Drk-mediated signaling to Rho kinase is required for anesthesia-resistant memory in *Drosophila*

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Anesthesia-resistant memory (ARM) was described decades ago, but the mechanisms that underlie this protein synthesis-independent form of consolidated memory in *Drosophila* remain poorly understood. Whether the several signaling molecules, receptors, and synaptic proteins currently implicated in ARM operate in one or more pathways and how they function in the process remain unclear. We present evidence that Drk, the *Drosophila* ortholog of the adaptor protein Grb2, is essential for ARM within adult mushroom body neurons. Significantly, Drk signals engage the Rho kinase Drok, implicating dynamic cytoskeletal changes in ARM, and this is supported by reduced F-actin in the mutants and after pharmacological inhibition of Drok. Interestingly, Drk–Drok signaling appears independent of the function of Radish (Rsh), a protein long implicated in ARM, suggesting that the process involves at least two distinct molecular pathways. Based on these results, we propose that signaling pathways involved in structural plasticity likely underlie this form of translation-independent memory.

ARM | Drk | Drok | memory | *Drosophila*

Temporal coincidence of an odor (conditioned stimulus, CS) with electric footshocks (unconditioned stimulus, US) elicits different types of aversive short- (STM), intermediate- (ITM), and long-term (LTM) memories in *Drosophila* (1). Multiple forms of coincident memories contribute to posttraining selective avoidance of the CS. ITM, for example, which is measured 3 h posttraining with 12 US/CS pairings, has been dissected into a labile component, sensitive to cold-induced amnestic treatment called anesthesia-sensitive memory (ASM) and an anesthesia-resistant (ARM) form. ARM lasting at least 24 h can also be induced by 5–10 consecutive sessions of 12 US/CS pairings (massed training) (1–3). ARM, unlike LTM, does not depend on protein synthesis but may involve modifications of preexisting proteins (1, 4) and is thought to be antagonistic to LTM (2, 4).

In contrast to ASM, the molecular pathways underlying ARM formation, storage, and recall remain poorly understood. Proteins with demonstrated roles in ARM formation include Radish (Rsh) (5); the constitutively active atypical PKC, PKM (6); the calcium channel Bruchpilot (Brp) (7); the d5HT1A serotonin and Oct2β2 octopamine receptors (8, 9); the Dop1R1, Dop2R dopamine receptors (10, 11); and Protein Kinase A (12). However, whether these molecules operate in one or more ARM-mediating signaling cascades is presently unclear.

ARM requires functional mushroom bodies (MBs), neurons essential for learning and memory in insects (13, 14). The MBs are bilateral neuronal clusters in the dorsal posterior brain extending dendrites ventrally to their somata and fasciculated axons projecting anteriorly and bifurcating to form the medial lobes (β/β′, γ) and dorsally to comprise the α/α′ lobes (15). Inhibition of synaptic output from α/β neurons impaired ARM (2), and this is consistent with the distribution of most proteins with known roles in this form of memory (5, 8), except for Oct2β2, which is required in α′ß′ (9). ARM formation appears to require octopaminergic input to the MB α′ß′ lobes from the Anterior Paired Lateral (APL) neurons (9, 16), whereas retrieval requires serotonergic input to the αβ lobes from the Dorsal Paired Medial (DPM) neurons. It appears then that at least two circuits and parallel molecular pathways contribute to ARM (16): an Oct2β2 receptor-mediated Rsh-independent in the α′ß′ lobes (9) and an Rsh-dependent, d5HT1A serotonin receptor-mediated in the αβ (8), which also receives Dop2R-mediated signals (11). Finally, Dop1R1 and Dop2R activities in the γ lobes have been suggested to contribute to ARM (10, 11). Drk, an SH2–SH3 domain adaptor protein orthologous to the mammalian Grb2, is also expressed preferentially in α/β neurons. Drk-mediated signaling to Ras and Raf is required for normal aversive learning signaling, whereas its role in ITM is independent of Ras activation (17). Because ITM comprises ASM and ARM (18), we investigated which of the two forms of memory is affected in *drk* mutant heterozygotes and revealed a specific role for the protein in ARM, mediated via the *Drosophila* homolog of Rho kinase, Drok, and apparently independent of the Rsh protein.

Results

Drk Reduction Selectively Affects ARM. Heterozygotes for loss-of-function alleles of *drk* learn at a slower rate, a deficit reversible by Ras1 or Raf activation (17). However, memory of the association was significantly reduced even if these mutant heterozygotes were trained equivalently to controls and their memory deficit was independent of Ras1 and Raf activities (17). Because 3-h memory consists of ASM and ARM, we sought to identify which memory form is affected in the mutants. We capitalized on the fact that conditioning the mutants with 12 US/CS pairings results in immediate memory (learning) equivalent to that of

Significance

Anesthesia-resistant memory (ARM) has been puzzling because unlike long-term memory (LTM), it is translation independent in *Drosophila*. Although the two forms of consolidated memory are housed within the mushroom body neurons, they seem to employ distinct molecular pathways, with those that underlie ARM largely unknown. Elucidation of these pathways is essential to understanding ARM, how it differs from LTM, and what underlies their apparent inverse relationship. We reveal a signaling pathway that underlies ARM. Collectively, our results and already published results lead us to propose that a molecular hallmark of ARM formation is activity-dependent localized structural and functional changes in the neuronal actin cytoskeleton that alter synaptic strength or properties stable enough to last at least 24 h.

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controls (Fig. 1*A*). To differentiate between the two types of memory (1), animals trained with one round of 12 US/CS pairings were subjected to cold shock anesthesia 2 h posttraining, and their performance was assessed 1 h later along with similarly conditioned non-cold shocked flies. As expected, 3-h memory post-cold shock was significantly reduced in control animals (compare filled bars), indicating abrogation of the labile ASM but persistence of ARM (Fig. 1*B*). Notably, however, ARM appeared nearly absent in $drk^{\Delta P24}$ heterozygotes (Fig. 1*B*, open bars), indicating that Drk reduction may selectively affect this form of aversive olfactory memory. This was better illustrated after fragmentation of 3-h memory to its components by subtracting the performance after cold shock of controls and mutants from the respective scores of untreated animals (7, 11). This verified that 3-h memory comprised nearly equal parts of ASM and ARM for controls (2), and whereas ASM seemed largely unaffected, ARM was severely attenuated for $drk^{\Delta P24}$/+ (Fig. 1*C*). Therefore, 3-h memory in *drk* mutant heterozygotes consists nearly exclusively of ASM.

To independently verify this conclusion, we elicited ARM using a different conditioning protocol, massed training (1, 2), consisting of five consecutive cycles of 12 US/CS. Again, $drk^{\Delta P24}$/+ and heterozygotes for an additional mutant allele, drk^{E0A}, presented deficient ARM (Fig. 1*D*). However, when conditioned with five spaced training cycles, which yield protein synthesis-dependent LTM (1, 2), the performance of both $drk^{\Delta P24}$/+ and drk^{E0A}/+ was indistinguishable from that of controls. These results strongly suggest that Drk is specifically required for normal ARM. Both these consolidated memory forms reside within the MBs (3, 8, 9) and have been hypothesized to be mutually exclusive and to engage distinct signaling pathways (2, 4). Although various signaling cascades and molecular pathways have been implicated in LTM formation, storage, and recall (19), such mechanisms remain largely elusive for ARM.

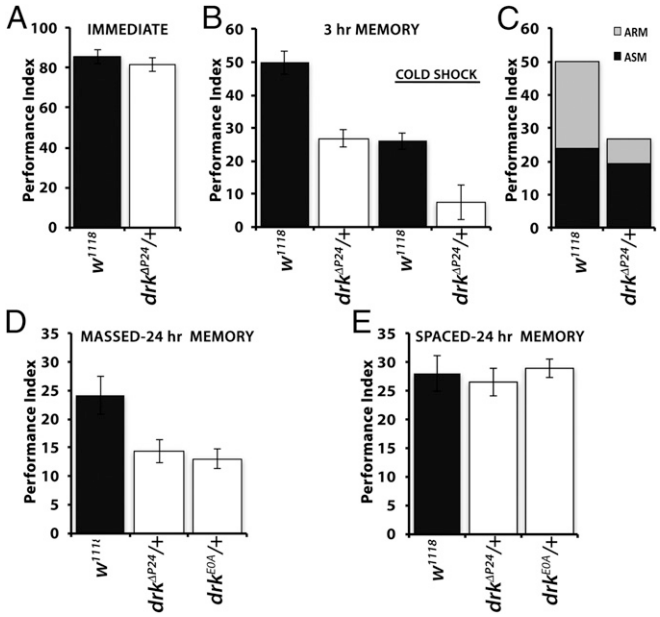

Fig. 1. ARM deficits in *drk* mutant heterozygotes. Performances of mutants are indicated by open bars and controls by black bars. The mean ± SEM are shown. Following ANOVA, potential differences among controls and mutants were assessed for significance with least square means contrast analyses. (*A*) Three-minute memory of $drk^{\Delta P24}$/+ animals after 12 US/CS conditioning once was not significantly different ($P = 0.23$, $n = 8$ each) than that of controls. (*B*) Three-hour memory (left side of the graph) of the null heterozygotes of single 12 US/CS session conditioning was significantly different from controls ($P < 0.001$, $n = 7$). Significant differences ($P < 0.0013$, $n = 7$) in 3-h ARM were also revealed following cold shock (right side of graph as indicated). (*C*) Partitioning the 3-h memory of $drk^{\Delta P24}$ heterozygotes and controls into ARM and ASM by subtracting from normal 3-h memory that after cold shock. ASM (black) is nearly identical, but ARM is highly reduced in $drk^{\Delta P24}$ heterozygotes. (*D*) ARM induced after 5× massed training and assessed 24 h later was significantly different in $drk^{\Delta P24}$/+ ($P < 0.007$, $n = 9$) and drk^{E0A}/+ ($P < 0.005$, $n = 7$) than controls. (*E*) In contrast, 24-h LTM induced by 5× spaced training was not affected ($P = 0.9$, $n = 14$ for $drk^{\Delta P24}$/+; $P = 0.78$, $n = 12$ for drk^{E0A}/+).

Drk Is Required in the α/β Lobes of the MBs for Normal ARM. Drk is detected in many adult brain structures, including the antennal lobe (AL), ellipsoid body, and prominently the α, β, and γ but not the α′ß′ lobes of the MBs (17), the anatomical site where the Rsh-dependent ARM trace is reported to reside (2, 5, 7). Therefore, we sought to determine whether Drk is required for ARM within the MBs or other adult brain structures. MB-specific Drk abrogation was achieved with transgenes (*drkR-1.2*), shown to effectively knock down its levels (17, 20) via RNA-mediated interference (RNAi).

Initially, we confirmed that 12 US/CS elicited normal immediate (3-min) memory in animals expressing *drkR-1.2* under two different MB αβ lobe-preferential Gal4 drivers (Fig. 2*A*), c772-Gal4 and c739-Gal4 (21). In contrast, expression of *drkR-1.2* under these drivers recapitulated the 3-h memory deficit of *drk* mutant heterozygotes (Fig. 2*B* and Fig. S1*A*), whereas cold shock appeared to abolish their 3-h memory (Fig. 2*B*), in accord with the ARM deficit of the mutants (Fig. 1*C*). The cold shock-induced ARM defect was further confirmed with the massed training protocol, which yielded highly compromised 24-h memory in *drkR-1.2*–expressing animals under both c772-Gal4 and c739-Gal4 (Fig. 2*C*). In agreement with the results for $drk^{\Delta P24}$/+ (Fig. 1*D*), LTM was unaffected in *drkR-1.2*–expressing animals (Fig. 2*D*), in strong support of the notion that Drk is required specifically for ARM. Because our data indicate that both conditioning protocols yield nearly identical results, henceforth we used the more robust mass training method unless otherwise specified.

Furthermore, because the expression pattern of c772-Gal4 and c739-Gal4 overlaps within the α/β MB lobes (21), these results strongly indicate that Drk is required specifically within these neurons for normal ARM. Because both of these Gal4 drivers are also expressed in the AL, which has been implicated in ARM (22), we used H24-Gal4 and NP1131-Gal4 to specifically abrogate Drk in the AL and γ lobes. We utilized Leo-Gal4, which is an MB-specific driver expressed in all neurons (23), as the positive control. Clearly, whereas Drk abrogation within γ neurons and the AL did not precipitate deficits, ARM was compromised under Leo-Gal4 (Fig. 2*E*), which is also expressed in the α′β′ neurons. However, as Drk is not expressed in α′β′ neurons, RNAi-mediated Drk abrogation in all MB neurons (Fig. 2*E*) appeared quantitatively similar to that limited to αβ lobes (Fig. 2*C*). Therefore, Drk is required for ARM within αβ neurons and appears to function independently of the octopaminergic signaling to the α′ß′ lobes.

Finally, to establish that deficient ARM does not result from developmental defects in the αβ lobes due to reduced Drk, we used TARGET (24, 25) to conditionally express *drkR-1.2* in adult MBs under c772-Gal4 (Fig. 2*F*) or Leo-Gal4 (Fig. S1*B*). Deficient 24-h ARM was observed only upon adult-specific induction of the Drk abrogating transgene, indicating that the ARM deficit does not originate from and underlie developmental deficits within MBs. Hence, Drk is specifically required within the postdevelopmental αβ MB neurons for normal ARM.

Rho Kinase Activation Restores ARM in *drk* Mutants. Since Drk does not signal to Raf1 for its function in memory (17), we searched for potential involvement of alternative signaling cascades. We were guided by its vertebrate ortholog GRB2, which engages a Rho kinase to maintain fear memory in rodents (26) to investigate the possibility that a similar pathway is involved in Drk-mediated ARM.

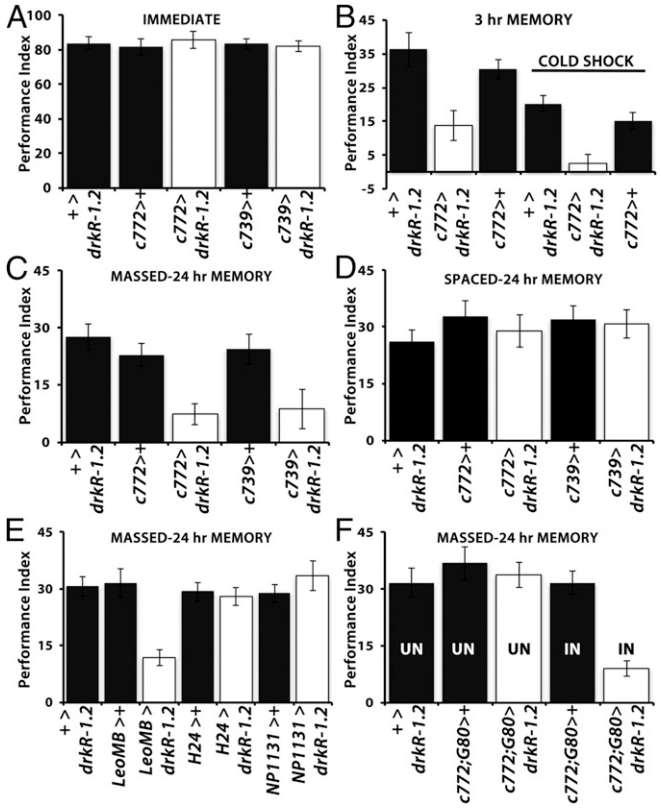

Fig. 2. Drk is required within the MB α/β lobes for normal ARM. The mean performance ± SEM is shown at the indicated times postconditioning. Controls are represented by black bars and animals with attenuated Drk via *UASdrkR1.2* (*drkR1.2*) expression by open bars. + denotes the presence of a w^{1118}-derived chromosome indicating either the lack of driver (+>) and heterozygosity for the transgene or heterozygosity for the Gal4 driver (i.e., c772>+). Potential differences were assessed with ANOVA and least square means contrast analyses. (*A*) Performance immediately after 12 US/CS conditioning of controls and animals with abrogated Drk in the indicated MB neurons. Loss of Drk in the MBs did not affect the performance relative to that of controls ($P = 0.634$, $n > 9$ per genotype). (*B*) Three-hour memory without and with cold shock of animals with abrogated Drk in 772 Gal4-marked neurons. The performance of *drkR1.2*-expressing animals was significantly different from that of both (+> *drkR1.2* and c772>+) controls without ($P < 0.0011$, $n > 7$) and with cold shock ($P < 0.002$, $n > 8$). (*C*) Abrogation of Drk within α/β neurons precipitated deficits in 24-h ARM either under the c772 Gal4 ($P < 0.003$, $n > 6$) or the c739 Gal4 driver ($P < 0.001$, $n > 8$). (*D*) LTM induced with spaced conditioning was not affected in animals with abrogated Drk in α/β lobes relative to controls ($P = 0.82$, $n > 7$). (*E*) Abrogation of Drk in all MB neurons under LeoGal4 resulted in a highly significant ($P < 0.001$, $n > 14$) deficit in 24-h ARM, while abrogation in the AL and γ-neurons (H24) Gal4 or γ-neurons (NP1131) only, did not yield significant differences from their respective controls ($P = 0.894$ and $P = 0.900$, respectively; $n > 10$). (*F*) Adult-specific abrogation of Drk in the MBs. Experimental flies (c772> *drkR1.2*) held under $Gal80^{ts}$-mediated suppression of Drk abrogation (UN) did not exhibit behavioral deficits compared with controls ($P = 0.899$, $n > 9$), whereas transgene induction (IN) precipitated deficient 24-h ARM ($P < 0.007$, n = 7).

Drosophila possesses a single Rho kinase ortholog, Drok, which like Raf is a serine/threonine kinase activated by the GTPase Rho1 (27).

If Drk signals engage Drok to mediate normal ARM, then a transgenic constitutively active form of the kinase, $Drok^{CAT}$ (28), may rescue the ARM deficit of $drk^{\Delta P24}$/+. To avoid complications because of the reported aberrant MB development precipitated by continuous $Drok^{CAT}$ expression (27–29), we expressed it conditionally and exclusively within adult $drk^{\Delta P24}$/+ αβ neurons (25). Significantly, $Drok^{CAT}$ expression in the αβ neurons fully restored 24-h ARM in *drk* heterozygotes (Fig. 3*A*). Full rescue was also achieved with an independent $Drok^{CAT}$ transgene on a different

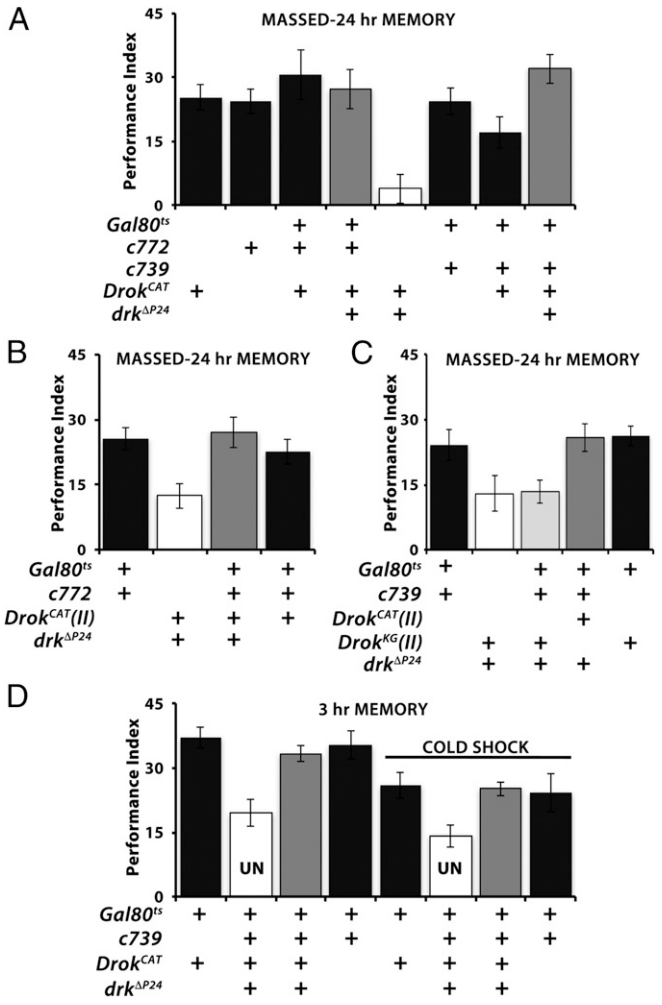

Fig. 3. Catalytically active Drok rescues the ARM deficits of *drk* mutant heterozygotes. Mean performances ± SEM are shown. Controls are represented by black bars, mutants by open bars, and mutant animals with potential behavioral rescue by gray bars. Following ANOVA, potential differences were assessed for significance with planned comparisons (least square means contrast analyses) as necessary. (*A*) Adult-specific expression of the constitutively active $UASDrok^{CAT}$ within α/β neurons rescued (gray bars) the ARM deficit of $drk^{\Delta P24}$/+ flies (open bar). The entire data shown are from animals induced in parallel. Whereas $drk^{\Delta P24}$/+; $Drok^{CAT}/Gal80^{ts}$ flies were significantly ($P < 0.0001$) deficient in ARM compared with any of the controls (black bars), $drk^{\Delta P24}/c772$; $Drok^{CAT}/Gal80^{ts}$ were not ($P = 0.9967$). Similarly, $drk^{\Delta P24}/c739$; $Drok^{CAT}/Gal80^{ts}$ flies performed equally well with controls ($P = 0.688$) but significantly different ($P < 0.0001$) from $drk^{\Delta P24}$/+; $Drok^{CAT}/Gal80^{ts}$ animals. $n > 7$ for all. (*B*) Rescue of the ARM deficit of $drk^{\Delta P24}$/+; $Drok^{CAT}/Gal80^{ts}$ animals (open bar) by induction of an independent $Drok^{CAT}$ transgene on chromosome II (gray bar). Whereas the performance of $drk^{\Delta P24}$, $Drok^{CAT}/c772$; $+/Gal80^{ts}$ was not different from that of controls ($P = 0.829$), it was significantly different from that of $drk^{\Delta P24}$, $Drok^{CAT}$/+; $+/Gal80^{ts}$ ($P < 0.0007$). (*C*) The catalytically inactive $Drok^{KG}$ transgene does not rescue (light gray bar) the ARM deficit of $drk^{\Delta P24}$, $Drok^{KG}$/+; $+/Gal80^{ts}$ ($P = 0.774$) under the c739 driver, which recues ($P = 0.731$) the $drk^{\Delta P24}$ deficit with the $Drok^{CAT}$ transgene ($drk^{\Delta P24}$, $Drok^{CAT}/c739$; $+/Gal80^{ts}$). $n > 7$. (*D*) Conditional expression of $Drok^{CAT}$ in α/β neurons rescues (gray bars) the 3-h memory deficit of $drk^{\Delta P24}$ heterozygotes after cold shock. Uninduced (UN) $drk^{\Delta P24}/c739$; $Drok^{CAT}/Gal80^{ts}$ animals were used as negative controls (open bars), and they performed significantly different from controls ($P < 0.0002$), while after induction they did not (gray bars, $P = 0.849$). Similarly, ARM after cold shock was significantly different in UN (open bars) $drk^{\Delta P24}/c739$; $Drok^{CAT}/Gal80^{ts}$ from controls ($^{+/+}$; $Drok^{CAT}/Gal80^{ts}$, or $+/c739$; $+/Gal80^{ts}$; $P < 0.003$ for both) and from the same flies after transgene induction (gray bar; $P < 0.0015$). $n > 7$.

chromosome (Figs. 3*B* and 4*C*). In contrast, expression of the catalytically inactive transgenic protein $Drok^{CAT\text{-}KG}$ (28) in the same MB neurons did not rescue the deficient ARM of $drk^{\Delta P24}$ heterozygotes (Fig. 3*C*). Conditional expression of either $Drok^{CAT}$ or $Drok^{CAT\text{-}KG}$ in wild-type adult MBs did not suppress or enhance ARM (Fig. 3 *A*–*C*), indicating that rescue did not result because of nonspecific effects of transgene overexpression. In addition, acute $Drok^{CAT}$ expression in αβ neurons under the TARGET system also restored 3-h cold shock-dependent ARM to levels exhibited by control animals (Fig. 3*D*), verifying that rescue was adult MB-specific. Similar results were obtained with the c772 driver (Fig. S1*C*).

Collectively, the results support a genetic Drk–Drok interaction within αβ neurons acutely required for normal ARM revealed after massed training or after cold shock.

Rho Kinase Activity Is Required for Normal ARM. Drok activity can be specifically required for Drk-mediated ARM, or it could function in both forms of consolidated memory. To differentiate between these possibilities, we conditionally abrogated the kinase within adult αβ neurons by *Drok RNAi*-mediating transgenes. Adult-specific attenuation of Drok within 772 Gal4-marked neurons did not affect 3-min memory (71.6 ± 2.46 for c772 Gal4, $Gal80^{ts}$>+; 74.2 ± 1.16 for *UAS-*$Drok^{RNAi\text{-}1}$>+; and 77.8 ± 3.08 for c772-Gal4, $Gal80^{ts}$ > *UAS-*$Drok^{RNAi\text{-}1}$; ANOVA $P = 0.2676$). However, it yielded significant deficits in ARM (Fig. 4 *A* and *B*) with two distinct RNAi-mediating transgenes. A similar deficit was also observed upon Drok abrogation within 739 Gal4-marked neurons (Fig. 4*C*). In contrast, LTM was not affected by Drok attenuation therein (Fig. 4*D*), strongly suggesting that the kinase plays a role specifically on the ARM form of memory.

In addition to its kinase activity, Drok contains a Rho GTPase binding site and a Pleckstrin domain (30), suggesting multiple ways that the protein could be involved in ARM. Because only constitutively active Drok rescued the deficit of *drk* mutants (Fig. 3), we hypothesized that ARM requires its kinase activity and not its Pleckstrin or GTPase domains. To differentiate between these possibilities and further validate the results with the kinase-dead transgene, we sought to inhibit the kinase activity without altering the levels of the protein itself and hence the dosage of these conserved domains. Because Rok family proteins are kinases of medical importance implicated in cancer, pulmonary hypertension, and neurodegenerative diseases (31), specific inhibitors are commercially available. We opted for the potent selective Rok inhibitor Fasudil hydrochloride (HA-1077), because it had been used on *Drosophila* before without apparent ill effects (32). Adult 2–3-d-old w^{1118} flies were fed the inhibitor (200 μM) for 16 h before conditioning. As illustrated in Fig. 4*E*, flies treated with the inhibitor presented little 24-h ARM after massed conditioning, but the drug did not affect LTM. Exposing the flies to the inhibitor only after conditioning did not affect ARM (Fig. S1*D*). Collectively then, the kinase activity of Drok is required for Drk-mediated ARM formation within αβ MB neurons.

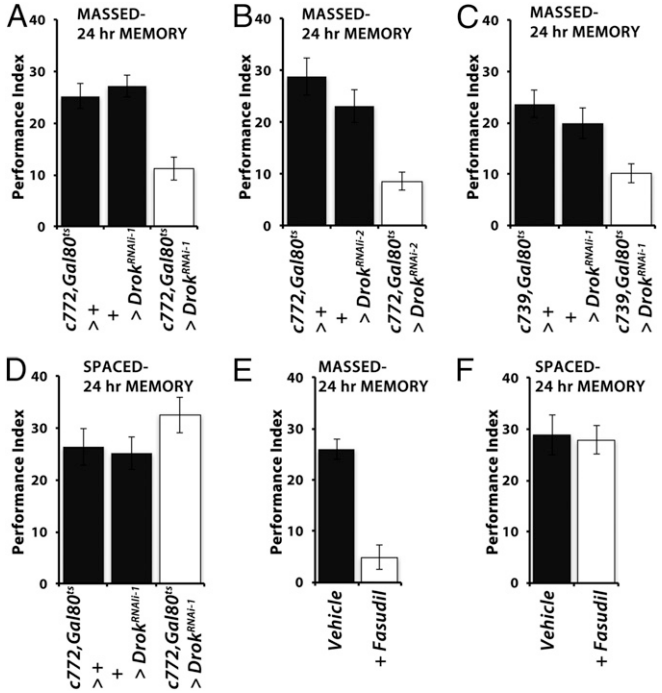

Fig. 4. Drok activity is required for ARM. Mean performances ± SEM are shown. Controls are represented by black bars and Drok attenuated flies by open bars. Following ANOVA, differences were assessed for significance with planned comparisons (least square means contrast analyses) as necessary. (*A*) Adult-specific attenuation of Drok in c772Gal4-marked neurons precipitated a significant deficit in 24-h ARM after massed conditioning relative to both controls ($P < 0.0001$, $n > 10$). (*B*) Adult-specific attenuation of Drok in c772Gal4-marked neurons with an independent abrogating transgene (*UASDrok*$^{RNAi\text{-}2}$) precipitated a significant deficit in 24-h ARM relative to both controls ($P < 0.0002$, $n > 10$). (*C*) A similar ARM deficit was observed upon Drok abrogation under the c739Gal4 driver ($P < 0.0007$, $n > 12$). (*D*) Adult-specific Drok attenuation in αβ neurons did not affect 24-h LTM ($P = 0.2631$, $n > 8$). (*E*) Adult-specific Drok activity attenuation in control w^{1118} animals with Fasudil precipitated a highly significant 24-h ARM deficit (ANOVA $P < 0.0001$, $n = 12$ each). (*F*) Spaced conditioning-induced LTM was not affected by Fasudil treatment in w^{1118} animals (ANOVA $P = 0.7541$, $n > 10$).

Rho Kinase Activation Does Not Restore ARM in *rsh* Mutants. The requirement of Drk within αβ neurons for normal ARM is in agreement with the preferential expression and functional requirement of Rsh, which is specifically implicated in this form of consolidated olfactory memory (5) within these neurons (8). The exact function of Rsh is still unclear but appears to possess a GTPase-activating domain (Flybase Curators, 2008; flybase.org/reports/FBgn0265597.html). ARM is specifically impaired in *rsh* mutants (5, 18) and appears to be independent of octopaminergic (9) inputs to the MBs for ARM formation. Therefore, we asked whether Drk and Rsh act in the same molecular cascade by investigating whether they interact genetically. Hence, we tested 24-h ARM after massed training in males hemizygous for rsh^1 and heterozygous for $drk^{\Delta P24}$ (rsh^1; $drk^{\Delta P24}$/+) and in doubly heterozygous rsh^1/+; $drk^{\Delta P24}$/+ females. Reducing Drk levels by 50% did not alter the ARM deficit of rsh^1 males (Fig. 5*A*), while ARM in the doubly heterozygous females was similar to those of $drk^{\Delta P24}$/+ females (Fig. 5*B*). Although the low performance levels of male flies potentially hindered resolution, the results suggest either that Drk is upstream of Rsh or the two proteins act in different molecular ARM-mediating pathways.

If Drk, Drok, and Rsh were in the same cascade, Drok activation could reverse the deficient ARM of rsh^1 males. This possibility is supported by the putative GTPase activator function of Rsh, which could be involved in Drok activation. Therefore, we introduced the $Drok^{CAT}$ transgene that rescued the $drk^{\Delta P24}$/+ ARM deficit into rsh^1 mutant flies. However, conditional expression of $Drok^{CAT}$ within the MBs of adult rsh^1 males under c772Gal4 or c739Gal4 failed to rescue their deficient ARM (Fig. 5*C* and Fig. S1*E*). The results suggest that Rsh does not act upstream of Drk or between Drk and Drok in a single signaling pathway. To test the alternative possibility that Rsh is downstream of Drk and Drok, we attempted to rescue the ARM deficit of $drk^{\Delta P24}$/+ flies with an inducible *rsh* transgene. Although the transgene was shown to rescue the deficient ARM of rsh^1 mutants (5) and was highly induced by a brief heat shock (Fig. S1*F*), it was unable to reverse the deficit of $drk^{\Delta P24}$ heterozygotes (Fig. 5*D*). These data strongly suggest that Drk and Rsh operate in distinct, potentially parallel molecular pathways serving ARM within αβ neurons.

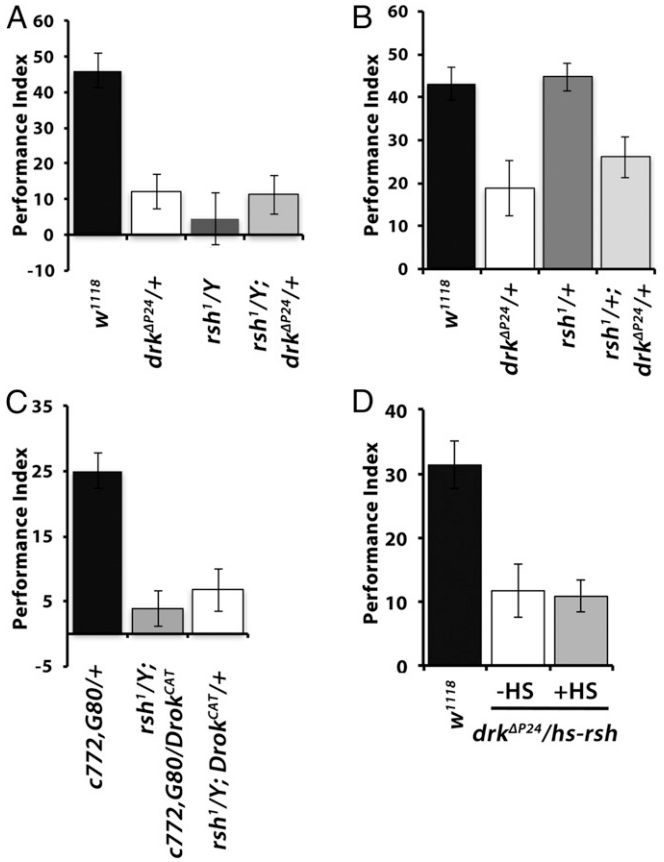

Fig. 5. Drk-mediated signaling to Drok for ARM does not engage Rsh. Mean performances ± SEM are shown for *A–D*. Following ANOVA, differences were assessed for significance least square means contrast analyses as necessary. (*A*) Twenty-four–hour ARM of control males (black bars) was significantly different ($P < 0.0001$) from that of $drk^{\Delta P24}$/+ (open bars), rsh^1 hemizygous males (dark gray bars), and rsh^1; $drk^{\Delta P24}$/+ (light gray bars). However, the performance of rsh^1 hemizygous males was not significantly different from that of $drk^{\Delta P24}$/+ ($P = 03428$) or rsh^1; $drk^{\Delta P24}$/+ ($P = 0.4023$). (*B*) Twenty-four–hour ARM of control females (black bars) was significantly different ($P < 0.0006$) from that of $drk^{\Delta P24}$/+ and rsh^1/+; $drk^{\Delta P24}$/+ ($P < 0.008$) but not from rsh^1/+ females ($P = 0.8318$, $n > 10$). (*C*) $Drok^{CAT}$ expression in their MBs does not rescue the 24-h ARM deficit of rsh^1 mutants; 24-h ARM of control (black bars) males was significantly different from that of rsh^1/Y; *c772 Gal4,*$G80^{ts}$/+ ($P < 0.0001$) but also from rsh^1/Y; *c772-Gal4,* $G80^{ts}$/*UASDrok*^{CAT} flies ($P < 0.0001$). In addition, the performance of rsh^1/Y; *c772 Gal4,*$G80^{ts}$/+ was not different from that of flies of the same genotype but was with those expressing $Drok^{CAT}$ ($P = 0.4944$, $n > 9$). (*D*) Twenty-four–hour ARM for $drk^{\Delta P24}$heterozygotes carrying a conditional heat shock-inducible *rsh* transgene. Induction of the transgene (+HS) did not improve ($P = 0.8527$) their performance over that of their siblings without induction (–HS), which remained significantly different from that of (not heat shocked) controls ($P < 0.001$, $n > 9$).

Because Drok-mediated signals likely engage actin and the actin cytoskeleton (26, 29, 31), we investigated whether changes in actin polymerization could be detected in animals with genetic or pharmacologically-induced attenuated ARM. We assessed filamentous actin (F-actin) levels in *drk* mutants and in control animals treated with the Drok inhibitor Fasudil. Brains were dissected from animals with strongly reduced Drk levels in the MBs [$drk^{\Delta P24}$/*LeoGal4*; *drkR-1.2*/+ (17)], stained with phalloidin, and quantification of the signal within the calyces revealed a significant reduction in filamentous actin compared with controls (Fig. 6*A*). A similarly highly significant reduction in filamentous actin levels was observed upon treatment with Fasudil before dissection (Fig. 6*B*), a treatment that nearly abolishes ARM (Fig. 4*E*), which collectively with the results from the mutants strongly implicate actin cytoskeleton dynamics in the process.

Discussion

Elucidation of the molecular pathways specific to ARM is essential to understanding this translation-independent consolidated memory form, how it differs from LTM, and what underlies their apparent inverse relationship (2, 4). Our evidence indicates that the small adaptor protein Drk/Grb2 is essential for ARM in a Rsh-independent manner. Genetically, Drk signals to Drok, but it is unlikely that they also interact physically. Drok lacks significant polyproline stretches targeted by the SH3 domain of Drk, and none of its regulatory phosphorylations are on Tyrosines residues, which could engage the SH2 domain (30).

Drk and Rsh are both present within MB neurons (5, 8, 17), but our evidence indicates that Drk/Drok-dependent ARM does not require Rsh. This is consistent with two signaling pathways serving ARM within αβ neurons, where Rsh expression overlaps that of Drk. These two cascades may be independent or converging downstream of Drok in a coincidence detection manner, but we favor the former based on the following: Serotonergic signals required for ARM formation in αβ neurons may engage Rsh (8), but not Drk/Drok required therein for structural plasticity as suggested below. Serotonergic signaling in Drk/Drok-mediated plasticity is currently under investigation. Another possibility consistent with independent cascades is that Rsh may only be involved in ARM retrieval within αβ neurons (16), while Drk/Drok in its formation or maintenance. In this model, *rsh* mutations will occlude rescue attempts with activated Drok, and Rsh overexpression will not rescue the *drk*/+ ARM deficits, as we describe (Fig. 5).

The ubiquitous Serine/Threonine kinase Drok is activated by the Rho1 GTPase, whose activation in turn requires Rho GTPase Activating Proteins (Rho-GAPs), and Drk physically interacts with multiple Rho-GAPs (33), likely bringing it in proximity with Drok. Finally, the Pleckstin homology domain of Drok indicates its membrane association and potential interaction with the actin cytoskeleton. Drk attenuation or inhibition of Drok activity precipitated reduction in F-actin and deficient ARM, suggesting that the deficit results from reduced ability to establish or maintain structural changes. In accord with its known functions (31), we propose that Drok mediates polymerization or stabilization, possibly of cortical actin filaments. We further propose that this activity-dependent cytoskeletal remodeling

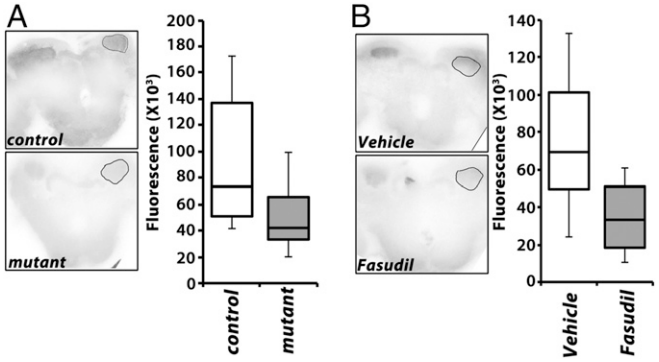

Fig. 6. Decreased filamentous actin in the MBs of *drk* mutants and upon acute pharmacological inhibition of Drok. (*A*) Representative confocal images of whole-mount brains at the level of the calyces used to quantify fluorescence from the marked regions of interest (ROI) after rhodamine-conjugated phalloidin staining. Control indicates *LeoGal4*/+, while mutant indicates the genotype $drk^{\Delta P24}$/*LeoGal4*; *drkR-1.2*/+. Quantification (*Right*) of multiple experiments revealed significant differences in fluorescence in the calyces control and mutant animals (Wilkoxon test, $\chi^2 = 12.4910$, $P < 0.0004$, $n > 20$). (*B*) Representative confocal images of whole-mount brains at the level of the calyces used to quantify fluorescence from the marked ROIs after rhodamine-conjugated phalloidin staining of vehicle and Fasudil-treated w^{1118} animals. Quantification (*Right*) revealed significant differences in fluorescence in the calyces control and mutant animals (Wilkoxon test, $\chi^2 = 22.6722$, $P < 0.0001$, $n > 28$).

alters synaptic strength or properties, which underlies ARM formation, a hypothesis currently under investigation.

The structural plasticity model for ARM we propose being dependent on Drk/Drok-mediated actin dynamics is supported by known functions of the Rho1/Rho-GAP/Drok module. Rho1, Rho-GAPp190, and Drok activities have been shown to transduce Integrin-originating signals to the cytoskeleton essential for axonal growth of MB α lobes during development (27). Interestingly, Integrins are known to regulate RTK signaling by recruiting adaptors such as Drk to the membrane (34). Upon RTK activation, Integrins and their associated signaling molecules colocalize at focal adhesion sites and signal to the cytoskeleton, mediating its remodeling (34), likely via the Rho1/Rho-GAP/Drok module. Rok/Rho-GAPp190 and Integrins are involved in neuroplasticity because they are essential for fear memory in the rat (26) and for fly olfactory learning (35), respectively. Furthermore, multiple reports detail neurotransmitter-mediated structural synaptic plasticity in adult vertebrate neurons via Rho and Ras GTPases (summarized in ref. 36). In addition, the reported role of dopamine and serotonin in ARM (8, 10, 11) is consistent with the proposed structural plasticity model, as both neurotransmitters have been implicated in spine dynamics in both insects (37) and vertebrates (38, 39).

Collectively then, our results and the biochemical and interactome evidence detailed above lead us to propose that a molecular hallmark of ARM formation is activity-dependent localized structural and functional changes in the neuronal cytoskeleton that alter synaptic strength or properties, stable enough to last at least 24 h. Testing this hypothesis will provide essential insights into understanding not only the nature and function of the ARM form of consolidated memory and its relationship to LTM in flies but also its analogous process in vertebrates.

Materials and Methods

Drosophila culture, strains, genetics, and conditioning have been described before (17) and along with strains used are detailed in *SI Materials and Methods*. Fasudil (HA-1077; Sigma-Aldrich) dissolved in water was used to abrogate ARM. Confocal microscopy was performed using standard methods. Untransformed (raw) data were analyzed parametrically with the JMP 7.1 statistical software package (SAS Institute Inc.) as before (17). Detailed methods are presented in *SI Materials and Methods*.

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