Ras acts as a molecular switch between two forms of consolidated memory in *Drosophila*

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Long-lasting, consolidated memories require not only positive biological processes that facilitate long-term memories (LTM) but also the suppression of inhibitory processes that prevent them. The mushroom body neurons (MBn) in *Drosophila melanogaster* store protein synthesis-dependent LTM (PSD-LTM) as well as protein synthesis-independent, anesthesia-resistant memory (ARM). The formation of ARM inhibits PSD-LTM but the underlying molecular processes that mediate this interaction remain unknown. Here, we demonstrate that the Ras→Raf→rho kinase (ROCK) pathway in MBn suppresses ARM consolidation, allowing the formation of PSD-LTM. Our initial results revealed that the effects of Ras on memory are due to postacquisition processes. *Ras* knockdown enhanced memory expression but had no effect on acquisition. Additionally, increasing Ras activity optogenetically after, but not before, acquisition impaired memory performance. The elevated memory produced by *Ras* knockdown is a result of increased ARM. While *Ras* knockdown enhanced the consolidation of ARM, it eliminated PSD-LTM. We found that these effects are mediated by the downstream kinase Raf. Similar to *Ras*, knockdown of *Raf* enhanced ARM consolidation and impaired PSD-LTM. Surprisingly, knockdown of the canonical downstream extracellular signal-regulated kinase did not reproduce the phenotypes observed with *Ras* and *Raf* knockdown. Rather, Ras/Raf inhibition of ROCK was found to be responsible for suppressing ARM. Constitutively active ROCK enhanced ARM and impaired PSD-LTM, while decreasing ROCK activity rescued the enhanced ARM produced by *Ras* knockdown. We conclude that MBn Ras/Raf inhibition of ROCK suppresses the consolidation of ARM, which permits the formation of PSD-LTM.

consolidation | memory | *Drosophila* | Ras | Raf

Consolidation is a process required for the formation of long-lasting memories (1–4). This process of converting memories that are initially sensitive to disruption from a variety of insults to more resilient ones is well conserved and many of its characteristics are shared across species. For example, memory in invertebrates and vertebrates lasts longer following multiple spaced training sessions (5–7), undergoes both molecular/cellular and systems consolidation (8, 9), and can be disrupted by inhibition of protein synthesis (6, 10).

The fruit fly *Drosophila melanogaster* forms two distinguishable types of consolidated aversive olfactory memory: 1) anesthesia-resistant memory (ARM), which reportedly decays to negligible levels by 4 d after conditioning, can be generated by a single training session; 2) protein synthesis-dependent long-term memory (PSD-LTM), which shows limited decay, requires spaced training (6). These two types of consolidated memory are not independent from one another. The formation of ARM impairs either the formation or expression of PSD-LTM (11, 12). Although circuit mechanisms possibly responsible for this relationship are beginning to be dissected (13), the molecular requirements in the mushroom body (MB), a brain region critical for the storage and retrieval of PSD-LTM and ARM (14–16), remain unknown.

The small GTPase Ras85D (Ras) is a *Drosophila* homolog of the mammalian Ras family genes KRAS, NRAS, and HRAS (17). Activated Ras proteins act as signaling switches, initiating signaling cascades through multiple downstream effector proteins (18). Precise induction and regulation of Ras activity is essential for mammalian synaptic plasticity and memory (19). Although upstream regulators of Ras, like NF1 and DRK, have been explored for their roles in *Drosophila* learning and memory (20, 21) Ras itself has not been thoroughly examined. A large RNA interference (*RNAi*) screen identified *Ras85D* as a memory suppressor gene but did not detail its specific role in memory suppression (22).

Here, we report that Ras activity in the MB acts as a switch between the two forms of consolidated memory, required both for PSD-LTM and inhibition of ARM. Increasing Ras activity dramatically reduced memory expression. We determined this effect was due Ras regulation of ARM. Knockdown of *Ras* enhanced the consolidation of ARM, leading to an overall increase in memory, while *Ras* knockdown eliminated PSD-LTM following spaced training. Although we found the effect of Ras on both ARM and PSD-LTM to be mediated by Raf, it is independent from the canonical downstream extracellular signal-regulated kinase (ERK). Instead, Ras/Raf-mediated inhibition of rho kinase (ROCK) suppresses ARM and is required for PSD-LTM.

Results

MB Neuron Ras Knockdown Enhances Memory Expression. A large *RNAi*-based genetic screen identified dozens of genes, including *Ras85D* (*Ras*), that suppress aversive olfactory memory (22). Pan-neuronal knockdown of *Ras* resulted in enhanced memory

Significance

The formation of long-lasting memories requires the suppression of factors that negatively regulate long-term memories (LTM). To date, research has largely focused on processes that positively regulate memory acquisition and consolidation. Here, we describe a signaling pathway that acts as a switch between two forms of memory, suppressing one in favor of the other. Inhibition of rho kinase (ROCK) by Ras/Raf activity suppresses anesthesia-resistant memory (ARM) and is required for the formation of protein synthesis-dependent LTM (PSD-LTM). Reduction in Ras/Raf and increased ROCK activity promotes the consolidation of ARM and prevents PSD-LTM. This work 1) defines an LTM suppressor signaling pathway that bidirectionally modulates consolidation and 2) provides a molecular mechanism for a previously observed antagonism between ARM and PSD-LTM.

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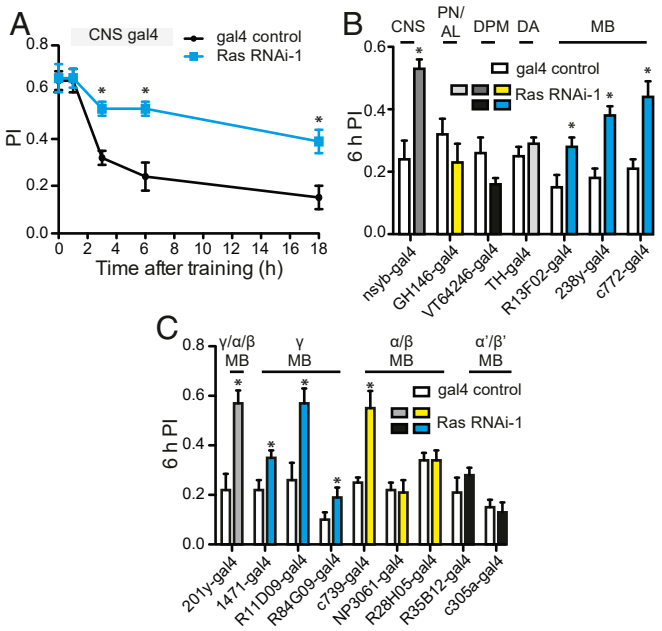

Fig. 1. Knocking down Ras in MBn enhances memory. (*A*) Knockdown of *Ras* expression with the pan-neuronal *nsyb-gal4* enhanced memory. *Ras-RNAi*–expressing flies exhibited elevated memory expression. Student's *t* test, $*P < 0.05$. $n = 6$ to 13. Error bars indicate SEM. (*B*) The *Ras-RNAi* line was crossed to the indicated *gal4* lines. *Ras* knockdown in MBn (*R13F02-Gal4, 238y-gal4, c772-Gal4*) enhanced memory tested at 6 h. There was significant variation in performance across the various *gal4* lines, so that each *gal4* line was used as a control for its corresponding *RNAi* group. Student's *t* test, $*P < 0.05$. $n = 6$ to 12. Error bars indicate SEM. (*C*) The *Ras-RNAi* line was crossed to the indicated *gal4* lines. *Ras* knockdown in both γ and α/β MBn (*201y-gal4*) and in γ MBn only (*1471-Gal4, R11D09-Gal4, R84G09-Gal4*) enhanced memory. Student's *t* test, $*P < 0.05$. $n = 13$ to 22. Error bars indicate SEM.

expression beginning at 3 h after single-cycle training and lasting until at least 18 h after training (Fig. 1*A*). There was no effect on memory performance immediately or 1 h after training. To resolve where *Ras* expression is required for normal memory, we expressed *Ras RNAi-1* with *gal4* lines that drive expression in specific brain regions (Fig. 1*B*). *RNAi* expression in MB neurons (MBn) with three different *gal4* drivers enhanced 6-h memory. Within the subdivided compartments of the MB, expression of *Ras RNAi-1* with three different γ MBn *gal4* drivers enhanced memory (Fig. 1*C*). All three Gal4s drove strong expression of green fluorescent protein in the main calyx and γmain (*SI Appendix*, Fig. S1*A*). *R84G09-Gal4* and *R11D09-Gal4* also drove expression in ventral accessory calyx and γd, while *1471-Gal4* did not. *R84G09-Gal4* was weakly expressed in α/β. Although *1471-Gal4* is expressed in many neurons outside of the MB, *R11D09-Gal4* and *R84G09-Gal4* expression patterns were much more restricted to the MB. There does not appear to be any overlap of non-MB expression between *R11D09-Gal4* and *R84G09-Gal4*, indicating the effect of *Ras RNAi* is due to knockdown in γ MBn. Expression with the α/β MBn driver *c739-gal4* enhanced memory, but two other α/β MBn *gal4* drivers did not. Each *gal4* driver expresses *gal4* at different levels and in different cells. It is possible that the two α/β MBn drivers that failed to produce a phenotype promoted insufficient expression of the *RNAi* to produce a measurable phenotype. Alternatively, expression of the *RNAi* in regions outside of α/β MBn by *c739-Gal4* could be responsible for the memory enhancement. *Ras RNAi* expression in γ MBn (*1471-Gal4*) enhanced memory compared to both *gal4*-only and *uas*-only control flies (*SI Appendix*, Fig. S1*B*), ruling out effects due to potentially unknown genetic differences in the stock lines. Expression of an MB-expressed *gal4* repressor, *MBgal80*, rescued the *Ras* knockdown-induced memory enhancement, confirming the phenotype is due to *RNAi* expression in MBn (*SI Appendix*, Fig. S1*C*). These results map the memory expression enhancement of *Ras85D* knockdown to γ MBn.

Memory Enhancement by MBn Ras Knockdown Is Not due to Effects on Development or Acquisition. Because neuronal Ras signaling is involved in both development (23) and the physiology of mature neurons (24), we used the TARGET ($gal80^{ts}$) system to temporally control expression of *Ras RNAi* in γ MBn (*1471-Gal4*). The $gal80^{ts}$ transgene restricts Gal4 activity at 18 °C but permits Gal4 activity at 30 °C (25). *Ras RNAi-1* had no effect on memory when expression was induced during development and restricted in adulthood (*SI Appendix*, Fig. S2*A*). Conversely, memory was enhanced with *RNAi* expression restricted during development and induced in adulthood (18 °C to 30 °C). Expression of two additional *Ras RNAi* transgenes specifically during adulthood increased memory performance (*SI Appendix*, Fig. S2 *B* and *C*), confirming the effect of *Ras* knockdown on memory expression occurs in the adult stage and is not due to a developmental effect. Sequences for *Ras RNAi-1* and *RNAi-2* partially overlap, with *RNAi-1* targeting exon 2 and *RNAi*-2 targeting exons 2 and 3. *Ras RNAi-3* has a unique target sequence located in exon 4. These results indicate that the effects on memory are due to *Ras85D* knockdown rather than an off-target effect. We confirmed that all three *Ras RNAi*s reduced Ras protein levels (*SI Appendix*, Fig. S2*D*).

Similar to pan-neuronal *Ras* knockdown (Fig. 1*A*), *Ras* knockdown in γ MBn (*1471-Gal4*) produced a long-lasting enhancement in memory but no difference immediately or 1 h after training (Fig. 2*A*), strongly suggesting that γ MBn Ras does not affect acquisition processes. However, it is possible a difference in initial memory formation was masked by high scores produced with the single-cycle, 12-shock training program. We avoided this ceiling effect by varying the shock number. There were no differences in memory expression between *Ras* knockdown and control flies using six, three, or even one shock pulse, confirming a lack of effect on acquisition (Fig. 2*B*). Additionally, *Ras RNAi* expression in γ MBn (*1471-Gal4*) did not alter naive avoidance of the two odors used at their working concentration or at a 100-fold dilution (*SI Appendix*, Table S1). Avoidance of electric shock pulses was also indistinguishable from the controls. Therefore, the memory enhancement caused by *Ras* knockdown is not attributable to enhanced acquisition of memory.

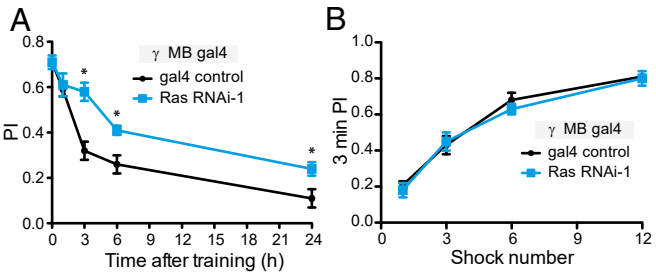

Fig. 2. *Ras* knockdown in MBn does not alter acquisition. (*A*) Knockdown of *Ras* expression with γ MBn *1471-Gal4* enhanced memory. *Ras-RNAi*–expressing flies performed similarly to control flies immediately and 1 h after acquisition but exhibited elevated memory at each subsequent time point tested. Student's *t* test, $*P < 0.05$. $n = 6$ to 12. Error bars indicate SEM. (*B*) Knocking down *Ras* expression with γ MBn *1471-Gal4* had no effect on memory acquisition. *Ras-RNAi*–expressing flies performed similarly to control flies immediately after acquisition when trained with the indicated number of shocks. Student's *t* test, $*P < 0.05$. $n = 6$. Error bars indicate SEM.

Ras Suppresses ARM and Is Required for PSD-LTM. We tested the effect of increased Ras signaling in γ MBn after acquisition by expressing optogenetically activatable Son of Sevenless (OptoSOS), a Ras activator (26). First, we confirmed that blue-light exposure is sufficient to stimulate Ras via OptoSOS in the MB. Flies were exposed to blue light for 10 to 30 min prior to brain dissection and fixation. Flies expressing OptoSOS in MBn (*R13F02-Gal4*) (Fig. 3 *A* and *B* and *SI Appendix*, Fig. S3*A* and Movie S1) or γ MBn (*1471-Gal4*) (*SI Appendix*, Fig. S3 *B* and *C* and Movie S2) had elevated MB phospho-ERK (pERK) levels only after blue-light stimulation, while *Gal4* control flies, which were not expressing OptoSOS, did not. There was no difference in pERK in control regions where no OptoSOS was expressed: antennal lobes for *R13F02-Gal4* and MB vertical lobes for *1471-Gal4*. Activation of OptoSOS by exposure to blue light for 20 min immediately after training produced a significant decrease in 3-h memory compared to the same genotype not exposed to blue light, or the same genotype exposed to blue light 20 min prior to training (Fig. 3*C*). Blue-light exposure had no effect on control genotypes. These results demonstrate Ras activity after training has a long-lasting effect on memory. Based on these post-acquisition effects by activated Ras, we predicted that the enzyme regulates memory consolidation in γ MBn.

In *Drosophila*, exposure to a posttraining cold shock eliminates labile memory but leaves consolidated ARM intact. *Ras RNAi*-expressing flies had elevated memory scores with and without cold shock, indicating that the memory enhancement resulting from *Ras* knockdown is due to increased ARM (Fig. 3*D*). The net increase in memory expression in both the "no cold shock" and "cold shock" conditions was similar, indicating that the memory enhancement is due primarily and perhaps exclusively to increased ARM rather than labile memory. We confirmed the effect of *Ras* knockdown on ARM using a second γ MBn driver (*R11D09-Gal4*) (*SI Appendix*, Fig. S3*D*). Memory 1 d after massed training is thought to be comprised of only ARM (6). *Ras* knockdown resulted in enhanced 24-h memory following massed training, confirming the role of Ras in ARM (*SI Appendix*, Fig. S3*E*). ARM consolidation is completed relatively fast, beginning immediately after training and reaching maximum levels by 1 h (1). To probe the time course of Ras involvement in ARM consolidation we measured the accumulation of ARM over time. Flies were subjected to cold shock at various times after training and ARM was measured 3 h after training. *Ras* knockdown resulted in enhanced accumulation of ARM by 2 h but not immediately or 1 h after training (Fig. 3*E*). This indicates that *Ras* knockdown does not alter the acquisition or accumulation rate of ARM but instead extends the ARM consolidation window from 1 h to at least 2 h after training. Combined with the evidence that Ras activity immediately after training reduces memory, we conclude that Ras activity normally limits the time window for ARM consolidation, perhaps by directing accumulated ARM into alternative memory expression/consolidation pathways.

In contrast to ARM, PSD-LTM depends on new protein synthesis and can be suppressed by inhibitors of transcription and translation. *Ras* knockdown with either *1471-Gal4* (Fig. 3*F*) or *R11D09-Gal4* (*SI Appendix*, Fig. S3*F*) resulted in no PSD-LTM 24 h after spaced training. Compared to the control genotype, where there was a pronounced effect of cycloheximide, *Ras* knockdown flies were insensitive to protein synthesis inhibition. This indicates that Ras is required for PSD-LTM following spaced training. Although *Ras* knockdown prevents the formation of PSD-LTM, these flies still generated robust 24-h memory. Based on our prior results indicating that *Ras* knockdown enhances ARM and on other studies showing that most or all of labile memory is eliminated by 24 h after training, we conclude that *Ras* knockdown promotes the formation of ARM at the expense of PSD-LTM. In other words, normal Ras activity promotes the formation of PSD-LTM at the expense of ARM.

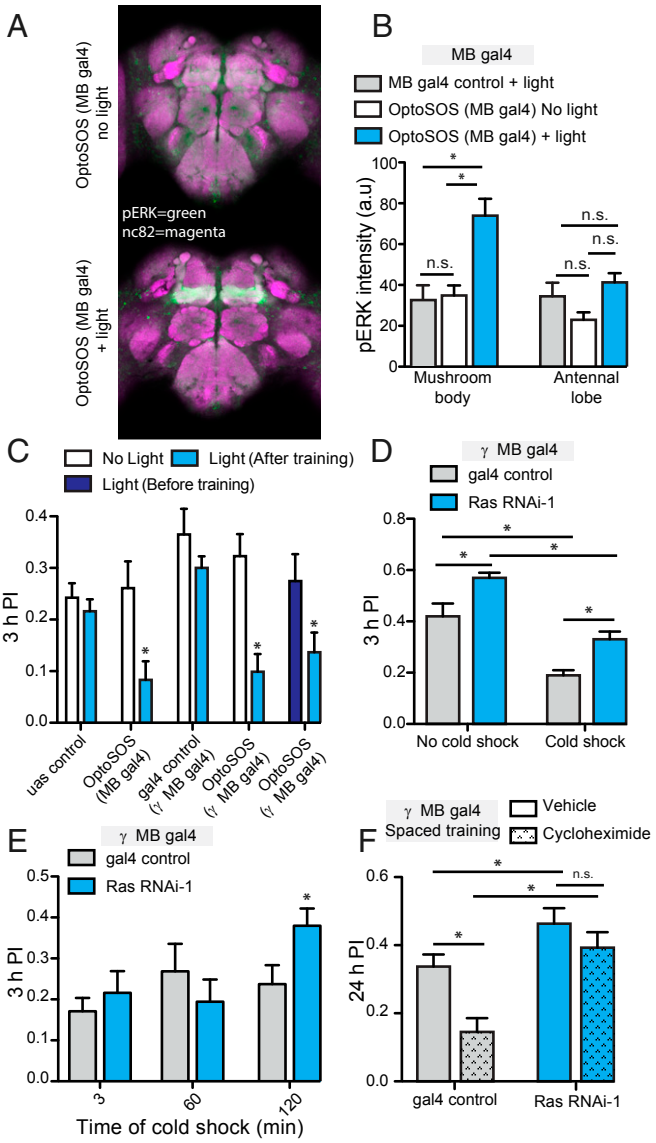

Fig. 3. Ras regulates consolidation. (*A*) Immunostaining for pERK following OptoSOS stimulation. Flies expressing *OptoSOS* in MBn (*R13F02-Gal4*) or *gal4* control flies were exposed to 448-nm blue light for 10 to 30 min prior to brain dissection and fixation under dim red light. Brains were stained with pERK and nc82. Images are average projections across the MB. (*B*) Blue-light stimulation of OptoSOS in MBn increased pERK levels. Fluorescent intensity was quantified in the MB horizontal lobes and center of the antennal lobes. One-way ANOVA with Bonferonni post hoc comparisons. $*P < 0.05$. $n = 12$. Error bars indicate SEM. (*C*) Activation of the Ras activator SOS after training reduced 3-h memory. Flies expressing *OptoSOS* in MBn (*R13F02-Gal4*) or in γ MBn (*1471-Gal4*) were exposed to blue light immediately after training, 20 min before training, or not at all. Flies were tested 3 h after training. Student's *t* test, $*P < 0.05$. $n = 6$ to 8. Error bars indicate SEM. (*D*) Knockdown of *Ras* expression with the γ MBn *1471-Gal4* enhanced 3-h ARM. At 2 h after training flies were subjected to 4°C for 2 min. Flies were tested 3 h after training. Two-way ANOVA with Bonferroni post hoc comparisons, $*P < 0.05$. $n = 11$ to 15. Error bars indicate SEM. (*E*) Knockdown of *Ras* expression with the γ MBn *1471-Gal4* prolonged the consolidation time for ARM. Flies were subjected to 4°C for 2 min at the indicated times posttraining. Flies were tested 3 h after training. Student's *t* test, $*P < 0.05$. $n = 7$ to 10. Error bars indicate SEM. (*F*) Knocking down *Ras* in γ MBn (*1471-Gal4*) reduced PSD-LTM following spaced training. Flies were fed 35 mM cycloheximide or vehicle overnight. Flies were trained with spaced conditioning five times and tested 24 h later. Two-way ANOVA with Bonferroni post hoc comparisons, $*P < 0.05$. $n = 10$. Error bars indicate SEM. n.s., not significant.

Ras/Raf Signaling Regulates Consolidation. Activated Ras signals through several defined pathways. RasV12 is a constitutively active form of Ras that increases signaling to all downstream effectors, including Raf and phosphatidylinositol 3-kinase (PI3K) (27, 28). Ras interacts with downstream effectors through different Ras binding domains and point mutations in these domains impair signaling through some effectors in favor of others (29). RasV12S35 and RasV12C40 are both constitutively active due to the V12 mutation, but RasV12S35 preferentially activates Raf while RasV12C40 preferentially activates PI3K (30, 31). To determine which pathway Ras signals through to regulate memory, we expressed constitutively active *Ras* mutants in all MBn (*R13F02-Gal4*). Adult expression of *RasV12*, which activates all downstream Ras pathways, impaired 3-h memory relative to its uninduced control (Fig. 4*A*). Raf-activating *RasV12S35* also impaired 3-h memory, while the PI3K-activating *RasV12C40* had no effect on memory scores. Restricting expression to γ MBn (*1471-Gal4*), we confirmed that *RasV12S35* impaired 3-h memory (*SI Appendix*, Fig. S4*A*). There was no difference for control flies between the uninduced and induced conditions indicating that shifting temperatures did not alter memory expression.

Expression of *Raf RNAi-1* in γ MBn (*1471-Gal4*) resulted in increased memory at 3 and 6 h after training (Fig. 4*B*) and this effect was observable when *RNAi* expression was restricted to adulthood (*SI Appendix*, Fig. S4*B*). *Raf* knockdown did not alter acquisition (*SI Appendix*, Fig. S4*C*) and there was no difference in avoidance of odors or electric shock pulses used for training (*SI Appendix*, Table S1). A second *RNAi* line, *Raf RNAi-2*, enhanced 3- and 6-h memory when expressed in γ MBn (*1471-Gal4*) (*SI Appendix*, Fig. S4*D*). γ MBn knockdown of *Raf* with *R11D09-Gal4* also caused enhanced 3-h memory (*SI Appendix*, Fig. S4*E*). Similar to *Ras* knockdown, *Raf* knockdown enhanced ARM (Fig. 4*C*). *Raf* knockdown extended the time for ARM consolidation beyond the normal 1-h posttraining period observed in control flies (*SI Appendix*, Fig. S4*F*). Moreover, *Raf* knockdown impaired PSD-LTM, demonstrated by the lack of effect from cycloheximide treatment (Fig. 4*D*). The effects of *Raf* knockdown on ARM and PSD-LTM are identical to those of *Ras* knockdown. Together with the results demonstrating that constitutive Ras/Raf signaling impairs memory, we conclude that Raf-dependent Ras signaling limits ARM and promotes PSD-LTM.

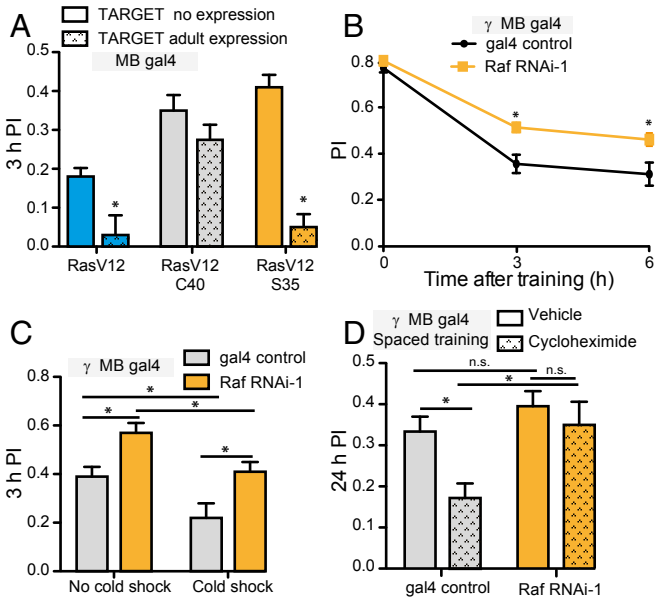

Fig. 4. Raf signaling mediates Ras consolidation effect. (*A*) Expressing constitutively active Ras (*RasV12*) or constitutively active Ras that only activates downstream Raf (*RasV12S35*) impaired 3-h memory relative to its control genotype. No effect was observed upon expressing the *RasV12C40* transgene. Expression in MBn (*gal80ts;R13F02-Gal4*) was restricted during development and either left uninduced (18-18 °C) or induced (18-30 °C) during adulthood. Student's *t* test, $*P < 0.05$. $n = 11$. Error bars indicate SEM. (*B*) Knockdown of *Raf* expression with the γ MBn *1471-Gal4* enhanced memory. *Raf-RNAi*–expressing flies performed similar to control flies immediately after acquisition but exhibited elevated memory at each subsequent time point tested. Student's *t* test, $*P < 0.05$. $n = 13$ or 14. Error bars indicate SEM. (*C*) Knockdown of *Raf* expression with the γ MBn *1471-Gal4* enhanced 3-h ARM. Two hours after training flies were subjected to 4°C for 2 min. Flies were tested 3 h after training. Two-way ANOVA with Bonferroni post hoc comparisons, $*P < 0.05$. $n = 12$ to 14. Error bars indicate SEM. (*D*) Knocking down *Raf* in γ MBn (*1471-Gal4*) reduced PSD-LTM following spaced training. Flies were fed 35 mM cycloheximide or vehicle overnight. Flies were trained with spaced conditioning five times and tested 24 h later. Two-way ANOVA with Bonferroni post hoc comparisons, $*P < 0.05$. $n = 6$. Error bars indicate SEM. n.s., not significant.

Consolidation Is Regulated by Downstream ROCK. Mitogen-activated protein kinase kinase (MEK) and ERK are downstream of Raf in the canonical Ras/Raf signaling cascade. To determine if Ras/Raf regulation of ARM consolidation occurs through MEK and ERK activity, we tested 3-h memory following *MEK* and *ERK* knockdown. Surprisingly, knockdown of neither *MEK* nor *ERK* (*SI Appendix*, Fig. S5 *A* and *B*) resulted in enhanced memory. In contrast to the memory enhancement produced by *Ras* and *Raf* knockdown, *ERK* knockdown reduced memory. We confirmed that the *ERK RNAi*s reduced ERK protein levels (*SI Appendix*, Fig. S5*D*). These results argue that Ras/Raf regulation of ARM is independent of ERK. Ras/Raf regulation of PSD-LTM also appears to be ERK-independent. Following spaced training, *ERK* knockdown had no significant effect on PSD-LTM (*SI Appendix*, Fig. S5*C*), indicating that, unlike Ras and Raf, γ MBn ERK does not play a significant role in PSD-LTM.

Several ERK-independent Raf signaling pathways have been established from prior studies. In mammalian cells, ROCK activity is inhibited through a direct interaction with activated Raf (32). Although it has not been demonstrated that Ras, Raf, and ROCK interact specifically in MBn, all three are expressed in γ MBn (33). If Ras/Raf activity suppresses ARM through the inhibition of ROCK, increasing ROCK activity should enhance memory. Adult expression of the constitutively active kinase domain of ROCK (*ROCK CA*) in γ MBn (*1471-Gal4*) significantly enhanced 3-h memory (*SI Appendix*, Fig. S5*E*). γ MBn expression of ROCK CA with *R11D09-Gal4* also enhanced 3-h memory (*SI Appendix*, Fig. S5*G*). Conversely, knockdown of *ROCK* reduced 3-h memory without affecting immediate memory (*SI Appendix*, Fig. S5*F*).

Like *Ras* and *Raf* knockdown, expression of *ROCK CA* in γ MBn (*1471-Gal4*) enhanced 3-h memory with and without cold shock, demonstrating ROCK activity promotes ARM (Fig. 5*A*). Expression of *ROCK CA* in γ MBn (*1471-Gal4*) also prevented the formation of PSD-LTM, indicated by the observation that memory of *ROCK CA* expressing flies was unaffected by protein synthesis inhibition (Fig. 5*B*). To more directly test if ROCK mediates the effects of Ras on memory, we measured the effect of reducing ROCK activity in *Ras* knockdown flies. *Ras* knockdown-mediated memory enhancement was eliminated by overnight treatment with the ROCK inhibitor fasudil (Fig. 5*C*). Similarly, expression of the catalytically inactive ROCK kinase domain (*ROCK KD*) reduced memory in *Ras* knockdown flies to a level similar to that of control (Fig. 5*D*). We conclude that γ MBn Ras activity drives the inhibition of ROCK, thereby suppressing ARM and promoting PSD-LTM.

Discussion

Discovering the molecular pathways leading to different forms of consolidated memory is important for a number of reasons. First,

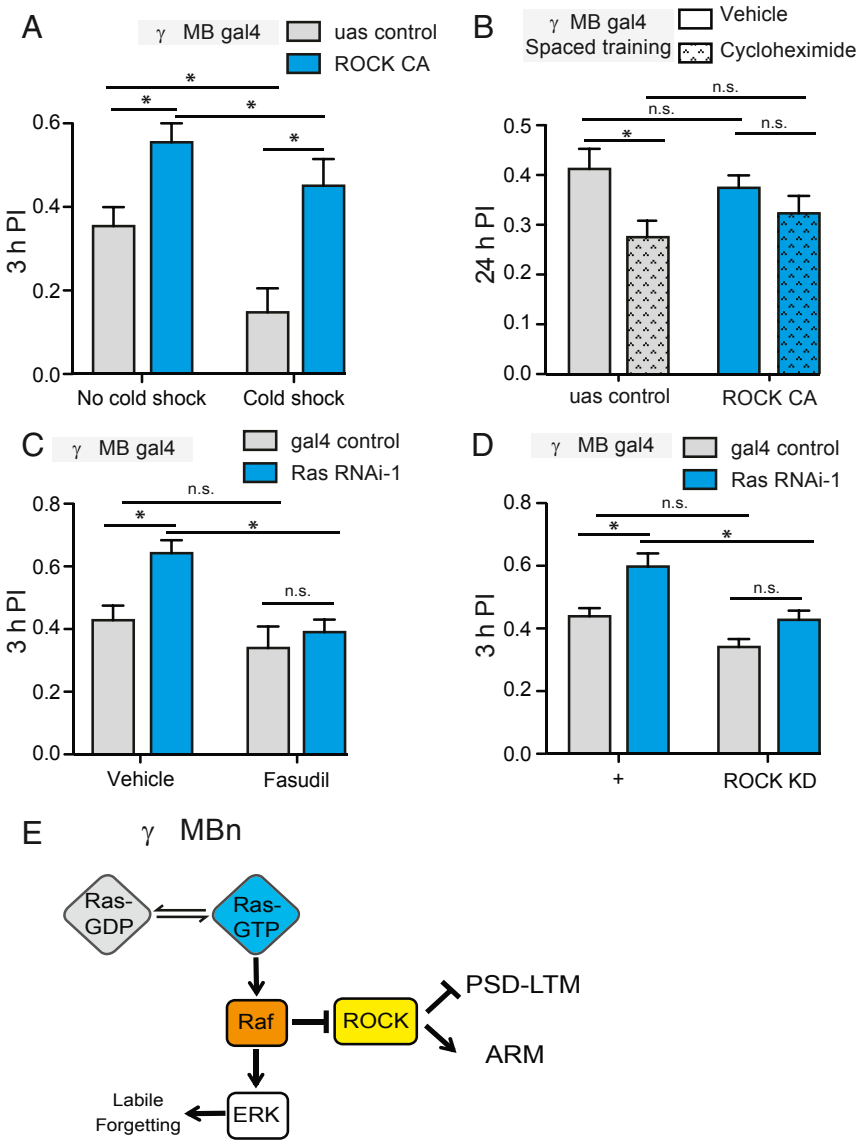

Fig. 5. Ras regulates consolidation through ROCK. (*A*) Expressing constitutively active ROCK with γ MBn *1471-Gal4* enhanced 3-h ARM. Two hours after training flies were subjected to 4°C for 2 min. Flies were tested 3 h after training. Two-way ANOVA with Bonferroni post hoc comparisons, $*P < 0.05$. $n = 12$ to 14. Error bars indicate SEM. (*B*) Expressing constitutively active ROCK in γ MBn (*1471-Gal4*) reduced PSD-LTM after spaced training. Flies were fed 35 mM cycloheximide or vehicle overnight. Flies were trained with spaced conditioning five times and tested at 24 h. Two-way ANOVA with Bonferroni post hoc comparisons, $*P < 0.05$. $n = 14$. Error bars indicate SEM. (*C*) Treatment with the ROCK inhibitor fasudil eliminated the enhanced memory due to expressing *Ras RNAi* in γ MBn (*1471-Gal4*). Flies were fed 400 μM fasudil or vehicle overnight. Two-way ANOVA with Bonferroni post hoc comparisons, $*P < 0.05$. $n = 7$ to 9. Error bars indicate SEM. (*D*) Expression of a kinase dead ROCK kinase domain eliminated the enhanced memory due to expression of *Ras RNAi* in γ MBn (*1471-Gal4*). Two-way ANOVA with Bonferroni post hoc comparisons, $*P < 0.05$. $n = 10$. Error bars indicate SEM. (*E*) Model for Ras regulation of consolidation. Our data suggest that training-induced Ras/Raf activity inhibits ROCK, thus limiting the consolidation of ARM. We speculate that spaced training enhances Ras/Raf inhibition of ROCK, resulting in a stronger suppression of ARM, thereby allowing for the formation of PSD-LTM. n.s., not significant.

this approach is likely to identify mechanisms that constrain LTM formation, an underexplored area of memory research. Here, we discovered that Ras→Raf →ROCK signaling suppresses ARM, permitting the formation of PSD-LTM. Inhibition of this pathway dramatically enhances ARM and eliminates PSD-LTM. Second, a more thorough understanding of the genetic requirements for consolidation in *Drosophila* is crucial to determine the relationship between ARM and mammalian memory. Ras, Raf, and ROCK can be added to a small but growing list of ARM-regulating genes that are established regulators of mammalian memory and neuronal plasticity.

Based on our results, we propose a model in which ARM consolidation is suppressed by a training-induced increase in Ras activity (Fig. 5*E*). Raf activity is increased in γ MBn following training (34), presumably through Ras, but the receptor(s) initiating this signaling are not known. Ras can be regulated through G-coupled protein receptors (35). It is possible that dopamine or an unknown coneurotransmitter released from dopaminergic neurons (DAn) during training initiates Ras signaling. This would provide a link between MP1 DAn, which are proposed to gate LTM (11), and Ras. The participation of ROCK in consolidation suggests that PSD-LTM and ARM are modulated by changes in the actin cytoskeleton (36) but does not directly indicate whether these changes occur in the pre- or postsynaptic compartments. Of the several genes known to be required for ARM, Bruchpilot (Brp) is the only one with a

well-established, specific subcellular compartmentalization (37). Brp is localized to presynaptic active zones and is required for normal presynaptic morphology and synaptic transmission, indicating that ARM may result from a form of presynaptic plasticity in the MB. Additionally, the DAn that are required for memory formation (38) innervate MB axons and modulate synaptic strength between MBn and downstream MB output neurons (39, 40). Our results demonstrating that artificial activation of Ras increases axonal pERK in γ MBn is evidence that Ras/Raf signaling participates in axonal signal transduction and is consistent with a previous report highlighting a role for presynaptic Raf activity in γ MBn (34). ROCK activity in mammalian axons is critical for a number of processes (41); however, it has not been tested whether ROCK signaling occurs in γ MBn axons.

The hypothesis that ARM inhibits the formation PSD-LTM was based on the observation that spaced training, which generates PSD-LTM, eliminates or precludes ARM (12). Subsequent research at the systems neuroscience level revealed that two sets of neurons, MP1 DAn (11) and serotonergic projection neurons (SPn) (13), appear to be responsible for the promotion of PSD-LTM through the suppression of ARM. The activity of these neurons is increased during spaced training. This activity reduces ARM, while inhibiting their activity enhances ARM. Blocking the activity of either set of neurons during spaced training does not prevent memory formation but prevents the formation of PSD-LTM. This suggests that without SPn and MP1 DAn activity, ARM occurs by default and is preferentially expressed at the expense of PSD-LTM. Ras fulfills the requirements as the intracellular and molecular switch regulating the inverse relationship between ARM and PSD-LTM. The suppression of ARM and formation of PSD-LTM both require Ras in γ MBn, which are downstream in the circuit from the ARM/PSD-LTM–gating MP1 DAn that synapse directly on to γ MBn (42, 43). A molecular model for consolidation is depicted in Fig. 5*E*.

The mammalian counterpart for ARM, if one exists, is unknown. Protein synthesis-independent ARM has been reported to be measurable up to 4 d after conditioning (6), while mammalian protein synthesis-independent memory lasts only hours (4). Despite the lack of a clear and direct mammalian counterpart to ARM, it is becoming apparent that many of the same genes that are involved in ARM also play a role in mammalian memory and plasticity. Ras, Raf, and CDC42 negatively regulate ARM (Figs. 3*B* and 4*D* and ref. 44) but in mammals are positive regulators of LTM (45, 46). Conversely, reduced *ROCK* (*SI Appendix*, Fig. S5*F* and ref. 47) or *dunce*, the latter purported to function through the SPn (34), impair ARM. In mammals, inhibition of ROCK or a mammalian ortholog of *dnc* (48), PDE4, enhances memory (49–51). It seems likely that discovering more genetic regulators of ARM will reveal previously unknown genetic regulators of mammalian memory. Based on the genes and their functions discussed here, it is possible that factors that promote ARM in *Drosophila* function in memory suppression in mammals.

The effect of ROCK on memory is not restricted to γ MBn. ROCK is also required in α/β MBn for ARM (47). In this neuron type, the effects of ROCK are not mediated by Ras but through Drk, the *Drosophila* homolog of Grb2. It is interesting to consider whether the ROCK substrate(s) mediating enhanced ARM in α/β and γ MBn are the same even though the upstream signaling components are distinct. Several ROCK targets have been established as important for normal memory, including cofilin (52) and nonmuscle myosin II (34).

A recent report indicates that ERK activity in γ MBn slows forgetting (34). Our results revealing that *ERK* knockdown reduces memory support this conclusion. However, this report finds that *Raf RNAi* expression in γ MBn reduces memory, which is at odds with our finding that *Raf RNAi* enhances memory. The most likely explanation for this discrepancy is the use of different *gal4*/*UAS-RNAi* combinations that produce different levels of gene knockdown. It is interesting to consider that Raf signaling in γ MBn might regulate three forms of memory: consolidated ARM and PSD-LTM through ROCK and labile memory through ERK (Fig. 5*E*).

Methods

A detailed description of the methods can be found in *SI Appendix*. Aversive olfactory conditioning experimental procedures were previously described (53). Briefly, adult flies were trained by pairing 90-V electric shocks with an odor (CS+) followed 30 s later by exposure to a second odor (CS–). Flies were tested in a T-maze allowing 2 min for flies to distribute between the two arms, one carrying the CS+ and the other carrying the CS– air stream. A half-Performance Index (PI) was calculated for each group using the formula (number of flies in CS– arm) – (number of flies in CS+ arm)/total flies in both arms. The two half-PIs were averaged to produce the final PI.

Data Availability. All relevant data are included in the paper and *SI Appendix*.

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