Genetic dissection of active forgetting in labile and consolidated memories in Drosophila

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Different memory components are forgotten through distinct molecular mechanisms. In *Drosophila*, the activation of 2 Rho GTPases (Rac1 and Cdc42), respectively, underlies the forgetting of an early labile memory (anesthesia-sensitive memory, ASM) and a form of consolidated memory (anesthesia-resistant memory, ARM). Here, we dissected the molecular mechanisms that tie Rac1 and Cdc42 to the different types of memory forgetting. We found that 2 WASP family proteins, SCAR/WAVE and WASp, act downstream of Rac1 and Cdc42 separately to regulate ASM and ARM forgetting in mushroom body neurons. Arp2/3 complex, which organizes branched actin polymerization, is a canonical downstream effector of WASP family proteins. However, we found that Arp2/3 complex is required in Cdc42/WASP-mediated ARM forgetting but not in Rac1/SCAR-mediated ARM forgetting. Instead, we identified that Rac1/SCAR may function with formin Diaphanous (Dia), a nucleator that facilitates linear actin polymerization, in ARM forgetting. The present study, complementing the previously identified Rac1/cofilin pathway that regulates actin depolymerization, suggests that Rho GTPases regulate forgetting by recruiting both actin polymerization and depolymerization pathways. Moreover, Rac1 and Cdc42 may regulate different types of memory forgetting by tapping into different actin polymerization mechanisms.

Results

SCAR/WAVE Complex Regulates Labile Memory Forgetting in the MB Neurons. We first tested whether SCAR/WAVE complex affects ASM forgetting using RNAi in *Drosophila*. To avoid developmental defects, we restricted RNAi expression to the adult flies using elav-GS, a pan-neuronal conditional expression driver that depends on RU486 feeding (21). We examined memory retention curves after a 1-session olfactory aversive conditioning (9). SCAR-RNAi-expressing flies (RU486+) showed memory performance index (PI) similar to the uninduced controls without RU486 feeding (RU486−) shortly after training (3 min and 1 h, Fig. 1A), but the memory was significantly higher at later time points (18, 19), and the downstream pathways that tie Rac1 and Cdc42 to actin remodeling and eventually forgetting are far from being fully elucidated. Rho GTPases can also affect actin polymerization pathways. For example, 2 WASP family proteins, SCAR/WAVE and WASp, are known to transduce Rac1 and Cdc42 activity to the activation of Arp2/3 complex to promote actin polymerization (18, 20); but it is unclear how they may contribute to the forgetting functions of Rac1 and Cdc42.

Significance

As a critical component of a healthy memory management system, forgetting has received increasing attention. Studies across multiple species support important roles of actin remodeling in forgetting. However, the underlying molecular mechanisms remain unclear. In *Drosophila*, Rac1 and Cdc42, 2 Rho GTPases that act as signaling hubs to coordinate actin remodeling, were reported to mediate the forgetting of labile and consolidated memories, respectively. Here, we showed that Rac1 and Cdc42 exert their effects on forgetting by acting through 2 different actin polymerization pathways, Rac1/SCAR/Dia and Cdc42/WASP/Arp2/3 complexes. These findings fill in the molecular landscape that links forgetting to actin remodeling at the cellular level and shed light on drug development that aims to tune forgetting to treat memory-related diseases.

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points (2, 3, and 6 h, Fig. 1A). Additional experiments using attenuated training intensity (SI Appendix, Fig. S1A) confirmed that SCAR knockdown does not affect initial learning (assayed at 3 min after training). For more stringent controls, SCAR-RNAi-expressing flies showed higher memory retention when compared with their parental controls (elav-GS/+ and UAS-SCAR-RNAi-1/+; RU486+, Fig. 1B and SI Appendix, Fig. S1B).

The phenotypes were again confirmed using an independent SCAR-RNAi line (SI Appendix, Fig. S1 C and D). The higher 3-h memory performance of SCAR-RNAi-expressing flies was blocked by cold-shock anesthesia (Fig. 1B and SI Appendix, Fig. S1E), suggesting that SCAR knockdown hampers the forgetting of the ASM component.

We also tested the effect of SCAR knockdown on interference-based forgetting using a protocol as previously shown (3) (Fig. 1C). After an initial learning session, the trained flies were subjected to a second learning session with a novel pair of odors and the flies were evaluated for 3-h retention of the first learning. The introduction of a second learning session (interference+) lowered memory performance in control flies (RU486−), but had no significant effect in SCAR-RNAi-expressing flies (RU486+) (Fig. 1C).

We next found 3 lines of experimental evidence supporting the notion that SCAR is one of the downstream effectors of Rac1 in ASM forgetting. First, Rac1 and SCAR function in the same brain locus. Rac1-dependent forgetting has been mapped to the intrinsic mushroom body (MB) neurons (3), which play a critical role in olfactory learning and memory in Drosophila (22). We found that expressing SCAR-RNAi in the adult MB neurons using a MB-specific, inducible driver (MB-GS) (23) resulted in higher 3-h memory retention, but left 1-h memory intact (Fig. 1D). Conversely, overexpression of SCAR in the adult MB neurons did not affect 1-h memory but reduced 3-h memory retention (Fig. 1E), and the reduction was also blocked by cold anesthesia (SI Appendix, Fig. S1F). Second, RNAi knockdown of other proteins (Sra1, Abi, and HSPC300) in the SCAR/WAVE complex (20) confirmed the memory phenotype of SCAR at 3-h after training (Fig. 1F). Third, we performed a genetic epistasis experiment by combining the expression of a constitutively active mutant (Rac1-CA) and the SCAR-RNAi-1 in the MB neurons. Consistent with our previous report (3), Rac1-CA expression decreased 3-h memory (MB-GS/UAS-Rac1-CA, RU486+, Fig. 1G). The decrement was dominated by SCAR knockdown (UAS-SCAR-RNAi-1/+; MB-GS, UAS-Rac1-CA/+; RU486+, Fig. 1G). We therefore conclude that SCAR/WAVE complex functions downstream of Rac1-mediated ASM forgetting (Fig. 1H).

### WASp Regulates ARM Forgetting in the MB Neurons

We also investigated the function of WASp in Cdc42-dependent ARM forgetting. To isolate ARM from ASM and to assay the retention of ARM (ARM PI) at different time points after training, we subjected flies to 2-min cold-shock treatment and then allowed flies to recover for 1 h at 25 °C before testing. We focus on the decay of ARM at 3 to 12 h after its formation reaches a plateau at about 2 h. Flies with conditional pan-neuronal WASp-RNAi expression (RU486+) showed slower ARM decay when compared with the RU486− controls. Their performance was higher when ARM was assayed at 6, 9, and 12 h after a 1-session training or at 24 h after a 4-session massed training (Fig. 2A). Note that the effect of WASp knockdown was specific to the later decay phase of ARM without affecting ARM retention at 2 and 3 h after a 1-session training (Fig. 2A) or at 2 h after attenuated training (SI Appendix, Fig. S2A), suggesting that WASp knockdown does not affect ARM formation. For more stringent controls, the higher ARM performance was confirmed by including the parental controls (Fig. 2B) and by using a second independent RNAi line (SI Appendix, Fig. S2C). ARM decay is likely not affected by WASp knockdown. WASp-RNAi-expressing flies had normal memory performance up to 3 h after a 1-session training (SI Appendix, Fig. S2B). Since ASM has considerable decay in the 3-h memory time window, the absence of effect of WASp knockdown

![fig1](image-url)
on 3-h memory indicates that the formation and decay of ASM are independent of WASp. For better visualization, we subtracted ARM from the intact memory (without cold-shock treatment) to generate the ASM component. Despite a consistent increase of intact memory and ARM by WASp knockdown at 6 and 9 h after a 1-session training, an effect on the ASM component was not observed (Fig. 2B and SI Appendix, Fig. S2D).

We also tested the requirement of WASp in interference-based forgetting of ARM using a retroactive interference paradigm used in our previous study (4). Flies received 4-session massed training, and immediately following the initial training, the trained flies were exposed to a second 4-session massed training with a novel odor pair. Testing of the ARM retention of the initial learning was performed at 3.3 h after the initial training. The interference learning reduced the performance in control flies (RU486−, Fig. 2C and SI Appendix, Fig. S2E), while such forgetting was suppressed in WASp-RNAi-expressing flies (RU486+, Fig. 2C and SI Appendix, Fig. S2E). Together, like Cdc42 (4), WASp is required for time-based and interference-based forgetting of ARM, and on the other hand, WASp is dispensable in ARM formation and ASM decay.

There are 2 additional lines of evidence supporting the idea that WASp functions downstream of Cdc42 in ARM forgetting. First, like Cdc42 (4), WASp-dependent forgetting also takes place in the MB neurons. Knockdown (Fig. 2D) and overexpression (Fig. 2E) of WASp in the adult MB neurons led to slower and accelerated ARM decay after a 1-session training. Second, we combined Cdc42 activation and WASp knockdown to test genetic interaction (Fig. 2F). Consistent with our previous finding (4), flies expressing constitutively active Cdc42 (MB-GS/UAS-Cdc42-CA, RU486+) had reduced ARM performance at 9 h after a 1-session training when compared with control flies (MB-GS+, RU486+). The reduction was reversed by coexpression of WASp-RNAi (MB-GS, UAS-Cdc42-CA/UAS-WASp-RNAi-1, RU486+). Flies expressing both Cdc42-CA and WASp-RNAi-1 had a performance level similar to flies expressing WASp-RNAi alone, and both groups were higher than the MB-GS+/+ control. The data suggest that WASp acts as a downstream effector of Cdc42 in ARM forgetting (Fig. 2G).

**Arp2/3 Complex Is only Required in Forgetting of ARM but Not ASM.**

Arp2/3 complex is a known downstream effector that ties the Rac1/SCAR pathway and Cdc42/WASp pathway to actin polymerization (18, 20). And it has been reported to be important for forgetting in *Caenorhabditis elegans* (24). We next tested the role of Arp2/3 complex in forgetting by knocking down Arp2 and Arp3, 2 major members of this complex (25), and by feeding flies with 20 μM of CK666, a specific inhibitor of Arp2/3 complex that stabilizes the inactive state of the complex (26). The inhibition of Arp2/3 complex by both genetic and pharmacological methods led to higher 6-h memory (Fig. 3D–F), but the 3-h memory was not affected (Fig. 3A–C). The higher memory retention at 6 h is specific to ARM, while the ASM component is spared (Fig. 3D–F). We additionally tested the dosage-dependent effect of CK666 feeding. Increased memory retention at 12 h was observed when flies were fed with CK666 higher than 5 μM (SI Appendix, Fig. S3B), while no effects were observed for memory retention at 3 h for all of the concentrations tested (up to 20 μM, SI Appendix, Fig. S3A). The specific effect on ARM forgetting indicates that Arp2/3 complex functions downstream of the Cdc42/WASp pathway. To test this, we combined the expression of constitutively active Cdc42-CA and WASp-RNAi with the pharmacological inhibition of Arp2/3 complex using CK666. ARM forgetting was accelerated by Cdc42-CA expression and slowed down by WASp-RNAi expression (Fig. 3G, CK666−−). However, in the presence of CK666 feeding, there are no differences among control flies and flies expressing Cdc42-CA and WASp-RNAi, suggesting that the effect of Arp2/3 complex inhibition dominates those induced by Cdc42-CA and WASp-RNAi expression. These data support the idea that Arp2/3 complex is specifically
required in Cdc42/WASp-mediated ARM forgetting but not in Rac1/SCAR-mediated ASM forgetting (Fig. 3H).

**Formin Dia Functions with Rac1/SCAR in ASM Forgetting.** To gain a better understanding of Rac1/SCAR-mediated ASM forgetting, we examined a number of interacting proteins of SCAR/WAVE complex (27–34) in a small-scale RNAi screen by knocking down these proteins in the MB neurons (SI Appendix, Fig. S4A). We found higher 3-h memory performance in flies expressing the RNAi of Diaphanous (dia), which encodes a formin family protein that induces linear actin polymerization (35).

We further tested Dia’s role in ASM forgetting. Compared with the RU486− control, dia-RNAi-expressing flies (RU486+) showed normal memory performance at 3 min after a 1-session training, but memory decay at 3 and 6 h was slower (Fig. 4A). Such Dia-dependent slower memory decay was abolished by cold anesthesia (Fig. 4A), suggesting that Dia is required only for ASM forgetting. We also fed flies with 2.5 μM SMIFH2, a small molecule inhibitor of formin-dependent but not Arp2/3 complex-dependent actin polymerization (36), SMIFH2 also led to higher memory at 6 h after training and the phenotype is sensitive to cold anesthesia (SI Appendix, Fig. S4B), which is consistent with RNAi knockdown of Dia. Conversely, acute expression of a constitutively active form of Dia (UAS-dia-C4), which lacks the N-terminal regulatory sequence and the C-terminal autoinhibitory domain (37), reduced 3-h memory. The effect of Dia-C4 was again blocked by cold anesthesia (Fig. 4B). Like SCAR, Dia is also required for interference-based forgetting (Fig. 4C). Thus, Dia bidirectionally regulates ASM forgetting in the MB neurons.

We next sought to determine the relationship between Dia and Rac1/SCAR-mediated ASM forgetting using both genetic and pharmacological manipulations. The accelerated memory forgetting observed in Rac1-CA-expressing flies (yellow bar, RU486+) was dominated by the Dia effect (red bar, RU486+) in flies expressing both Rac1-CA and dia-RNAi (orange bar, RU486+) (Fig. 4D). Consistently, pharmacological inhibition of Dia also slowed down memory decay and masked the accelerated forgetting induced by Rac1-CA and SCAR overexpression (Fig. 4E). These data indicate that Dia functions downstream of Rac1/SCAR-mediated ASM forgetting in the MB neurons (Fig. 4F).

**Rac1/SCAR/Dia-Dependent Forgetting Functions in the MB γ-Neurons.** The MB neurons are divided into 3 major types: the γ-, α/β′-, and α/β-neurons (SI Appendix, Fig. S5A), which have distinct roles in different phases and processes of olfactory memory (22). The MB-GS is a broad MB driver, which covers both the γ- and α/β-neurons (38) and is not suitable for differentiating different MB types. We hereby turned to the TARGET system (39) and used a temperature shift from 18 °C to 30 °C to inactivate the pan-MB Gal4 lines (40), a γ-neuron-specific Gal4 line (5-HT1B-GAL4) (41), a γ-neuron driver, 5-HT1B-GAL4 (41), also led to slower memory decay that lasted up to 24 h (Fig. 5A and SI Appendix, Fig. S6C). In the MB, 5-HT1B-GAL4 drives expression exclusively in the γ-neurons; whereas weak expression can still be found elsewhere in the ellipsoid body and some scattered neurons in the antennal lobe and the optical lobe (Fig. 5A). However, the integration of a MB-Gal80 transgene (42) suppressed the expression specifically in the MB (Fig. 5A) and also blocked the memory increment at 24 h (Fig. 5B). We note that 5-HT1B-GAL4 has a higher expression level in the γ-neurons, which may explain the discrepancy as to why similar phenotypes were not observed in the initial study with 3 other γ-neuron drivers, 1471, NP1131, and 201Y. Consistent with the previous data, we did not detect a phenotype even when a
Identification of Dia as a downstream effector of Rac1/SCAR in ASM forgetting.

**Discussion**

There are three major findings. First, 2 WASP family proteins, SCAR/WAVE and WASp, act as downstream effectors of Rac1-mediated ASM forgetting and Cdc42-mediated ARM forgetting, respectively. Second, although the Arp2/3 complex is a well-established effector that links activation of WASP family proteins to actin polymerization, it is only required in Cdc42/WASp-mediated ARM forgetting. Instead, formin Dia functions together with Rac1/SCAR in ARM forgetting. Third, feeding inhibitors of the Arp2/3 complex and Dia to fruit flies led to rather specific effects on ARM and ASM forgetting, raising the possibility of developing drugs on these molecular targets to treat memory-related diseases.

The effect of Rac1 on ASM forgetting has been tied to the activation of an actin depolymerization regulator cofillin presumably through a PAK/LIMK phosphorylation cascade (3, 17). However, actin dynamics is a balanced play that requires continuous turnover between polymerization and depolymerization (43). It is not known whether signaling pathways regulating actin polymerization also play a role. There are 3 families of proteins that nucleate and promote actin polymerization, Arp2/3 complex, WH2-domain proteins, and formin (18, 20). Our finding that Arp2/3 complex and formin Dia function in ARM and ASM forgetting suggests that both actin polymerization and depolymerization pathways contribute to forgetting. How Arp2/3 complex and Dia separately contribute to ARM and ASM forgetting remains an open question. It is yet to be determined whether these proteins have different expression or subcellular locations in the MB neurons. However, it is interesting that Arp2/3 complex and formins are specialized in different types of actin polymerization (18, 20, 44).

In our working model, Cdc42 activates Arp2/3 complex via a canonical pathway (Cdc42/WASp/Arp2/3 complex), while Rac1-mediated ASM forgetting depends on SCAR/WAVE complex. This complex, in addition to SCAR/WAVE, includes at least 4 other members: Sra-1, Abi, HSPC300, and Kette (20). These additional members are thought to hold SCAR/WAVE in the inactive state, until GTP-bound Rac1 binds to Sra-1 and relieves the inhibition (20). On the other hand, the intact complex is essential for the stability of the SCAR/WAVE protein as well (i.e., failure to keep the intact complex can lead to SCAR degradation) (45). This latter effect may explain our observation that RNAi knockdown of SCAR complex members has the same effect on inhibiting forgetting as the knockdown of SCAR. As a WASP family protein, SCAR/WAVE is able to associate with and activate Arp2/3 complex through its C-terminal region (46). However, RNAi knockdown of Arp2 and Arp3 and pharmacological inhibition of Arp2/3 complex specifically affects ARM forgetting, while no effects on ASM retention were observed. We therefore propose that Rac1/SCAR may function through Arp2/3 complex-independent mechanisms (47). SCAR/WAVE complex is reported to physically associates with Dia through one of its members, Abi, to regulate actin dynamics (48, 49). Our behavioral characterization of Dia knockdown and overexpression, as well as the genetic epistasis experiment, support the idea that Dia could be downstream of Rac1/SCAR in ASM forgetting. Details about the functional coordination between SCAR/WAVE and Dia therefore await further clarification.

**Materials and Methods**

**Fly Strains.** Flies were reared at 25 °C and 60% relative humidity on a cornmeal medium under a 12/12 h light/dark cycle, except that flies in experiments using the TARGET system were reared at 18 °C. For details, see SI Appendix, SI Materials and Methods.
Fig. 5. Function of Rac1/SCAR/Dia forgetting pathway in the MB γ-neurons. (A) Expression patterns of 5-HT1B-GAL4. Besides the strong expression in the MB γ-neurons, the Ga4 also labels neurons outside the MB, including the ellipsoid body in the central complex (arrow). MB-Gal80 suppressed the expression of 5-HT1B-GAL4 in the MB neurons specifically. Green, mCD8-GFP reporter; magenta, nC82. (Scale bar, 50 μm.) (B) Memory retention curves. Conditional expression of Rac1-DN in the adult MB γ-neurons with the 5-HT1B-Gal4 driver was sufficient to slow down memory decay, and the effect was blocked by MB-Gal80 (24 h). n = 6 to 10. (C) Consistent with Rac1-DN, RNAi knockdown of SCAR/WAVE complex members in the MB γ-neurons led to higher memory retention at 6 h. n = 8 to 10. (D) Knockdown of Dia in the adult MB γ-neurons resulted in higher memory retention at 6 h. n = 6. Data are means ± SEM. *P < 0.05, n.s., nonsignificant.

Behavioral Assays and Related Treatments. Averisc otary conditioning was performed as previously described (3, 4). For details, see SI Appendix, SI Materials and Methods.

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