized the distinct roles of different MB subtypes during memory processes (Keene and Waddell, 2007). Thus, to further clarify the involvement of different MB neurons in the suppressive effect of Drac1(N17) on memory decay, we surveyed more MB-Gal4s, including *17d* ($\alpha\beta$), *C739* ($\alpha\beta$), *C305a* ($\alpha'\beta'$), *1471* (γ), *NP1131* ($\gamma+\alpha'\beta'$), *201Y* ($\gamma+\boldsymbol{\alpha\beta}$), *D52H(f)* ($\gamma+\alpha\beta$), *NP65* ($\boldsymbol{\alpha'\beta'+\alpha\beta}$), and *C320* ($\gamma+\boldsymbol{\alpha'\beta'+\alpha\beta}$) (Figure 3B; bolding indicates weak expression; f represents female; for characterization of their expression patterns, see Aso et al., 2009). Among these additional nine MB-Gal4s examined (Figure 3B), memory enhancement was observed only with *D52H(f)*, which strongly labels the γ and $\alpha\beta$ neurons but not the $\alpha'\beta'$ neurons (around 2700 out of 4000 MB neurons; see Aso et al., 2009). Expression of Drac1(N17) in a smaller fraction of $\gamma+\alpha\beta$ neurons (*201Y*), strongly in most of $\alpha\beta$ neurons (*C739*), or strongly in most of γ neurons (*NP1131*), had no detectable effects on 3 hr memory. Thus, Rac likely functions in a large population of $\gamma+\alpha\beta$ MB neurons to regulate early memory decay.

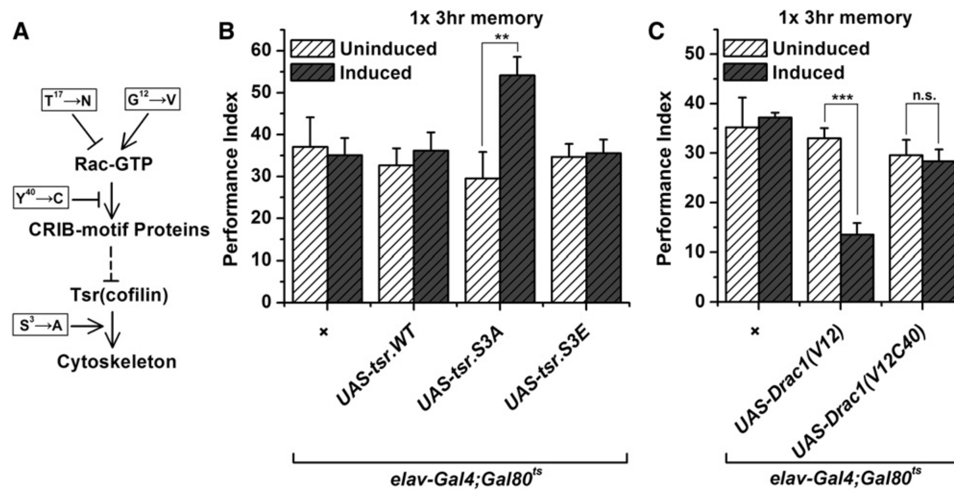

Figure 4. Phenotypic Characterization of Rac Downstream Signals

(A) Rac can signal through cofilin to regulate actin cytoskeleton remodeling.

(B) Three hour memory was significantly improved with neuronally induced expression of persistently active cofilin (Tsr.S3A) (ANOVA, $p < 0.01$ compared to uninduced group) but not wild-type (Tsr.WT) or inactive cofilin (Tsr.S3E). n = 6–7, means ± SEM.

(C) Three hour memory was diminished with neuronally induced expression of Drac1(V12) (ANOVA, $p < 0.001$) but not the double mutant Drac1(V12C40) (ANOVA, $p > 0.7$). n = 6–8, means ± SEM.

See also Figure S4 for additional data to address specificity.

Increased Rac Activity Accelerates Memory Decay

Given that Rac inhibition resulted in slower memory decay, we expected that increased Rac activity might hasten memory decline. Indeed, heat-shock-induced expression of constitutively active Drac1(V12) in the adult MB (*Gal80^{ts}/+; UAS-Drac1(V12)/+; OK107/+*) led to accelerated memory decay as compared to the uninduced control (Figure 3C). Notably and in accordance with those observed in Drac1(N17)-expressing flies, the immediate memory at 3 and 15 min after training was not affected. The observed memory decline was specific to Drac1(V12) expression, since heat shock treatment did not accelerate memory loss in parental controls (Figure S3H). In addition, sensorimotor responses (Table S1) and MB gross morphologies (Figure 3D) were not altered by Drac1(V12) expression. In conclusion, memory decay can be bidirectionally regulated through genetic manipulation of Rac activity in the adult MB neurons.

Slower Memory Decay in Rac Downstream Target Cofilin Mutant

To verify the relevance of Rac to the observed phenotypes, we tested the effects of genetic perturbation of Rac downstream components. Cofilin, a potent actin depolymerizing factor (Bamburg, 1999), is known to play a crucial role in mediating the cytoskeleton remodeling activity of Rac. In one of the well-established pathways (Figure 4A), Rac activity triggers sequential activation of PAK and LIMK, which in turn phosphorylates cofilin at Ser3 and inhibits its actin depolymerization activity (Arber et al., 1998; Edwards et al., 1999; Yang et al., 1998). *twinstar* (*tsr*) encodes the *Drosophila* homolog of cofilin (Gunsalus et al., 1995). Here, we utilized two Tsr point mutations, with the nonphosphorylatable Tsr.S3A being persistently active and the phosphorylation-mimicking Tsr.S3E being inactive (Ng and Luo, 2004). Neuronal expression of Tsr.S3A, but not Tsr.S3E or wild-type Tsr, significantly enhanced 3 hr memory performance (Figure 4B). The observation that cofilin hyperactivation gives rise to the same phenotype as seen with Rac inhibition argues that the above findings in dominant Rac mutants are not likely consequences of nonspecific effects.

To further demonstrate specificity, we tested a double mutant variant of Rac, Drac1(V12C40) (Kim et al., 2003). As a result of the effector loop mutation Y40C (Joneson et al., 1996; Lamarche et al., 1996; Ng et al., 2002), this constitutively active mutant loses the ability to bind with PAK as well as other Cdc42/Rac1 interactive-binding (CRIB)-motif effector proteins and is therefore unable to inhibit cofilin through the PAK/LIMK pathway. In contrast with intact Drac1(V12), expression of Drac1(V12C40) did not accelerate memory decay (Figure 4C). Therefore, the Rac-regulated PAK/LIMK/cofilin pathway might be critical in influencing memory decay.

Suppression of Interference-Induced Forgetting in Drac1(N17)-Expressing Flies

Thus far, we have described the effects of Rac on passive memory decay. In this section, we describe experiments in which we attempted to test interference-induced forgetting, which has been historically viewed as a major cause of forgetting (Jonides et al., 2008; Wixted, 2004). In our interference learning paradigm (Figure 5A; see also Experimental Procedures), retroactive interference was introduced at 1.5 hr after the initial learning by training flies to acquire a novel odor-electric shock association (Figure S5). The choice of 1.5 hr parallels the time course of Rac's effects on memory decay (Figures 1A and 3C). Consequences of interference were evaluated by assaying 3 hr memory retention of the prior learning. As shown in Figure 5, interference learning consistently caused memory decline in control

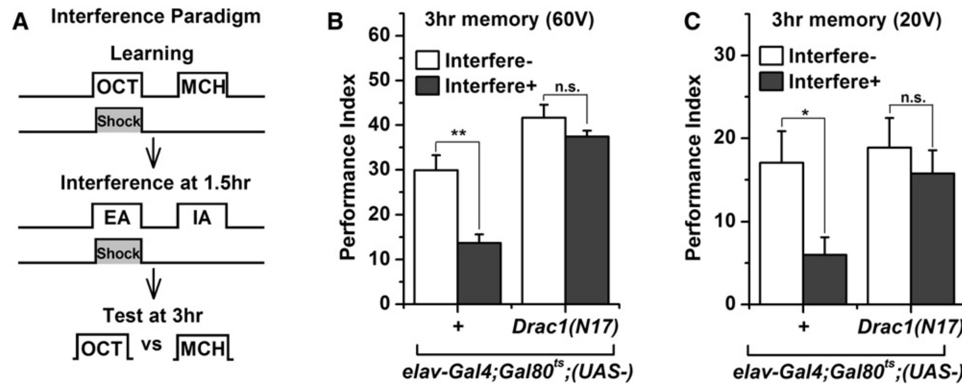

Figure 5. Suppression of Interference-Induced Memory Loss in Drac1(N17)-Expressing Flies

(A) Interference effects of new learning (EA/IA) on 3 hr memory retention of the prior learning (OCT/MCH) were tested.

(B and C) Retention of the prior memory, either strong (B) or weak (C), was attenuated by interference learning in control flies (ANOVA, $p < 0.05$) but not in Drac1(N17)-expressing flies (ANOVA, $p > 0.2$). n = 6 or 8, means ± SEM.

See also Figure S5.

but had no apparent effects in Drac1(N17)-expressing flies. Thus, besides passive memory decay, interference-induced forgetting is also suppressed by inhibition of Rac activity.

Rac-Dependent Forgetting in Reversal Learning

The passive memory decay and interference learning we have described above take a time course of several hours for removal of the "disused" memories. To determine whether the same mechanism might be recruited within a much shorter timescale to eliminate "inappropriate" memories in a changing behavioral context, we employed a reversal learning paradigm (Quinn et al., 1974; Tully et al., 1990), in which the odor-electric shock contingency was reversed in each training session (Figure 6A; see also Experimental Procedures).

Take Reversal × 1 as an example. It consisted of two training sessions with reciprocal odor paired with punishment (Figure 6A). On testing, flies were given a choice between the two reversely trained odors, OCT and MCH. Notably, it takes less than 15 min to finish the experiment, during which no obvious passive memory decay shall occur (see Figure 1A). Thus flies after training, theoretically, shall display no bias in the testing choice because aversive memories to the two trained odors are equally strong. Nevertheless, the actual data showed that trained flies selectively avoided the odor most recently punished, i.e., the one paired with shock in the last session (Figure 6B; see also Experimental Procedures for calculation of reversal learning PI).

We reasoned that earlier acquired conflicting aversive memory was likely to be removed during reversal learning. On the basis of this notion, it was expected that inhibition of forgetting would make the two trained odors equally aversive and therefore significantly reduce reversal learning performance. Toward this end, we showed that Drac1(N17)-expressing flies had a performance level that was significantly lower than those in control flies and close to zero at the second reversal (Figure 6B). Conversely, flies with acute expression of Drac1(V12) in the adult MB displayed an elevated performance level in reversal learning (Figure 6C). These results thus suggest that the Rac-regulated forgetting mechanism might be activated and contribute to the removal of earlier acquired inappropriate memory in reversal learning.

To demonstrate the effect of impeded forgetting more directly, we conducted a third-odor test of reversal learning, wherein flies were given a choice between one of the trained odors, OCT, and a previously unexposed odor, benzaldehyde (Ben) (Figure 6D; see also Experimental Procedures). As expected, when OCT was paired with electric shock in the initial session (Learning), strong avoidance of OCT over Ben was obtained for all the groups (Figure 6E). At the first reversal (Reversal × 1), the avoidance was reduced dramatically in control flies (Figure 6E; see *elav-Gal4*/+*; Gal80*^{ts}/+ and uninduced group of *elav-Gal4*/+*; Gal80*^{ts}/+*; UAS-Drac1(N17)*/+), suggesting that the aversive memory to OCT was weakened or removed in response to the change in odor-electric shock contingency. Further, at the second reversal (Reversal × 2) when the pairing relationship between OCT and electric shock was restored, these control flies showed strong avoidance of OCT again (Figure 6E). However, for Drac1(N17)-expressing flies, OCT avoidance was maintained at a similar level through Learning, Reversal × 1, and Reversal × 2 (Figure 6E; see induced group of *elav-Gal4*/+*; Gal80*^{ts}/+*; UAS-Drac1(N17)*/+). Such data are consistent with the idea that the aversive memory to OCT is unable to be removed as a result of impeded forgetting.

Training-Evoked Rac Activity in Correlation with Forgetting

Given the above behavioral phenotypes, we expected that endogenous Rac might be activated with a temporal pattern paralleling behavioral changes. Therefore, we sought to monitor training-induced Rac activation by measuring relative levels of Rac-GTP through the PBD pull-down assay of whole head extracts (see Experimental Procedures). To confirm the sensitivity of the assay, we showed that Rac activation was readily detected in response to acute expression of Drac1(V12) in the MB neurons (driven by OK107; Figure 7A).

We first examined Rac activation in association with acute memory removal in reversal learning. There were two groups of

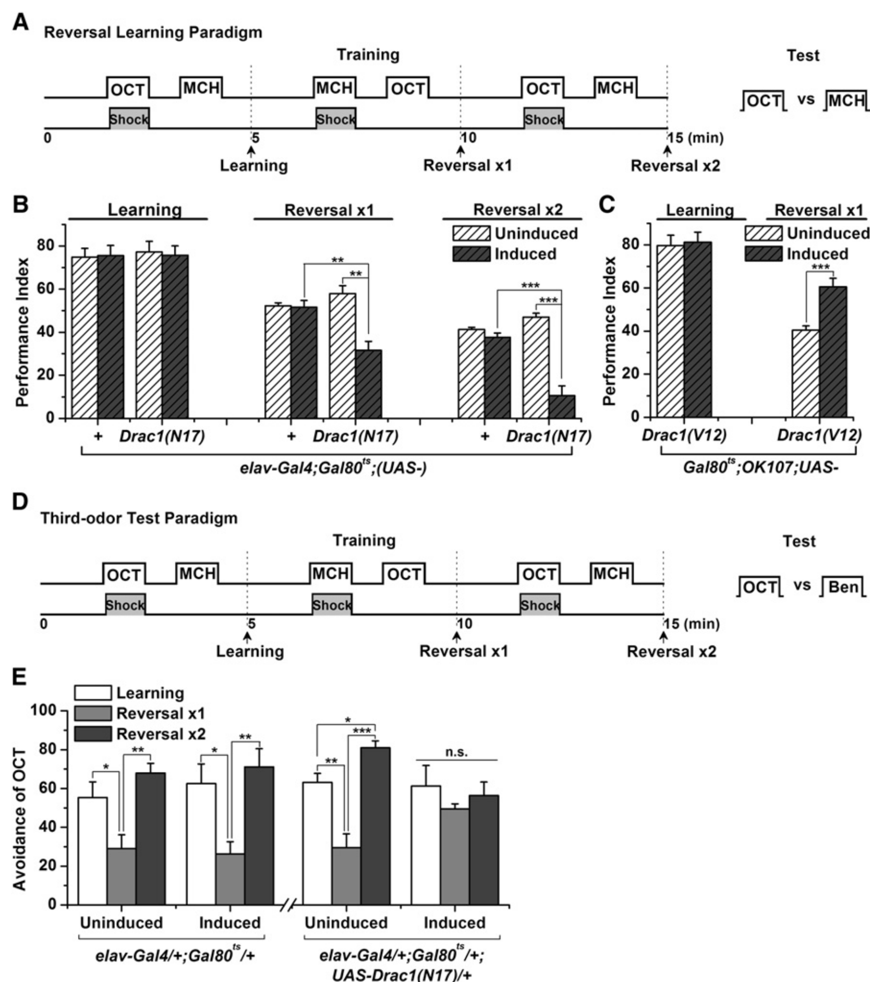

Figure 6. Performance in Reversal Learning Is Altered by Expression of Drac1(N17) and Drac1(V12)

(A) In reversal learning, pairing relationships between electric shock and the two trained odors (OCT/MCH) were reversed in every training session.

(B) In spite of what they might have learned in the previous training sessions, flies tended to avoid the odor paired with punishment most recently. However, the performance of Drac1(N17)-expressing flies in reversal learning was significantly worse than controls (ANOVA, $p < 0.01$ for "Reversal × 1," $p < 0.001$ for "Reversal × 2" compared to the uninduced control). n = 6, except 4 for "Learning," means ± SEM.

(C) Conversely, the reversal learning performance of flies expressing Drac1(V12) in the adult MB is superior to the corresponding uninduced control (ANOVA, $p < 0.001$). n = 6–8, means ± SEM.

(D) Instead of a choice between the two trained odors, OCT versus MCH, flies in third-odor test were given a choice between OCT and a previously unexposed odor, Ben.

(E) Avoidance of OCT in the course of reversal learning is shown for Drac1(N17)-expressing flies and *elav-Gal4/+; Gal80*^{ts}/+ control. Avoidance scores after different training experience were compared and analyzed by ANOVA. Statistical significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) or nonsignificance (n.s.) is indicated. n = 6–8, means ± SEM.

wild-type flies: one received an increasing number of reversal learning sessions whereas the other received the same number of repetitive learning sessions. As shown in Figure 7B, Rac activity was remarkably elevated in reversal learning as expected. By contrast, a progressive decrement was observed in the repetitive learning group, which experienced the equal levels of sensory input and training intensity but not the odor-electric shock contingency reversal. The later observation was not initially expected, particularly with respect to the decrement of up to 50% that of naive group after "Learning × 3." The extent of change was surprisingly large if we took into account the behavioral mapping of Rac functions to the MB neurons (even with the fact of a large proportion; Figure 3). A putative assumption that the MB harbors the majority of Rac activity out of the whole brain during resting state might help to explain the bidirectional changes. Alternatively, there also exists the possibility that the observed changes encompass other neurons undergoing Rac activity regulation but are not intimately related to behavioral output. Regardless of the mechanism, the suppression of Rac activity in repetitive learning is not inconsistent with Rac's putative role in forgetting, as it is quite likely that forgetting inhibition is one of the multiple mechanisms recruited to obtain a stronger memory after repetitive learning. Thus, an increase in Rac activity correlates with the demand for "inappropriate" memory removal (reversal learning), whereas a decrease likely contributes to stronger memory retention (repetitive learning).

Encouraged by the correlation observed in reversal learning, we then sought to determine the time course of Rac activation during passive memory decay. The retention curve of wild-type flies (Figure 7C) was similar to the parental controls shown in Figure 1A, and thus we assayed three time points (0 hr, 1 hr, and 3 hr) to correspond to the time window of Rac's behavioral effects. In parallel with the memory decay curve (Figure 7C), we observed an increase of Rac activity at 1 hr (Figure 7D). This result, together with the evoked Rac activation in reversal learning, strongly supports the notion that Rac activation regulates the forgetting process during memory decay and removal.

DISCUSSION

In the current study, investigation of the functions of the small G protein Rac in memory formation leads to the conclusion that memory decay consists of an active forgetting component caused by Rac activation. We arrive at this conclusion from the following two categories of supporting evidence.

The first category shows that the effects of Rac on memory decay are independent of memory acquisition. First, genetically induced inhibition or elevation of Rac activity do not affect the first 30 min of memory but alter later memory decay (Figures

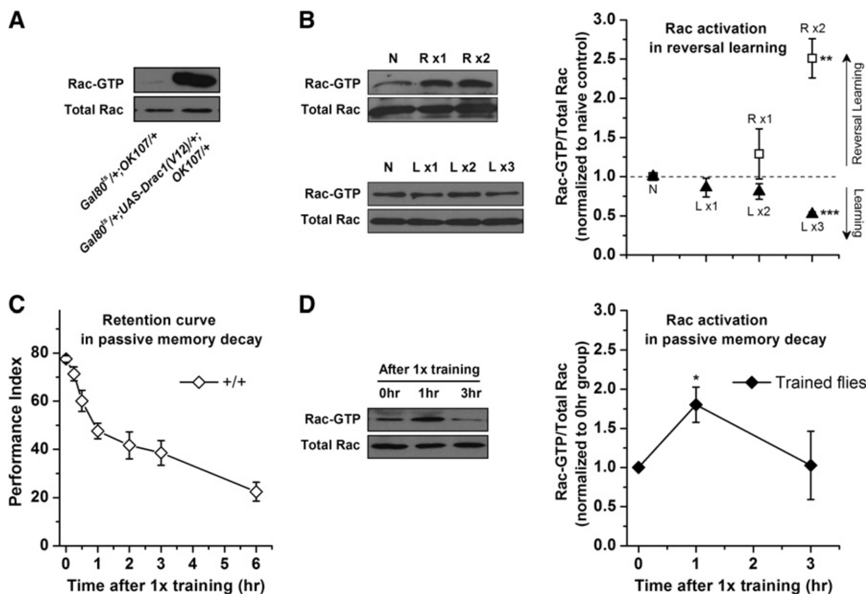

Figure 7. Endogenous Rac Activation Correlates with Forgetting

(A) Levels of Rac-GTP and total Rac in whole head extracts from heat-shock-treated flies of the indicated genotypes.

(B) Representative western blots and group data showing Rac activation in heads of naive flies and flies subjected to various training experiences. N, Naive. "R × 1, × 2" indicate "Reversal × 1, × 2," as shown in Figure 6A. "L × 1, × 2, × 3" indicate "Learning × 1, × 2, × 3" repetitive learning with indicated number of training sessions. Statistically significant differences from the Naive group were detected for the "R × 2" and "L × 3" groups (paired-samples t test, $p < 0.01$ and 0.001, respectively). Group data represent means ± SEM. n = 6 and 5 independent experiments for "reversal learning" and "repetitive learning," respectively.

(C) A typical memory retention curve of wild-type flies after one-session training. n = 8–10, means ± SEM.

(D) Rac activation in heads of trained flies at various retention intervals (0, 1, and 3 hr). Flies at 1 hr after training showed higher Rac activity compared with the "0 hr" group (paired-samples t test, $p = 0.04$). Group data represent means ± SEM. n = 4 independent experiments.

1A and 3C). Second, the acquisition curves generated by varying training intensity are not affected by inhibition of Rac activity (Figure 1B). Finally, the slower memory decay after Rac inhibition is still observed in the 20 V weak training (Figure 1C) and in the *rut* mutant background (Figure 1D), and both show much reduced initial memory acquisition and thus rule out ceiling effect as an explanation for the slower decay. Taken together, Rac affects memory decay rather than acquisition.

The second category of evidence supports a specific role of Rac in forgetting. First, the prolonged memory after Rac inhibition (Figure 1A) stands apart from the known consolidated memory forms but retains the fragile nature of early memory (Figure 2), raising the possibility of suppression of early memory decay. Second, inhibition of Rac activity blocks interference-induced forgetting (Figure 5). Third, acute removal of inappropriate memory in reversal learning is altered by genetic manipulation of Rac activity (Figure 6). Fourth, the Rac-dependent forgetting mechanism appears to be independent of Rut-mediated memory formation mechanisms (Figure 1D).

These two categories of evidence together lead to the conclusion that inhibition of Rac activity suppresses forgetting whereas an increase in Rac activity accelerates forgetting. This conclusion derived from genetic and behavioral analysis is further supported by biochemical assays of training-evoked Rac activation in wild-type fly heads, wherein rising of endogenous Rac activity spanned from a few minutes to hours, corresponding to acute memory removal in reversal learning and gradual memory loss in passive memory decay.

Involvement of the Rac Pathway in Memory Processes

We revealed the role of Rac in active forgetting through spatially and temporally confined expression of dominant Rac mutants. These dominant mutant-expressing approaches, although providing invaluable information, also result in concerns related to specificity (Feig, 1999; Hakeda-Suzuki et al., 2002), as the dominant mutants function by targeting the upstream activators or downstream effectors while these targets are usually shared by several closely related Rho family GTPase members (Johndrow et al., 2004). To address the specificity of our results, we also examined effects on 3 hr memory by similar induction of UAS-driven dominant mutants of three other Rho GTPases, including Dcdc42, DrhoA, and DrhoL (Figure S4). For dominant-negative mutations, only Dcdc42(N17) was also found to enhance 3 hr memory (Figures S4A and S4B). However, expression of the constitutively active form, Dcdc42(V12), did not accelerate memory decay (Figures S4A and S4B), as observed in the case of Drac1(V12). Moreover, as an additional support to the specificity of Drac1(V12) effect, the double mutant Drac1(V12C40) that is incapable of activating PAK as well as other CRIB-motif effector proteins failed to accelerate memory decay (Figure 4C). Therefore, Rac is thought to be responsible for the observed phenotypes, which is further supported by the consistent changes in endogenous Rac activity after different training experiences.

How might Rac be related to forgetting at the cellular level? One clue comes from the examination of a well-established Rac downstream pathway wherein we found that hyperactivation of cofilin enhanced 3 hr memory performance as observed with Rac inhibition (Figure 4B). Cofilin belongs to a family of F-actin depolymerizing factors that are known to sever actin filaments and promote actin turnover (Bamburg, 1999). In vertebrates, the actin-depolymerizing activity of cofilin has been shown to be important for synaptic plasticity and activity-dependent modification of spine morphology (Fukazawa et al., 2003; Zhou et al., 2004). Therefore, the Rac-forgetting mechanism might employ cofilin to modulate actin cytoskeleton remodeling,

which in turn may facilitate physiological or morphological changes necessary for erasing memory.

Independent Molecular Mechanisms for Memory Formation and Forgetting

The *Drosophila* olfactory memory curve consists of many components, with most notable features including a rapid forgetting of transient early memory and a gradual formation of consolidated late memory (DeZazzo and Tully, 1995). There is an increasing body of reports that investigate memory formation, demonstrating the involvement of synaptic plasticity and activation of various signal transduction pathways, such as Ca^{2+}, cAMP, and transcription factor CREB-dependent cascades (Davis, 2005; Margulies et al., 2005). These molecular and cellular mechanisms seem to be highly conserved in other species (Elgersma and Silva, 1999; Kandel, 2001). Although much less is known about whether multiple biologically active processes also contribute to the rapid forgetting of transient early memory, our study of Rac suggests that the molecular mechanisms of forgetting might be very different from those of memory formation.

rut-encoded adenylyl cyclase plays an essential and conserved role in memory formation (Davis, 2005; Kandel, 2001). Accordingly, several memory forms including STM, MTM, and LTM are all found to be impaired in *rut* mutants (Blum et al., 2009; Dubnau and Tully, 1998; Zars et al., 2000). However, the Drac1(N17)-induced suppression of memory decay is intact in the background of *rut* mutation (Figure 1D). Notably, the observed behavioral effect is derived from expression of Drac1(N17) presumably only in *rut*-expressing neurons (driven by the rut^{1047} Gal4 line), whereas both Rut and Drac1(N17) retain their respective effects on initial memory acquisition and forgetting. It is therefore of considerable interest to determine whether the molecular basis for memory formation and forgetting are independent in general.

Time-Based Decay versus Interference Theory of Forgetting

In the literature of psychology, there is a long history of debate on the nature of forgetting, in terms of whether it is caused by time-based decay or by interference from irrelevant information (Jonides et al., 2008; Wixted, 2004). In the current study, we were able to examine several forms of forgetting with the help of genetic manipulation, including passive memory decay, interference learning, and reversal learning. In the case of passive memory decay, no overt interference was present, but we still observed that Rac was activated at 1 hour later, after training in parallel with the advent of a forgetting component. The result implies that forgetting might be an intrinsic characteristic of initially acquired memory, with training inducing not only memory formation but also forgetting, albeit in different time domains. In this regard, the operation of the forgetting mechanism does not necessarily involve interference but is likely boosted or evoked by heightened interference as indicated in the cases of the interference-learning-induced memory loss (Figure 5) and reversal-learning-induced memory removal (Figure 6). Thus, the two seemingly different theoretical explanations of forgetting, time-based decay and interference, might share the same mechanism at the molecular level.

EXPERIMENTAL PROCEDURES

Fly Stocks

Strains from the Bloomington Stock Center are as follows: *UAS-Drac1(V12)* (#6291), *UAS-Drac1(N17)* (#6292), *UAS-Dcdc42(V12)* (#6287), *UAS-Dcdc42(N17)* (#6288), *UAS-DrhoA(V14)* (#7330), *UAS-DrhoA(N19)* (#7328), *UAS-DrhoL(V20)* (#4851), *UAS-DrhoL (N25)* (#4849), $Drac1^{EY05848}$ (#15461), $Drac2^{\Delta}$ (#6675), Mtl^{Δ} (#6676), *UAS-tsr.WT* (#9235), *UAS-tsr.S3A* (#9236), *UAS-tsr.S3E* (#9239), *UAS-mcd8GFP* (#5130), *tublin-Gal80^{ts}* (#7019). *UAS-Drac1(V12C40)* was from Dr. Akira Chiba. rut^{1047} was generated by standard transposon mutagenesis. All the above flies were outcrossed for at least five generations with w^{1118} *(isoCJ1)* wild-type flies or balancers with the wild-type genetic background. Gal4 drivers used were either extant stocks in our lab or kindly provided by Dr. Hiromu Tanimoto. *MBGal80* and *OK107; UAS-mcd8GFP* were gifts from Dr. Scott Waddell and Dr. Liqun Luo, respectively.

Heat Shock Regimen

When Gal4 together with Gal80^{ts} were used to drive expression, crosses were raised in 18°C. Two- to four-day-old progeny were collected and divided into two groups. The induced group was transferred to a 30°C incubator for 3 days, whereas the uninduced control group was kept at 18°C. Both groups were allowed to recover at 25°C for at least 1 hr before behavioral experiments.

Behavioral Assays

Pavlovian Olfactory Aversive Conditioning

Training and test were performed as described previously (Tully et al., 1994; Tully and Quinn, 1985) in a 25°C room with 70% relative humidity.

During training, around 100 flies were exposed sequentially to two aversive odors (3-octanol [OCT] and 4-methylcyclohexanol [MCH], Fluka, 1.5×10^{-3} and 1×10^{-3} dilution in heavy mineral oil, respectively) for 60 s with 45 s flush of fresh air after each odor. Flies received unconditioned stimulus (US) (twelve 1.5 s pulses of 60 V electric foot shock) during the presence of the first odor (conditioned stimulus [CS]+) but not the second (CS−). This procedure constituted a typical training session. In some of the experiments, the US intensity was modified to make the conditioning nonmaximal, either by lowering shock voltage from the regular 60 V to 20 V, 10 V or by reducing the shock pulses from the regular 12 to 6, 2. When the number of shock pulses was reduced, the durations for odor exposures were also correspondingly decreased. Occasionally, repetitive spaced trainings with intersession interval of 15 min were used to yield longer memory.

To assay memory, trained flies were allowed to choose between CS+ and CS− in a T-maze for 120 s. A performance index (PI) was calculated from the distribution of flies in the two T-maze arms (Tully et al., 1994). A PI of 0 indicated a distribution of 50:50 (no learning), whereas a PI of 100 indicated that all the flies avoided the negatively reinforced CS+ (perfect learning). To eliminate odor bias, each experiment (n = 1) consisted of two reciprocal groups, with one trained to associate OCT with shock and the other to associate MCH with shock. The final PI was the average of PIs from the two groups.

For 3 min memory, flies were tested immediately after training. For measurement of longer memory retention, they were placed in food vials for the duration of a particular retention interval (at 25°C for retention up to 3 hr, at 18°C for that longer than 3 hr) before transferring to T-maze for testing at 25°C.

Interference Learning

Retroactive interference was introduced at 1.5 hr after the initial learning (OCT/MCH) by exposing flies to new learning with a novel pair of odors (ethyl acetate [EA] and isoamyl acetate [IA], Alfa Aesar, 2×10^{-3} dilution) as CS+/CS−. Interference effects were evaluated by comparing 3 hr memory retention of the prior learning (OCT/MCH) in flies with or without new learning (referred to as "Interfere+" and "Interfere−," respectively). In the experiment, the initial learning was trained with both regular (60 V) and weak (20 V) training protocols, but the interference learning only utilized regular (60 V) protocol.

Reversal Learning

In "Reversal × 1," after a regular one-session training, flies were retrained by another session but with the CS-US contingency reversed, i.e., the odor (either OCT or MCH) that was paired with shock in the first session was not paired with shock in the second session and vice versa. For "Reversal × 2," an additional reversal session was included, thus the CS-US contingency was reversed again and the same as that in the first session. A 90 s interval existed in between each reversal session. Immediately after the last training session, flies were tested for choice between the two trained odors, OCT versus MCH. PI was calculated as stated above, except that the odor paired with shock in the last training session was taken as the "CS+." Each experiment also consisted of two reciprocal groups as above.

In third-odor test, flies after reversal learning (OCT paired with shock in the initial session) were given a choice between OCT and a previously unexposed odor (benzaldehyde [Ben], Fluka, 2×10^{-3} dilution). A half PI was generated accordingly (see above and Tully et al., 1994). At the same time, a group of untrained flies was tested to control for naive odor bias. The score of the untrained group was subtracted from that of the conditioned group to get a final index that indicated avoidance of OCT in the course of reversal learning.

Cold-Amnesia and Drug Feeding

The procedures were as described previously (Tully et al., 1994). For cold-amnesia, flies were transferred to empty vials and cooled in ice water for 2 min. After the treatment, flies were allowed to recover in fresh food vials for 1 hr before memory test. For CXM feeding, at the last day of heat-shock induction, flies were fed with 35 mM cycloheximide (Sigma) and 5% glucose dissolved in 3% ethanol (CXM+) or vehicle alone (CXM−) for 12–14 hr at 30°C and then subjected to behavioral training. After training, the drug feeding was continued at 18°C until memory retention was tested 24 hr later.

Rac Activity Assay

Relative levels of GTP-bound Rac were determined by PBD pull-down assay (Upstate Biotechnology) according to manufacturer's procedure. Briefly, heads from around 400 flies were isolated and homogenized in Mg^{2+} lysis buffer. Large cuticular debris was removed by centrifugation at 12,000 g for 10 min at 4°C. After being precleared with glutathione-agarose (Santa Cruz), the GTP-bound Rac was precipitated from the cell lysates through binding to the p21-binding domain (PBD) of PAK-1 fused to GST (Upstate Biotechnology). PBD-associated Rac, as well as total Rac in the lysates, was examined by western blot with a mouse anti-human Rac1 monoclonal antibody (BD Transduction Laboratories, 1:2000 dilution). Intensities of the detected bands in western blots were quantified in NIH Image J software.

Statistics

Unless stated otherwise, the data are shown as means ± standard error of the mean (SEM) and analyzed by ANOVA with Bonferroni corrected pairwise comparisons in SPSS 11.0 (Chicago, IL, USA). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; n.s., nonsignificance ($p > 0.05$).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, five figures, and one table and can be found with this article online at doi:10.1016/j.cell.2009.12.044.

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