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Supplementary Information for

Genetic Dissection of Active Forgetting in Labile and Consolidated Memories in *Drosophila*

Yang Gao, Yichun Shuai, Xuchen Zhang, Yuwei Peng, Lianzhang Wang, Jing He, Yi Zhong, Qian Li

Qian Li
Email: liqian8@tsinghua.edu.cn
Yi Zhong
Email: zhongyi@tsinghua.edu.cn

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Supplementary Information Text

Materials and Methods

Fly Strains.

Flies were reared at 25℃ and 60% relative humidity on a cornmeal medium under a 12/12 hr light/dark cycle, except that flies in experiments using the TARGET system were reared at 18℃. *elav-GS* and *MB-GS* were gifts from Ronald L. Davis. *UAS-SCAR* was from Jennifer A. Zallen. *4-59* and *5-66a* were from Ulrike Heberlein. *pJFRC81-10XUAS-IVS-Syn21-GFP-p10* was from Gerry Rubin. *247-dsRed* (II) was from André Fiala. The following stocks were from the Bloomington Stock Center: *UAS-Rac1-CA* (#6291), *UAS-Rac1-DN* (#6292), *5-HT1B-Gal4* (#27637), *Gal80^{ts}* (#7017 and #7019), *UAS-WASp* (#39724), *UAS-dia-CA* (#27616), *13F02* (#48571), *71G10* (#39604), *pJFRC12-10XUAS-IVS-myr::GFP* (#32197). RNAi stocks were from Tsinghua Fly Center and the targeting sequences can be found on the website (http://fly.redbux.cn): *UAS-SCAR-RNAi-1* (TH02179.N), *UAS-SCAR-RNAi-2* (THU1743), *UAS-WASp-RNAi-1* (THU2125), *UAS-WASp-RNAi-2* (TH02180.N), *UAS-Sra1-RNAi* (THU3810), *UAS-Abi-RNAi* (THU1798), *UAS-HSPC300-RNAi* (THU1680), *UAS-Arp2-RNAi* (THU2791), *UAS-Arp3-RNAi* (THU1112), *UAS-Cip4-RNAi* (TH02174.N), *UAS-Ena-RNAi* (THU1895), *UAS-Abl-RNAi* (TH04329.N), *UAS-kug-RNAi* (THU4120), *UAS-drk-RNAi* (THU5426), *UAS-Arf79F-RNAi* (THU2017), *UAS-dock-RNAi* (THU2815), *UAS-dia-RNAi* (THU0408). *w1118 (isoCJ1)* is used as wild type. *OK107*, *C739*, and *201Y*, flies were extant stocks in our lab.

Transgene Induction

GeneSwitch and TARGET were used for temporally and spatially controlled transgene expression. To induce transgene expression using GeneSwitch, flies were fed with RU486 for two days (see “Drug Feeding” section for details). In TARGET experiments, for the “30℃” groups, adult flies within 2 days of eclosion were transferred from 18℃ to a 30℃ incubator and kept in 30℃ or 3 days; while for the “18℃” groups, flies were kept in 18℃ for the same time period. Both the “18℃” and “30℃” groups were allowed to acclimate to 25℃ for at least 1 hr before the behavioral experiments.

Drug Feeding

Flies of the control groups (RU486-, CK666- and SMIFH2-) were fed with vehicle alone (5% glucose and 3% ethanol), while those of the experimental groups (RU486+, CK666+ and SMIFH2+) were fed with 500 μM RU486 (Mifepristone, J&K Scientific), 20 μM CK666 (Sigma-Aldrich) and 2.5 μM SMIFH2 (Sigma-Aldrich) dissolved in 5% glucose and 3% ethanol respectively. All feeding started 2 days before behavioral training and lasted until the termination of testing.

Behavioral Assays

Two- to five-day-old flies were used for behavioral experiments using a Pavlovian olfactory aversive conditioning procedure. Before experiments, flies were allowed to acclimate to the behavioral room at 25℃ and 60% relative humidity for at least 30 min. During training, a group of about 100 flies were presented with the conditional stimulus odor (CS+) for 60 s and in the meantime received 12 pulses of 1.5-s, 60-V electric shock delivered at a 5-s interpulse interval. The flies were then presented with a second odor (CS-) for 60 s but without electric shock. Following each odor presentation, there was a flushing of fresh air for 45 s. Odors used were 3-octanol (OCT, Sigma-Aldrich) and 4-methylcyclohexanol (MCH, Fluka) diluted in heavy mineral oil, 1.5×10^{-3} and 1.0×10^{-3} for OCT and MCH respectively. To test memory, trained flies were transferred to a T-maze and allowed to choose between the CS+ and CS- odors for 2 min. Performance index (PI) was calculated from the distribution of flies in the two odors at the end of the choice, i.e. $PI = (fraction_{CS-} - fraction_{CS+})\times100$. A PI of 100 indicates that all flies avoid CS+, while a PI of 0 indicates a 50:50 distribution of flies between CS+ and CS- odors. To remove odor bias, each PI was the mean of two reciprocal groups, where OCT and MCH were used as CS+ respectively. Massed training consisted of four repetitive training sessions as above without an interval in between sessions. In experiments using weakened training, the number of electric shock pulses was reduced from 12 to 6 and 2 or the voltage was reduced from 60 to 20 and 10 V. When the number of shock pulses was reduced, the durations for odor exposures were also correspondingly decreased. For 3 min memory, flies were tested

immediately after training. For longer memory retention, flies were placed in vials with the same content as before training until the memory testing.

For the "Interference" experiments, flies were subjected to an initial learning session (OCT/ MCH), either one-session (Fig. 1C) or four-session massed (Fig. 2C) training, and then were given retroactive interference learning using a novel pair of odors, ethyl acetate (EA, Alfa Aesar) and isoamyl acetate (IA, Avocado Research Chemicals) diluted in heavy mineral oil at a concentration of 2.0×10^{-3}. Following previously reported protocols, interference learning was either at 1.5 hr after the initial one-session learning (Fig. 1C) or immediately follows the termination of the four-session massed training (Fig. 2C).

Reversal learning was performed as in our previous study. The odor (either OCT or MCH) paired with shock in the first session was not paired with shock in the second session and vice versa. Flies were immediately tested for a choice between the two reversely trained odors. PI was calculated as in normal learning, except that the odor last paired with shock was taken as the "CS+". In the trace conditioning protocol, a 30-s stimulus-free period was inserted between the termination of "CS+" and the US". It is assumed that the successful learning flies to hold a short-lasting internal representation of the "CS+" to bridge the temporal gap between CS+/US.

Cold Shock

Trained flies were transferred to a pre-chilled empty glass vial and immersed in an ice-water mixture for 2 min. The anesthetized flies were then allowed to recover in vials (with the same content as before training) at 25℃ for 1 hr before testing for memory.

Gal4 expression patterns

Examination of Gal4 expression patterns followed a protocol described previously. In comparison of 5-HT1B-Gal4 expression without and with MB-Gal80, GFP antibody (rabbit polyclonal to GFP, 1:1000, Invitrogen) and nc82 (1:25, Developmental Studies Hybridoma Bank) counterstaining were used. In the estimation of expression levels of different MB Gal4 lines, the Gal4 driver were crossed to *MB247-dsRed*; *10XUAS-IVS-Syn21-GFP-p10,* which bear both a GFP reporter and dsRed under the control of a

mushroom body promoter (1). 2- to 6-day old female adult female offspring were dissected. The brains were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) in PBS for 1 hr, and were later mounted without antibody staining. Images were acquired on a Zeiss LSM510 confocal microscope using a 20× objective (NA = 0.75). The image data were processed and adjusted for brightness and contrast in Fiji/ImageJ and Matlab.

Statistics.

Statistical analysis was performed in GraphPad Prism 6. Two-group comparisons were analyzed using two-tailed t tests. Multiple-group comparisons were analyzed by one-way or two-way ANOVAs followed by Bonferroni's multiple comparisons. $p < 0.05$ were considered statistically significant and are marked with an asterisk, while n.s. indicates non-significant differences ($p > 0.05$).

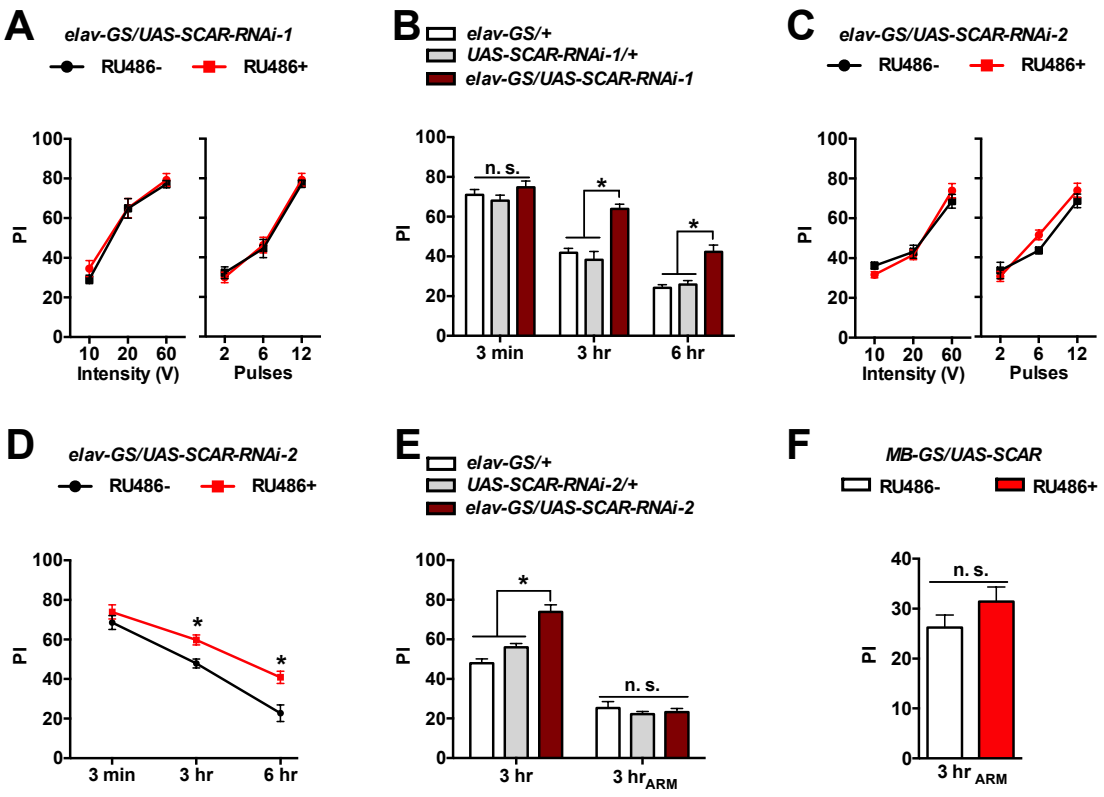

Fig. S1. Additional behavioral characterization of the effects of SCAR on ASM.

(A) No effect on initial learning by SCAR knockdown as assayed with weakened training protocols. Memory performance was measured immediately (within 3 min) after one-session training with reduced electric shock intensity or a varied number of electric shock pulses.

(B) *SCAR*-RNAi expressing flies (*elav-GS/UAS-SCAR-RNAi-1*) showed slower memory decay at 3 and 6 hr after one-session training when compared with the parental controls (*elav-GS/+ and UAS-SCAR-RNAi-1/+*) without affecting 3-min memory.

(C) Normal performance in initial learning for a second *SCAR*-RNAi. No differences were found between the group with *SCAR*-RNAi-2 expression (*elav-GS/UAS-SCAR-RNAi-2*, RU486+) and the "RU486-" control group.

(D) Memory retention curve for *SCAR*-RNAi-2. For flies with *SCAR*-RNAi-2 expression, 3-min memory was normal, while 3-hr and 6-hr memory were higher.

(E) *SCAR*-RNAi-2 expression increased 3 hr memory, without affecting the ARM component.

(F) Overexpression of SCAR in the adult MB neurons (*MB-GS/UAS-SCAR*, RU486+) did not affect 3 hr_{ARM} after one-session training.

Data are means ± SEM. *$p < 0.05$. n.s., non-significant. $n = 8$.

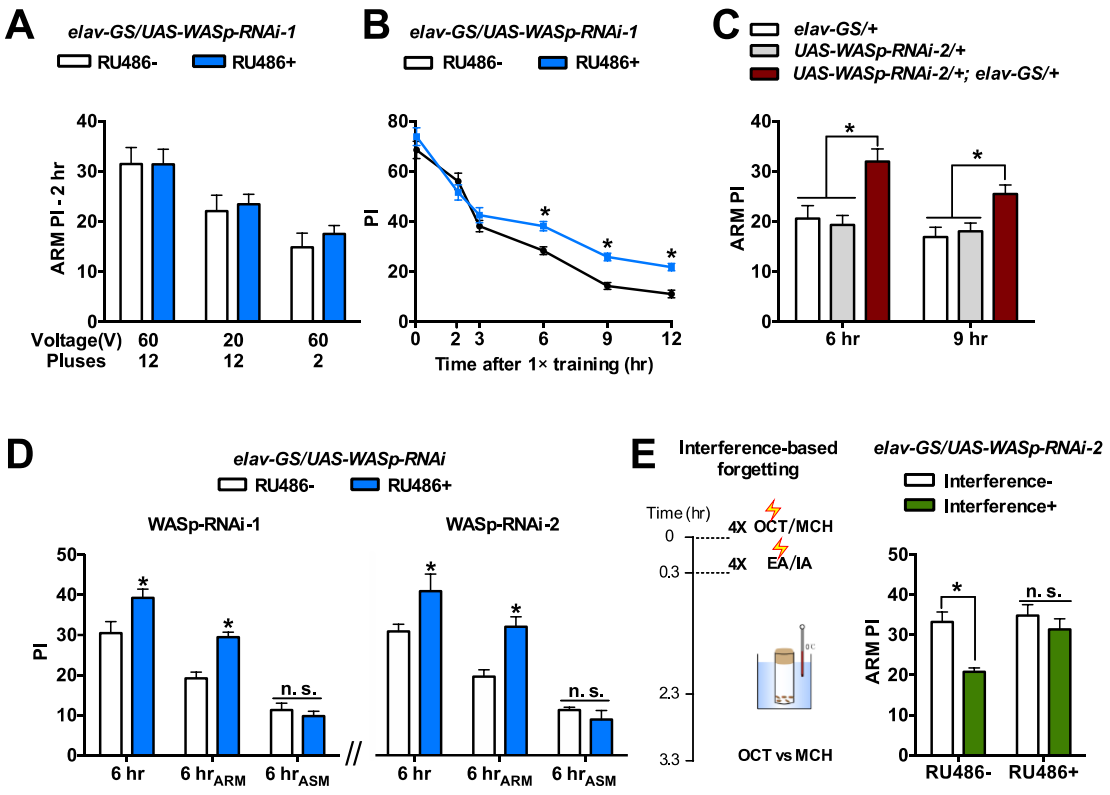

Fig. S2. Additional behavioral characterization of the effects of WASp on ARM.

(A) WASp RNAi expression did not affect 2 hr_{ARM} after one-session training. Training was weakened by reducing electric shock intensity or decreasing the number of electric shock pulses. There were no statistically significant differences between the "RU486+" and "RU486-" groups.

(B) Memory retention curve of *WASp*-RNAi-expressing flies after one-session training without cold shock. Statistically significant differences were observed only at later time points.

(C) *WASp*-RNAi-expressing flies (*elav-GS/UAS-WASp-RNAi-2*) had higher ARM performance at 6 and 9 hr after one-session training when compared with their parental controls (*elav-GS/+ and UAS-WASp-RNAi-2/+*).

(D) WASp knockdown specifically affected ARM.

(E) Confirmation of the effects on interference-based forgetting with *WASp*-RNAi-2. The paradigm was as in Fig. 2C. Comparison was between groups without and with interference learning.

Data are means ± SEM. *$p < 0.05$. n.s., non-significant. $n = 8$.

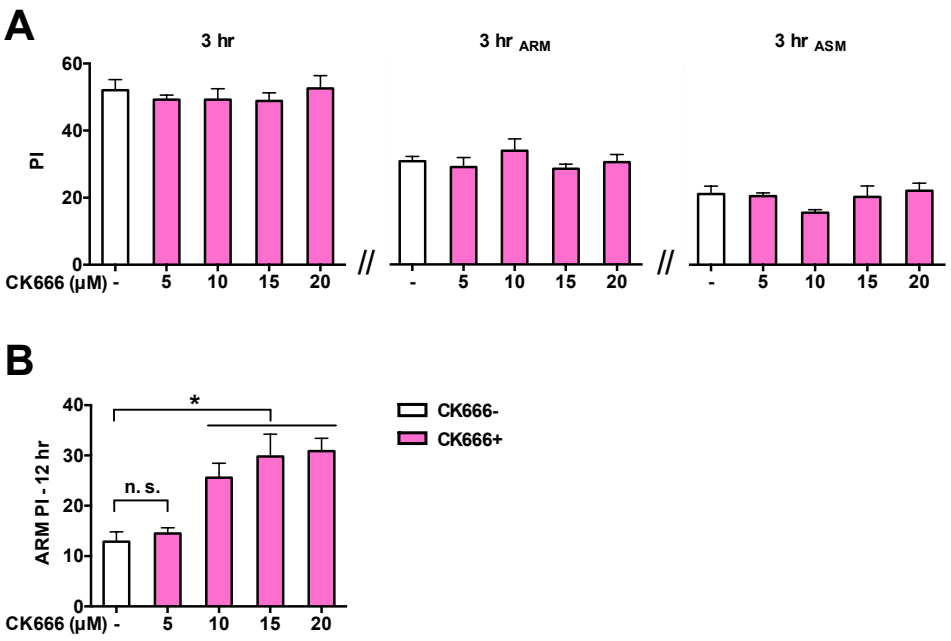

Fig. S3. Dosage-dependent effect of Arp2/3 complex inhibitor on ARM.

(A) Flies were fed with vehicle alone, or different concentration of the Arp2/3 complex inhibitor CK666. There were no statistically significant differences in 3-hr memory, either total, ARM or ASM. For all ARM measurement, flies received cold-shock at 1 hr before testing. ASM was inferred from the subtraction of ARM from the intact memory.

(B) Dosage-dependent effect of CK666 on ARM at 12 hr.

Data are means ± SEM. *$p < 0.05$. n.s., non-significant. $n = 8$.

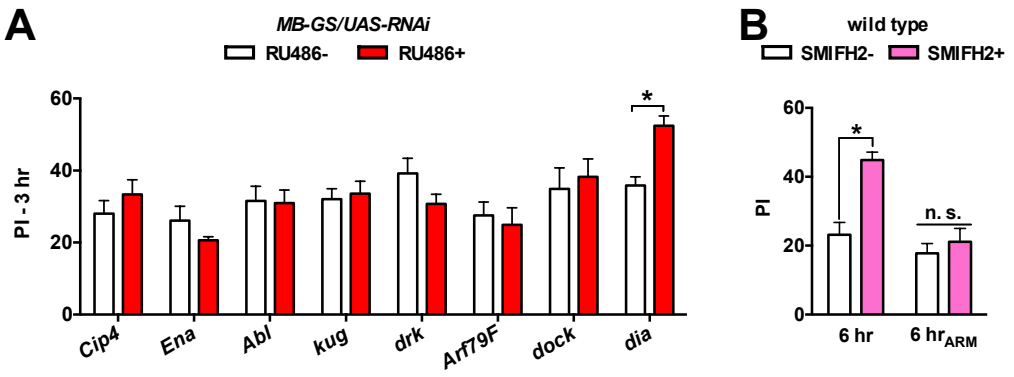

Fig. S4. RNAi screen of the interacting proteins of SCAR/WAVE complex and the test of SMIFH2.

(A) RNAi was expressed in the adult MB neurons and 3 hr memory was examined. Among the RNAi lines screened, only Dia RNAi resulted in a higher 3 hr memory.

(B) Feeding flies with 2.5 μM SMIFH2, a formin inhibitor, increased 6 hr memory without affecting ARM.

Data are means ± SEM. *$p < 0.05$. n.s., non-significant. n = 8.

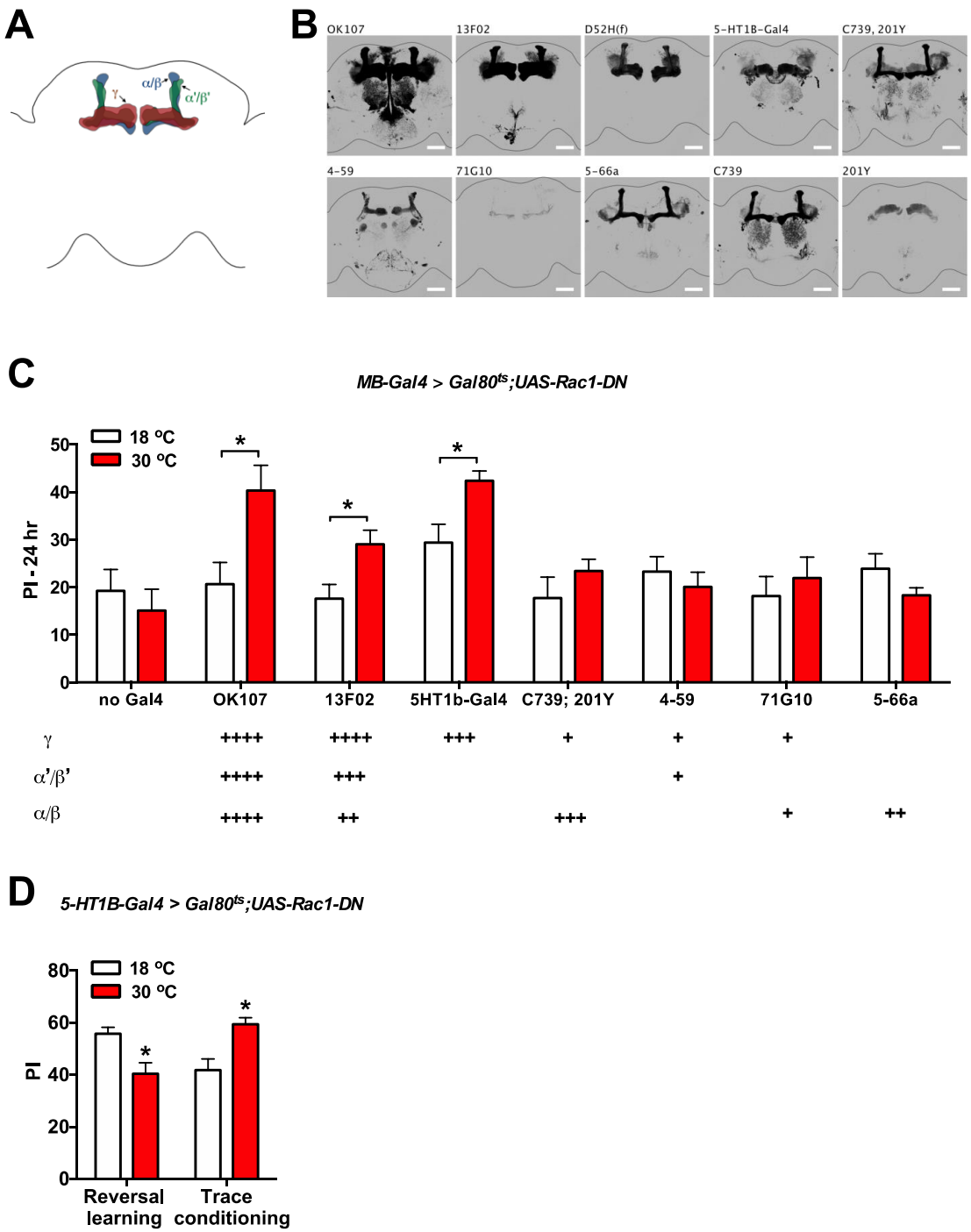

Fig. S5. Expression of dominant-negative Rac1 in the MB γ neurons is sufficient to suppress forgetting.

(A) The three types of MB neurons send axons to the anterior side of the brain to form different lobes of the MB: γ, α'/β', and α/β.

(B) Validation of Gal4 expression patterns. Images are maximum z-projections with *10XUAS-IVS-Syn21-GFP-p10* as a reporter. Gal4 lines used in the behavioral experiments include OK107 and 13F02 that label all three MB types, 5-HT1B-Gal4 that labels only the γ type, 5-66a that labels only the α/β type, and 4-59 that labels the α'/β' type and weakly the γ type. In addition, 71G10 carries an enhancer fragment from *sif*, which encodes a GEF protein that activates Rac1. The expression pattern of 71G10 covers both the γ and α/β types, albeit very weakly. Some Gal4 lines used in the previous study were also shown. D52H(f) have broad expression in the γ and α/β types and show memory increment when used to drive dominant-negative Rac1 expression. C739 and 201Y have primary expression in the α/β and γ types respectively; both Gal4 lines did not show a phenotype in driving dominant-negative Rac1 expression. The combined Gal4 line C739, 201Y integrate both transgene into the same fly. Scar bar, 50 μm.

(C) Effects on 24 hr memory after one-session training by driving expression of dominant-negative Rac1 (*UAS-Rac1-DN*) in different subsets of MB neurons. Statistically significant differences were found for OK107, 13F02 and 5-HT1B-Gal4; for all the other groups. The expression levels in different MB neuron types were estimated using MB247-dsRed as a reference and were normalized to the level in OK107.

(D) Effects of Rac1-DN expression on reversal learning and trace conditioning with 30-s trace interval.

Data are means ± SEM. *$p < 0.05$. n = 6-10.

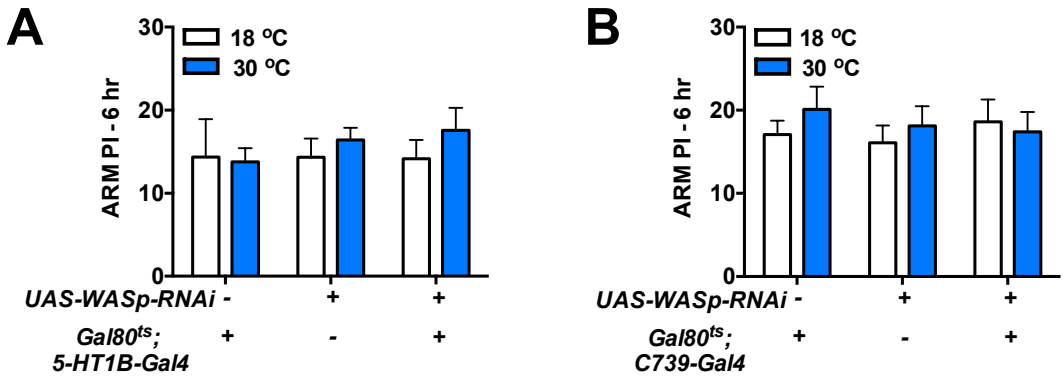

Fig. S6. Testing the effects on 6 hr$_{ARM}$ by restricting WASp RNAi expression in subpopulations of the MB neurons.

WASp-RNAi-1 was conditionally expressed in the adult MB γ neurons (A) or α/β neurons (B) with 5-HT1B-Gal4 and C739 drivers respectively. There were no statistically significant differences in the 6 hr$_{ARM}$ performance of the "18°C" and the "30°C" groups.

Data are means ± SEM. n.s., non-significant. n = 8.

References

1. Pech U, Pooryasin A, Birman S, & Fiala A (2013) Localization of the contacts between Kenyon cells and aminergic neurons in the Drosophila melanogaster brain using SplitGFP reconstitution. *J Comp Neurol* 521(17):3992-4026.