A dual role for the adaptor protein DRK in Drosophila olfactory learning and memory

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Abstract

Participation of RAS, RAF and MAPK in learning and memory has been demonstrated in a number of studies, but the molecular events requisite for cascade activation and regulation have not been explored. We demonstrate that the adapter protein DRK which is essential for signaling to RAS in developmental contexts, is preferentially distributed in the adult mushroom bodies, centers for olfactory learning and memory. We demonstrate that *drk* mutant heterozygotes exhibit deficits in olfactory learning and memory, apparent under limited training conditions, but are not impaired in sensory responses requisite for the association of the stimuli, or brain neuroanatomy. Furthermore we demonstrate that the protein is required acutely within mushroom body neurons to mediate efficient learning, a process that requires RAF activation. Importantly, 90-minute memory remained impaired, even after differential training yielding equivalent learning in animals with compromised DRK levels and controls, and did not require RAF. Sustained MAPK activation is compromised in *drk* mutants and surprisingly is negatively regulated by constitutive RAF activity. The data establish a role for DRK in *Drosophila* behavioral neuroplasticity and suggest a dual role for the protein, first in RAF activation-dependent learning and additionally in RAF-inhibition dependent sustained MAPK activation essential for memory formation or stability.

Keywords

learning; memory; DRK; RAS; RAF; signaling

Introduction

Accumulating evidence established the essential role of signaling through the typically oncogenic RAS/RAF/MAPK cascade in vertebrate (Kelleher et al., 2004; Sweatt, 2004; Chen et al., 2006; Davis and Laroche, 2006; Shalin et al., 2006; Chwang et al., 2007) and invertebrate (Bailey et al., 2004; Sharma and Carew, 2004) learning and memory. Cascade regulation is relevant for human cognition as loss of the RAS regulator Neurofibromin (NF) results in the mentally retarding condition Neurofibromatosis 1 (Weeber and Sweatt, 2002). However,

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although RAS/RAF/MAPK signaling is clearly essential for neuroplasticity, mechanisms that activate and regulate this pathway in neurons are largely unclear.

Neuronal RAS can be activated via Receptor-Tyrosine Kinases (RTKs), neurotransmitter-activated G-protein-coupled or NMDA-glutamate receptors, voltage-gated calcium channels and cell adhesion molecules (Grewal et al., 1999; Mazzucchelli and Brambilla, 2000; Weeber et al., 2002; Krapivinsky et al., 2003). Adapter proteins like GRB2/DRK link RAS to receptors (Pawson and Saxton, 1999) and confer some signaling selectivity and specificity (Pawson and Saxton, 1999; Luschnig et al., 2000). Typically, activated RAS activates a MEK-kinase, one of which is RAF, at the plasma membrane. Among other actions, RAF activates another kinase MEK, which activates yet another kinase MAPK. MAPK modulates transcription factor activity in the nucleus, but also targets cytoplasmic and membrane proteins (Martin et al., 1997; Sweatt, 2001). Recent findings suggest that neurons utilize these molecules in alternative context-specific signaling routes to achieve different specific end results (Kolch, 2005; Douziech et al., 2006; Shalin et al., 2006), adding complexity to this cascade and its regulation.

Drosophila has been instrumental in identifying genes involved in learning and memory (Skoulakis and Grammenoudi, 2006). It offers a powerful system to explore RAS/RAF/MAPK signaling and its regulation in neuroplasticity as was done for eye and embryonic development (Dickson et al., 1996; Baek and Lee, 1999). Unlike vertebrates however and except the potential RAF-regulating 14-3-3ζ protein Leo (Skoulakis and Davis, 1996; Li et al., 1997; Philip et al., 2001) and possibly dNF1 (Guo et al., 2000), typical cascade members have not been implicated in Drosophila learning and memory. However, the α-integrin VOL (Grotewiel et al., 1997) and the adhesion molecule FASII (Cheng et al., 2001) are required for olfactory learning and memory and may be involved in RAS/RAF/MAPK signaling (Martin and Kandel, 1996; Ruoslahti, 1999; Stork, 2003). Significantly, these proteins and LEO accumulate preferentially in the Mushroom Bodies (MBs), centers for olfactory learning and memory (Heisenberg, 2003; Davis, 2004). The MBs are bilateral neuronal clusters in the dorsal posterior brain cortex, extending dendrites ventrally forming the calyces and fasciculated axons anteriorly, bifurcating to form β, β' and γ medial and α, α' vertical lobes (Crittenden et al., 1998; Heisenberg, 2003).

To systematically study RAS/RAF/MAPK signaling in Drosophila learning and memory, we aimed to identify cascade members with roles in these processes based on their presence in the MBs (Skoulakis and Grammenoudi, 2006). Here we report on DRK, a protein with striking distribution in the MBs, which is essential for transmission of RTK signals to RAS/RAF/MAPK (Perrimon et al., 1995; Raabe et al., 1995).

Materials and Methods

Drosophila culture, strains and genetics

Drosophila were cultured as described previously (Mershin et al., 2004). The transposon insertions mutant alleles drk^{10626} (hereafter drk^{P1}) (Simon et al., 1993), and drk^{k02401} (hereafter drk^{P2}) (Roch et al., 1998), were obtained from the Bloomington Stock Center. The deletion allele $drk^{ΔP24}$ was obtained from N. Perrimon (Hou et al., 1995a). The two point mutations within the SH2 domain drk^{EoA} (amino acid 106 H to Y) and drk^{R1} (amino acid 67 A to H), reported to yield non-functional protein with respect to its ability to bind and support signaling originating from a receptor tyrosine kinase, were obtained from M. Simon and E. Hafen respectively (Simon et al., 1991; Olivier et al., 1993; Simon et al., 1993). All strains were normalized to an isogenic ry^{506} strain (Skoulakis et al., 1993; Skoulakis and Davis, 1996) as follows: For the drk^{P1} transposon allele carrying the ry^+ marker, free recombination was allowed with the ry^{506} second chromosome and ry^+ animals were selected each generation for 6 generations. Point mutations and the $w+$-bearing transposon allele drk^{P2} were crossed to a strain carrying second chromosomal balancers in a ry^{506} genetic background and individual

females carrying the CyO balancer over the mutant allele were used to establish three lines after six further backcrosses to the isogenic balancer strain. One backcrossed line was used for detailed behavioral analyses following pilot experiments demonstrating equivalent learning performance among the lines. *UAS-Raf^{gof}* was obtained from A. Brand (Brand and Perrimon, 1994). The *eya^{ph}* strain (Choi and Benzer, 1994) was obtained from the Choi laboratory. *c739, Tub*GAL80^{ts} were obtained from G. Roman.

Germline transformants for behavioral rescue were generated from a full length *drk* cDNA (Simon et al., 1993), placed under heat shock promoter control in a *ry$^+$*-bearing vector (Roman et al., 1999) and injected into *ry^{506}* isogenic embryos. Two independent third chromosome lines, *T1*and *T5.3* were selected for ease of genetic manipulations. A transgene capable of generating double stranded *drk* RNA was generated by cloning a BamHI – EcoRI fragment from the full length cDNA (Simon et al., 1993) into the BglII – EcoRI sites of the sympUAST vector (Giordano et al., 2002) and the resulting construct was injected into *w^{1118}* embryos carrying isogenized Canton S-derived chromosomes. Two independent lines were selected, *drkRNAi1.2* (*drkR-1.2*) on the second and *drkRNAi2* (*drkR-2*) on the third chromosome. Because, the genetic background of the *drkRNAi*-generating transformants is *w^{1118}*, all relevant *drk* mutant alleles were introduced to that genetic background. To generate *UAS-Raf^{WT}* a HindIII/XbaI fragment containing the entire *Raf* ORF (Rommel et al., 1997) was subcloned into pUAST and germline transformants were obtained in the Canton S- *w^{1118}* genetic background using standard methods. *drk^{ΔP24}* mutant heterozygotes harboring *UAS-Raf^{gof}*, *UAS-Raf^{WT}*, or *UAS-drkRNAi* transgenes and flies harboring both *drkR-1.2* and *drkR-2* were generated by standard genetic crosses. The strain carrying the UAS-*Ras^{V12S35}* effector loop mutant was obtained from Dr. V. Budnik (Koh et al., 2002) and the transgene was introduced into the *w^{1118}* genetic background with repeated backrosses and then recombined onto the second chromosome bearing the *drk^{ΔP24}* mutation in the same genetic background.

For behavioral experiments, non-balancer bearing progeny from crosses of CyO-balanced *drk*, males to *ry^{506}* females were used. To generate flies for behavioral rescue, balanced *drk* mutant flies, homozygous for the transgene on the third chromosome were crossed to homozygous transgene bearing stocks and non-balanced progeny were selected for testing. For conditional behavioral rescue, the transgenes were induced by three 20 minute heat shocks delivered every 6 hours, followed by a 4 hour rest period at 25°C prior to training and testing. To abrogate DRK levels, *Elav*GAL4, c772GAL4, c739GAL4, 201YGAL4, MB247GAL4, c232GAL4 females were crossed *en masse* to *drkR-1.2* and *drkR-2* males. Progeny of the cross between *w^{1118}* females and *drkR-1.2* and *drkR-2* males were used as controls. Flies were placed at 29°C for 24 hours for maximal transgene induction and allowed 45 minutes recovery at 25° C prior to training. For rescue experiments with *UAS-Raf^{gof}* transgenes, c772GAL4;*Tub*GAL80^{ts} (McGuire et al., 2003) and c739GAL4,*Tub*GAL80^{ts} (Ferris et al., 2006) and control *w^{1118}* females were crossed *en masse* to *drk^{ΔP24}*/CyO;*UAS-Raf^{gof}*, or *UAS-Raf^{gof}* males. Progeny was reared at 18°C and resultant non-balanced 4-6 day old flies were used for behavioral experiments. To maximally induce the transgenes, flies were moved from 18°C to 31°C for 16-20 hrs followed with recovery at 25°C for 45 minutes prior to conditioning. Because *UAS-Raf^{WT}* resides on the first chromosome, males carrying *UAS-Raf^{WT}* in wild type and *drk* mutant background were crossed to c772GAL4 and *w^{1118}* females and non-balanced females only, were used for rescue experiments.

Immunohistochemistry

Immunohistochemistry and histology were performed as described previously (Skoulakis and Davis, 1996; Crittenden et al., 1998). The DRK distribution pattern was determined using two different rabbit polyclonal sera, one from M. Simon (Crittenden et al., 1998) and one generated for this study. The antibody we generated, was raised in rabbits against bacterially expressed

full length DRK protein fused at its amino-terminus with Glutathione-S-Transferase (GST). Its specificity was initially tested against homozygous embryos, which were found to lack signal (Supplemental Figure 5). Both antibodies were used at 1:1500, while the secondary at 1:1000. For DRK detection in heads of mutant flies and flies expressing *drkRNAi* under c772GAL4, 6μm paraffin sections of control and mutant animals mounted on the same slide were obtained and challenged with a-DRK antibody used at 1:2000 dilution and secondary a-rabbit HRP at 1:2000 to increase the sensitivity of the assay. The HRP reaction was allowed to proceed for 30 seconds for all slides. Images from control and experimental head sections were obtained in the same session using the same acquisition settings for each photograph.

RT-PCR and Western blot analysis

For detection of transgene specific transcripts, RNA was extracted within 30 minutes after the last induction as previously described (Philip et al., 2001; Mershin et al., 2004). Transgene specific transcripts were selected with a reverse primer specific for the unique SV40 derived sequence at the 3′ of the vector (Roman et al., 1999) and a forward primer specific to the *drk* cDNA. 10% of each RT was subjected to PCR. As a qualitative control of the RT, 200 ng of either rp49 or leo6.2 reverse primer was used, followed by PCR with *rp49*, or *leo*-specific primers respectively (Philip et al., 2001). For detection of DRK in western blots, total protein extract equivalent of ¼ of an adult fly head was loaded per lane of 10% acrylamide gels, transferred to PVDF membranes and probed with the a-DRK antibody at 1:4000 and mouse anti-syntaxin (DSHB) at 1:2500. For detection of pMAPK and total MAPK levels, extract equivalent to 1 adult head was loaded per lane and the primary antibodies were used at 1:500 for mouse a-pMAPK, (Sigma), and 1:2000 for rabbit a-MAPK (Cell Signalling). Mouse a-tubulin (DSHB) at 1:2500 was used as an internal loading control. Four independent experiments were scanned, the band intensities were determined using ImageQuant 5.0 (Molecular Dynamics) and used to calculate ratios of pMAPK/MAPK. To assay the level of p-MAPK after training, w^{1118}, $drk^{\Delta P24}$ and drk^{e0A} heterozygotes were trained with 6 US/CS pairings as detailed below and immediately frozen on dry ice in pre-chilled 15 ml tubes. The tubes were rapidly vortexed to separate fly body parts and five heads were quickly selected and homogenized in Laemmli buffer containing protease inhibitor mix (Roche) and 1mM Sodium orthovanadate. Blots to probe the level of p-MAPK after induction of transgenes in control and mutant backgrounds utilized extracts from dissected brains. Brains were dissected in cold PBS supplemented with the protease mix (Roche) and 1mM Sodium orthovanadate and then transferred and lysed in similarly supplemented Laemmli buffer.

Behavioral analyses

All experiments were performed in a balanced design, where all genotypes involved in an experiment were tested per day. The experimenter was blind to the genotype in experiments testing learning of mutant heterozygotes (Figure 2). Behavioral experiments were replicated at least once with flies from different crosses and a different time period (biological replicates). Although the absolute performance scores varied somewhat between biological replicates, the relative difference in the performance of mutants and controls remained the same.

Olfactory learning and memory in the negatively reinforced paradigm coupling aversive odors as conditioned stimuli (CS+ and CS-) with the electric shock unconditioned stimulus (US) (Tully and Quinn, 1985) was performed essentially as described previously (Philip et al., 2001; Mershin et al., 2004). The timing of stimulus delivery was kept proportional to that for the full 12 CS/US pairing protocol, such that 3 shocks were delivered in 15 seconds of continuous CS+ presentation, 6 pairings within 30 seconds and so on. Conditioning with discrete stimuli was performed according to Beck *et al* (Beck et al., 2000). Olfactory and shock avoidance assays and the non-associative odor/shock pre-exposure (Preat, 1998) experiments were performed as described (Mershin et al., 2004; Acevedo et al., 2007a). We refer to the "3-

minute memory" earliest post-training performance assessment as learning, or immediate memory (IM) (Skoulakis and Davis, 1996). Data were analyzed parametrically with the JMP statistical package (SAS Institute Inc., Cary, NC) as described before (Philip et al., 2001; Mershin et al., 2004) and detailed in the text or figure legends.

Results

Preferential expression of drk in mushroom body neurons

The *drk* gene encodes a protein of 211 amino acids consisting entirely of a central RTK-binding SRC-Homology domain 2 (SH2) flanked by two SH3 domains (Pawson and Gish, 1992) and is the ortholog of the vertebrate GRB2 (Olivier et al., 1993; Simon et al., 1993; Raabe et al., 1995). The protein binds to the guanine exchange factor SOS via its amino-terminal SH3 domain forming a complex essential for RAS activation. The carboxy-terminal SH3 domain binds to DISABLED, which may link SRC-like tyrosine kinase signaling to the RAS/RAF/MAPK cascade (Le and Simon, 1998). Therefore, DRK plays an essential role in the initiation of multiple signaling cascades that potentially lead to MAPK activation.

Investigation of the role of DRK in olfactory learning and memory were initiated by the observation that reporter β-galactosidase in *drk^P1* heterozygotes accumulated preferentially in Kenyon cells (not shown). We confirmed this observation and examined in detail the distribution of DRK in adult brain with the polyclonal antibody we generated. DRK was found preferentially distributed in MB axons, the pedunculus and the α, β and γ lobes (Figure 1). The protein was undetectable in cell bodies (Figure 1A), modest in the R4 neurons of the ellipsoid body (Figure 1B), antennal lobe glomeruli (Figure 1C, D, E and F) and very low in the calyces (Figure 1A, E and F) and α' and β' lobes (Figure 1C, E and F). This distribution is in agreement with previously published results using a different antibody (Crittenden et al., 1998). In addition, sections challenged with pre-immune sera did not yield any staining and staining was significantly reduced in head lysates from mutants and animals with RNAi-mediated reduction of the protein (see below). These results demonstrate the specificity of the antibody and its usefulness as a highly specific marker for axons of α, β and γ MB neurons. Similar to DRK, GRB2 accumulates in the hippocampus and amygdala (Kunlin et al., 2001;Lamprecht et al., 2002), centers of learning in vertebrates.

drk heterozygous mutants learn inefficiently

The striking distribution in the MBs suggested that as with other proteins highly enriched in these neurons (Heisenberg, 2003; Davis, 2004, 2005; Skoulakis and Grammenoudi, 2006; Berry et al., 2008), DRK may be required for olfactory learning and memory. However, homozygotes for *drk* mutations die at the larval or pupal stages (Olivier et al., 1993; Simon et al., 1993; Hou et al., 1995a). Nevertheless, we reasoned that since the protein does not possess enzymatic activity, yet is essential for signal transduction of RTK-initiated signals (Lowenstein et al., 1992; Hou et al., 1995a; Cheng et al., 1998; Feller et al., 2002), reduction in its amount may affect learning and memory detectably.

To determine whether reduction in DRK affects behavioral neuroplasticity, we subjected the deletion heterozygotes *drk^ΔP24* expected to harbor 50% of the protein, to olfactory associative conditioning. Initial performance measurements are possible approximately 3 minutes after initial association of the stimuli, but we will call this immediate memory "learning", for consistency with published olfactory conditioning experiments. Initially we used the standard 12 CS/US pairings for training (Tully and Quinn, 1985), but although learning of *drk^ΔP24*/+ animals was lower than that of controls (Figure 2A), it was not different statistically. However, reducing the number of pairings to 8, 6, or 4 (see Materials and Methods) revealed highly significant learning deficits in the mutants. Similar results were obtained in multiple

independent experiments with $drk^{\Delta P24}/+$ heterozygotes in both ry^{506} and w^{1118} genetic backgrounds (see below) and $drk^{P1}/+$ animals (not shown). These results demonstrate that the 20% decrease in learning after training with 6 or less pairings, represents a *bona fide* learning impairment reflecting the reduction in DRK dosage. This pairing-specific effect is independent of genetic background and *drk* allele and represents a highly significant reduction in learning, considering the multiple biochemical pathways subserving learning and memory (Skoulakis and Grammenoudi 2006). Thus it appears that under our experimental conditions, 12 CS/US pairings train control flies to "saturation", or a performance plateau. These apparently over-training conditions, allow *drk* mutant heterozygotes to nearly overcome the limitation presented by reduced DRK and "catch up" with controls, yielding nearly similar performance indices. In contrast, reducing the pairings such that robust, but sub-plateau learning was attained by controls, revealed the deficit in the heterozygotes, suggesting that reduction in DRK affects the efficiency of learning, but not learning ability *per se*. We used the 6 pairing protocol for all subsequent experiments because it consistently yielded maximal differences between mutants and controls.

To confirm the importance of DRK levels for efficient learning, we examined the performance of a collection of transposon insertions and point mutant heterozygotes (see Materials and Methods). Again, compared to ry^{506} controls, all mutant heterozygotes exhibited consistent and highly significant reductions in learning of the same magnitude as that of $drk^{\Delta P24}/+$ animals (Figure 2B). This ascertained that the deficit maps genetically to the *drk* gene and was not associated only with the deficiency-bearing chromosome. Because the learning deficit was similar for all heterozygotes, we selected the point mutant drk^{EoA}, in addition to $drk^{\Delta P24}$ for subsequent experiments. Furthermore, the inefficient learning phenotype of *drk* heterozygotes was independently verified using a discrete stimulus conditioning paradigm (Beck et al., 2000; Cheng et al., 2001; Mershin et al., 2004). Mutants performed significantly lower than equivalently trained controls, but importantly and in support to the inefficient learning notion, improved their performance upon additional pairings nearly the same as controls (Supplemental Figure 1A). Nevertheless, mutants always appeared to perform similar to control animals trained with one less pairing. These results are consistent with the interpretation that reduction in functional DRK results in inefficient learning, which is exaggerated upon reduced training.

To determine how inefficient learning affects memory, retention of 6 CS/US training was assessed up to 360 minutes later. Mutant heterozygotes exhibited significantly lower performance 30, 60, and 90 minutes after training ($p < 0.001$). Maximal performance difference from controls was consistently observed 90 minutes post-training, while at 180 and 360 minutes the difference was smaller and significant at the $p < 0.05$ level (Figure 2C). Interestingly, 90-minute memory after 12 pairings, which produced learning approximately equivalent to ry^{506} and 8 CS/US, were significantly different from that of controls (Supplemental Figure 1B). These data suggest that although overtraining can effectively rescue the learning deficit of *drk* heterozygotes it does not affect memory of the association.

The learning deficits of *drk* mutant heterozygotes may reflect a reduction in the efficiency of DRK mediated signaling, or developmental deficits that disallow performance equivalent to that of controls. However, histological and immunohistochemical examination of the heads of mutant heterozygotes with multiple antigenic markers did not reveal any gross structural anomalies in the brain (Supplemental Figure 2, 1-6). Moreover, avoidance of the odors used as CS and electroshock (US) were not different from that of controls (Table 1), indicating that compromised perception of the CS and/or US stimuli could not account for the learning and memory deficits. In addition, the heterozygous mutants exhibited normal odor avoidance after pre-exposure to electric shock (Acevedo et al., 2007c) (Supplemental Table 1), indicating that differential response to odor-shock exposure does not underlie the learning and memory

deficits. Collectively, the results of the behavioral and histological analyses indicate that reduced functional DRK does not alter normal brain development or result in reduced sensory perception of the stimuli used for conditioning. Thus, the behavioral deficits of the mutant heterozygotes are *bona fide* learning and memory deficits.

Conditional rescue of the learning and memory deficits of *drk* heterozygotes

We attempted conditional rescue of the behavioral deficit by regulated expression of *drk* transgenes to unequivocally demonstrate that the inefficient learning and memory of mutants does not have a developmental origin and to investigate whether the protein is required acutely for these processes. Two independent heat inducible *drk* transgenes, *T1* and *T5.3*, were crossed into the $drk^{\Delta P24}$ and drk^{EoA} mutant backgrounds and upon induction, transgene-specific mRNAs were detected in heads of animals that bear them (Figure 2D). Accumulation of *T1* or *T5.3* transcripts in wild type animals did not result in enhanced learning or memory (not shown). We concentrated on attempting to reverse the learning and the 90-minute deficits because memory at this interval appeared affected maximally in $drk^{\Delta P24}$ heterozygotes relative to controls (Figure 2 C). Learning (Figure 2E) and 90-minute memory (Figure 2F) of transgene-bearing mutant heterozygotes were indistinguishable from that of controls after induction. The effect was observed with both transgenes and both $drk^{\Delta P24}$ and drk^{EoA} heterozygotes. In contrast, similarly treated mutant heterozygotes or uninduced transgenes in the mutant background did not rescue the learning and memory deficits. Transgene induction did not result in deficits or improvements in learning, or changes in sensorimotor behaviors (Table 1). The data strongly indicate that the associative learning and memory deficits do not originate in aberrant development below histological detection and DRK is required acutely for learning and memory formation.

DRK is required within the MBs for normal learning

The preferential distribution of DRK within the MBs suggested that the learning and memory deficits of the mutants are precipitated by reduced levels of the protein within these neurons. To address this issue directly and independently demonstrate that neuroplasticity deficits are not the outcome of altered development, we adopted a strategy of spatial-specific, RNA-interference (RNA-i)-mediated abrogation of the protein. Thus, we generated independent strains (*drkR-1.2* and *drkR-2*, see Materials and Methods) harboring transgenes able to generate double stranded interfering RNAs by symmetric transcription (Giordano et al., 2002).

Pan-neuronal expression with *Elav* of either independent RNAi-mediating transgene, or both simultaneously, did not result in lethality. Simultaneous pan-neuronal expression of *drkR-1.2* and *drkR-2* resulted in highly significant reduction of DRK, equivalent to that in heterozygotes of the null allele $drk^{\Delta P24}$. Further reduction in DRK was achieved by expressing one of the silencing transgenes in the presence of $drk^{\Delta P24}$ (Figure 3A). These results suggest that expression of the silencing transgenes yields an effect on DRK approximately equivalent to a heterozygote for a hypomorphic mutation, while expression of both transgenes appeared equivalent to a null heterozygote. The reduction of DRK in $drk^{\Delta P24}$ heterozygote head lysates was in fact reflected in the level of the protein within the α/β and γ lobes of the MBs (Figure 3B, 1 and 2 versus 3 and 4). To drive the transgenes within the MBs, we used the c772 driver because it is expressed developmentally late in pupal MBs (Armstrong et al., 1998) and because it is expressed in α, β and γ lobes (Mershin et al., 2004), in a pattern closely resembling that of DRK. In addition, we used the α/β lobe specific driver c739. A significant reduction of DRK in the MBs, particularly evident in the α/β lobes, was detectable upon expression of *drkR-2* with c772 in otherwise normal (Figure 3B, 5 and 6) or $drk^{\Delta P24}$ heterozygous mutant background (Figure 3B, 7 and 8). Similar results were obtained using whole mount dissected brain preparations and confocal microscopy (Supplemental Figure 1C). Therefore, the notable difference between the null heterozygotes and animals with RNAi-mediated abrogation of

DRK is that only MB neurons where the protein accumulates preferentially are affected in the latter, instead of the entire brain in the former.

Reduction of DRK within the MBs with expression of either RNAi-mediating transgene under c772 or c739 did not alter sensorimotor responses to electric shock and aversive odors (Table 1), task-relevant non-associative behaviors (Supplemental Table 1) or the gross structure of the MBs (Supplemental Figure 2), in accord with results obtained from the mutant heterozygotes. In contrast, DRK abrogation within the MBs resulted in a CS/US pairing-specific reduction in learning. Whereas learning of animals expressing either transgene was indistinguishable from that of controls after 8 CS/US pairings, significant differences were uncovered after limiting the pairings to 6 or 4 (Figure 3C and D). Similar results were obtained with animals expressing *drkR-1.2* under the MB-specific MB247 and the more broadly expressed, but enriched within a subset of α and γ lobe neurons 201Y GAL4 driver (Supplemental Figure 1D). These results indicate that DRK is required within the MBs, especially the αβγ neurons, for normal learning and are consistent with the notion that reduction of the protein within these neurons underlies the inefficient learning of the heterozygous mutants. The relatively milder reduction of DRK upon expression of either transgene compared to the 50% reduction in null heterozygotes, is the most likely explanation for the manifestation of learning deficits after training with 6 or less pairings (Figure 3C and D) and not with 8 as with $drk^{ΔP24}$ heterozygotes (Figure 2A). Consistent with this, two copies of the RNAi-mediating transgenes yielded a larger deficit than that precipitated by either transgene, or the heterozygous null mutation and similar to that of the null heterozygote combined with one transgene (Figure 3E). Thus, the inefficient learning phenotype is proportional to the level of DRK reduction within the MBs in consequence to the number of RNAi-mediating transgenes expressed in these neurons.

In contrast, DRK abrogation in ellipsoid body neurons where low levels of the protein were observed did not affect learning (Figure 3F). This result supports the specificity of the learning deficit to DRK abrogation within the MBs. The negligible contribution of DRK in neurons outside the MBs in the learning and memory impairments is also supported by the fact that although null heterozygotes expressing an RNAi-mediating transgene in the MBs apparently harbor the least DRK (Figure 3A), their learning deficit was not more severe than animals simultaneously expressing *drkR-1.2* and *drkR-2* in the MBs (Figure 3 E). Therefore, we have revealed a similar learning deficit in *drk* mutant heterozygotes and by limiting the amount of DRK specifically within the MBs, demonstrating a dose-dependent essential role of protein for normal olfactory learning.

A role for DRK in memory

Heterozygous *drk* mutants exhibited compromised 90-minute memory proportional to their learning deficits (Figure 1C), which were rescued by conditional transgene expression (Figure 2F). To determine whether a similar phenotype was exhibited by animals with abrogated DRK within MBs, we examined 90-minute memory in animals expressing the RNAi-mediating transgenes. To get robust memory, we trained animals with 8 CS/US instead of the usual 6 used for assessment of learning (Figure 3). As anticipated, learning was not significantly different in controls and animals expressing the *drkR-2* or *drkR-1.2* transgenes (Figure 4A and Supplemental Figure 3). In contrast, 90-minute memory of the association was significantly different in animals with RNAi-mediated DRK abrogation than equivalently trained controls and the difference was consistently more pronounced under c772 (Figure 4A and Supplemental Figure 3). A similar phenotype was observed with both $drk^{ΔP24}$/+ and drk^{E0A}/+ animals trained with 12 CS/US (Supplemental Figure 1B). Therefore, although intensive training largely eliminated the differences in learning/immediate performance, 90-minute memory remained deficient. This may indicate that although performances were not statistically different, training

with 8 CS/US did not actually yield equal learning in controls and mutants. This learning difference became apparent as memory deficits because this measure afforded better resolution. Alternatively, 8 CS/US-training resulted in equivalent learning as suggested by the immediate performance scores, but the memory deficit was revealed because DRK is required for memory independent of its role in learning and memory is more sensitive to abrogation of the protein in the MBs than learning is.

To investigate whether the differential effect on 90-minute memory resulted from decreased learning in animals with reduced DRK, we trained control animals with 6 CS/US, while animals with lower amounts of DRK in the MBs were trained more intensely with 8 CS/US. Although this differential training regime clearly yielded equal learning, 90-minute memory of the more intense training was still significantly reduced in *drkR-2*-expressing animals compared to that of lesser trained controls (Figure 4B). This phenotype was not particular to RNAi-mediated abrogation of DRK because we applied differential training to *drk^{ΔP24}* heterozygotes in their original *ry^{506}* genetic background with similar results (Figure 4C). In congruence, mutant heterozygotes trained with 3 discrete pairings displayed reduced 30-minute memory from that of control flies trained to approximately equivalent levels with 2 CS/US (Supplemental Figure 1A). These results indicate that in addition to its role in learning, DRK appears to independently affect 90-minute memory formation or stability within the MBs.

DRK engages RAF for learning, but not for 90-minute memory

Signals that engage DRK are known to eventually activate MAPK (Perrimon et al., 1995), and pivotal members of the classical RAS/RAF cascade including MAPK are expressed in the adult central brain (Supplemental Figure 4A). Hence, to investigate whether DRK reduction affects signalling through this pathway, we determined the level of activated, di-phosphorylated MAPK (pMAPK) in head lysates of mutant heterozygotes and animals expressing *drkR-2* and *drkR-1.2*. Compared to total MAPK, the phosphorylated protein was reduced approximately 50% in all animals with reduced DRK levels (Figure 5A). Therefore, reduction in DRK appears to affect the levels of phosphorylated MAPK, but not the amount of total MAPK, suggesting impairment in signalling possibly through RAS and RAF.

Since RAS and RAF have been reported essential for learning in vertebrates (Costa et al., 2002; Weeber et al., 2002; Chen et al., 2006), we aimed to determine whether these molecules are engaged by the DRK-initiated signal within the MBs. To that end, we investigated whether the learning and memory deficits of *drk* mutant heterozygotes could be ameliorated or reversed by temporally controlled expression of constitutively active *ras* and *raf* transgenes within the MBs. Tissue and temporal specific expression of such transgenes was achieved with the TARGET system (McGuire et al., 2003; McGuire et al., 2004b). We used the c772 and c739 because they were the most restrictively expressed MB Gal4 drivers that yielded an effect with the *drkRNAi* transgenes. Initially, we attempted rescue with the constitutively active RAS^{V12} protein (Szuts et al., 1997). However, its conditional accumulation within MBs of adult control animals and *drk^{ΔP24}* heterozygotes resulted in severe learning deficits, without apparent structural changes in these neurons (A. Moressis and Skoulakis E. M. C., in preparation). Although the results suggest that constitutive activation of RAS in the MBs perturbs normal learning, they disallow conclusions regarding potential DRK/RAS interactions within these neurons. However, RAS^{V12} has been reported to constitutively activate not only RAF, but also PI3-kinase and Ral-GDS (Karim and Rubin, 1998; Koh et al., 2002) and thus the abovementioned learning deficit could result either from simultaneous perturbation of all three signaling pathways, or one of them alone. To specifically probe the effect of Ras on MAPK phosphorylation, which is decreased in *drk* mutants and RNAi-expressing animals, we utilized animals bearing the *Ras^{V12S35}* double mutant transgene, which activates constitutively only RAF (Karim and Rubin, 1998; Koh et al., 2002). The transgene insertion alone, or combined

with the c772 driver but kept silent by GAL80^{ts} (McGuire et al., 2004a) did not rescue the deficit of $drk^{\Delta P24}$/+ animals, or affect learning adversely (Figure 5B). In contrast, expression of the Ras^{V12S35} transgene by inactivation of the GAL80^{ts} suppressor restored normal learning to $drk^{\Delta P24}$ heterozygotes, while it did not appear to affect learning in control animals (Figure 5C). These results suggest that impaired DRK signaling to RAF via RAS likely underlies the learning deficits of *drk* heterozygotes.

To examine the potential involvement of RAF in the DRK mediated signal directly, we utilized a transgene encoding a constitutively active RAF kinase (Raf^{gof}) because it lacked the amino-terminal regulatory domain (Brand and Perrimon, 1994). While keeping the transgene silent did not alter the learning deficit of $drk^{\Delta P24}$/+ (Supplemental Figure 4D), conditional expression of Raf^{gof} in adult MBs reversed the learning deficit of $drk^{\Delta P24}$ heterozygotes (c772/$drk^{\Delta P24}$; *UAS-Raf^{gof}*/*Tub*GAL80^{ts}), while expression of the transgene in control animals (c772/ +; *UAS-Raf^{gof}*/*Tub*GAL80^{ts}) did not result in impaired or enhanced learning (Figure 5D). Similar results were obtained under c739 (Supplemental Figure 4C). Thus, rescue of the $drk^{\Delta P24}$ heterozygote impairment was not a consequence of enhanced learning upon Raf^{gof} expression resulting in a performance plateau prior to that of control animals. This interpretation was confirmed by limiting CS/US pairings to three. This would be expected to exaggerate any potential enhanced learning of Raf^{gof} expressing animals, thus explaining the apparent "rescue", but yielded similar performances for both strains (Supplemental Figure 4B). In addition, we attempted to rescue the learning impairment of $drk^{\Delta P24}$ heterozygotes with a full length *Raf* transgene (*UAS-Raf^{WT}*). Unlike Raf^{gof}, paneuronal or MB-restricted expression of the wild type protein did not result in lethality, thus it was not necessary to use the TARGET system to restrict its expression temporally. Rather, we drove it in the MBs with the late-expressing c772 (Armstrong et al., 1998; Mershin et al., 2004). Accumulation of the wild type RAF in MB neurons improved immediate performance of $drk^{\Delta P24}$ heterozygotes significantly, but not to the level of equivalently trained controls (Figure 5E). It appears then that although it accumulates nearly equivalently with RAF^{gof} (not shown), activation of the wild type protein was limited, likely due to the 50% DRK reduction in $drk^{\Delta P24}$ heterozygotes. In contrast, elevation of the constitutively active kinase yielded full rescue because it does not require DRK for activation. Similarly, MB-specific accumulation under *c772* of both Raf^{gof} and Raf^{WT} resulted in full reversal of the learning deficit of drk^{EoA} /+ flies (Supplemental Figure 4E). Full rescue of the drk^{EoA}/+ learning deficit with Raf^{WT} expression is consistent with the mutant being a strong hypomorph compared to the more demanding rescue of the $drk^{\Delta P24}$ null allele.

Surprisingly, neither RAF^{gof}, nor RAF^{WT} accumulation in the MBs rescued 90-minute memory (Figure 5F and G). Since the transgenic proteins accumulate in the MBs in addition to the endogenous kinase, it is unlikely that activated RAF, at least in the case of the constitutively active protein, does not suffice to support 90-minute memory, unless it is unstable and falls below a critical threshold quickly after training. Moreover, the full rescue of the $drk^{\Delta P24}$ /+ learning defects with RAF^{gof} predicts that memory would be normal if it simply was a consequence of learning. However, our results support the alternative hypothesis that RAF activation via DRK is essential for normal learning only and that parallel or subsequent engagement of signaling molecules other than RAF is required for memory. This hypothesis is further supported by the ability of DRK to engage different molecules by its two SH3 domains (Rubin, 1991; Raabe et al., 1996; Le and Simon, 1998; Feller et al., 2002).

Deficient MAPK activation in *drk* mutant heterozygotes

To substantiate independently the results of the behavioral analyses and explore the potential mechanism(s) underlying the apparent dual effects of DRK reduction on learning and memory, we investigated pMAPK levels in adult brains for two reasons. First, they were significantly reduced in animals with reduced DRK (Figure 5A). Second, we hypothesized that memory,

but not learning may be affected by the altered pMAPK levels since persistent MAPK activation has been linked to memory formation (Weeber and Sweatt, 2002; Sweatt, 2004; Thomas and Huganir, 2004).

The dynamics of MAPK activation in the brains of adult flies were investigated at three particular time points following 6 US/CS associative training (Figure 6A). At two minutes post-training, roughly coincident with behavioral assessment of learning, pMAPK levels were substantially elevated in control animals and *drk* mutant heterozygotes. However, pMAPK levels in controls were not significantly different from those in $drk^{\Delta P24}$/+ and drk^{E0A}/+ animals (Figure 6B). In contrast, 15 minutes post-training, pMAPK remained elevated in controls, but was not different than the level in naïve animals for $drk^{\Delta P2}$ and drk^{E0A} heterozygotes. Furthermore, although pMAPK appeared to return to naïve levels 90 minutes post-training, in $drk^{\Delta P24}$/+ and drk^{E0A}/+ it was significantly further reduced not only compared to controls, but even below the level in naïve animals (Figure 6B). These results indicate that 50% reduction in DRK does not affect MAPK activation acutely after training, but rather impairs significantly the sustained activation of the kinase, particularly evident at 90 minutes. This suggests that in agreement with previous reports (Hoeffer et al., 2003; Sweatt, 2004; Thomas and Huganir, 2004), failure to sustain pMAPK levels likely underlies the 90-minute memory deficit. Furthermore, considering the behavioral rescue of learning but not 90-minute memory by *raf* tranagenes in the MBs, these results further support the notion that RAF activity is involved in acute elevation of pMAPK post-training, but not for its sustained activation. Therefore, the 90-minute memory deficit of *drk* heterozygotes is likely independent of RAF as indicated by the behavioral analyses.

To further support these conclusions, we aimed to determine pMAPK levels in naïve control and $drk^{\Delta P24}$/+ animals following induction of the *Raf* transgenes. As expected, conditional pan-neuronal accumulation of RAF^{gof} resulted in substantial elevation of pMAPK in the brains of control animals while total MAPK levels appeared unchanged (Figure 6C). Similarly, pan-neuronal accumulation of RAF^{WT}, elevated pMAPK levels, albeit as expected to a somewhat lesser degree compared to the effect of the constitutively active RAF^{gof}. Surprisingly, pan-neuronal accumulation of RAF^{gof} and RAF^{WT} in $drk^{\Delta P24}$ drastically decreased pMAPK levels, without altering MAPK (Figure 6C). In fact, quantification of multiple blots demonstrated that in $drk^{\Delta P24}$ heterozygotes accumulating RAF^{gof}, pMAPK levels were 15% of those in control flies. Similarly, pMAPK levels were reduced 50-55% in $drk^{\Delta P24}$/+ accumulating RAF^{WT} (Figure 6C). Therefore the reduction in pMAPK levels was more prominent upon accumulation of the constitutively active RAF^{gof}. These results indicate that prolonged RAF activation suppresses MAPK activation, or sustained pMAPK levels when DRK is reduced by 50%. Because pMAPK levels in $drk^{\Delta P24}$/+ and drk^{E0A}/+were not different from controls after behavioral training, normal DRK levels do not appear essential for MAPK activation, but rather to maintain sustained pMAPK levels, perhaps by suppressing RAF activity.

Therefore, consistent with our behavioral observations, learning depends on RAF activity, but MAPK activation does not appear essential for this process. In contrast, sustained MAPK activation appears requisite for 90-minute memory formation, but in *drk* mutant background it is further suppressed by transgenic RAF over-accumulation. This then is unlikely to result in RAF-dependent rescue of the 90-minute memory deficit in the heterozygous mutants. Collectively our data suggest that DRK plays a dual role, one dependent on RAF activation for learning and an independent role in memory, dependent on sustaining pMAPK levels, possibly by antagonizing RAF activity. Furthermore, our results extend the known functions of DRK to post-developmental neuronal processes which underlie MB-mediated olfactory learning and memory.

Discussion

Analyses of their roles in the development of the Drosophila embryonic body plan (Perrimon et al., 1995; Gelb and Tartaglia, 2006) and the compound eye (Wassarman et al., 1995; Doroquez and Rebay, 2006) have been essential for understanding the *in vivo* functional relationship and signaling among members of the RAS/RAF/MAPK cascade. We extend this analysis to olfactory learning and memory with this initial demonstration that DRK accumulates in the MBs with a highly specific pattern and is required within these neurons for learning/acquisition and 90-minute memory. These results support the proposed role of 14-3-3ζ/LEO, a protein with acute role(s) in olfactory learning and memory (Skoulakis and Davis, 1996; Philip et al., 2001) in regulation of RAF activity (Skoulakis and Davis, 1998). Consistently, a role in neuroplasticity has also been reported for the DRK rat ortholog GRB2, which is involved in long-term fear memory formation in the lateral amygdala (Lamprecht et al., 2002).

Our data suggest that DRK likely engages distinct signaling molecules and cascades required for learning and memory perhaps because as in developmental contexts (Hou et al., 1995b; Le and Simon, 1998; Feller et al., 2002), it interacts with different molecules with each SH3 domain. Clearly, the DRK mediated signal engages RAS1 and surprisingly signals specifically to RAF for efficient learning. Congruently, conditional knockout of B-RAF in the murine hippocampus resulted in deficient spatial and contextual learning (Chen et al., 2006), in accord with the learning deficits of other members of the cascade in mice (Brambilla et al., 1997; Giese et al., 2001; Ohno et al., 2001; Costa et al., 2002). However, as in the murine model, the molecular mechanism(s) employed by RAF for learning is currently unclear and will require identification and characterization of additional molecules involved in the process. Since in adult MBs no RTK has been identified to date, the nature of the extracellular signal transduced via DRK is unknown. However, since both DRK-SH2 domain mutants drk^{R1} and drk^{E0A}, exhibited deficits in learning, involvement of at least one RTK in learning and memory is probable. Interestingly, the tyrosine-phosphorylated, tyrosine phosphatase CSW and notably both SRC family members have been identified as DRK interactors in genetic screens (Cooper et al., 1996; Zhang et al., 1999; Firth et al., 2000). SRC could mediate an integrin-originated signal to RAF (Stork, 2003; Kinbara et al., 2004) via DRK. RAF activation via SRC has been recently demonstrated in Drsosphila embryogenesis (Xia et al., 2008) and may be operant in the MBs as well. Interestingly, the integrin VOLADO accumulates preferentially in the MBs and mutants exhibit learning and memory deficits (Grotewiel et al., 1997). This provides an experimentally tractable indirect alternate route for the DRK-mediated signal to RAF.

DRK-mediated signals appear to be required for 90-minute memory even under training conditions yielding normal learning underscoring the proposed dual role of DRK in these processes. This signal(s) is required for maintenance of MAPK activation, known to be essential for memory formation (Adams and Sweatt, 2002; Weeber and Sweatt, 2002; Bozon et al., 2003; Shalin et al., 2004), as illustrated by post-training pMAPK levels in the Drosophila brain. Surprisingly, prolonged RAF activity such as yielded by the constitutively active enzyme appears inhibitory to sustained MAPK activation. Interestingly, these results suggest that RAF activation does not inexorably lead to MAPK activation. Importantly, DRK appears to be required for inhibition of prolonged RAF activity and this is at least one likely role of the protein in 90-minute memory formation, independent of its RAF-activation-dependent role in learning. The precise mechanism of DRK-dependent RAF inhibition is unknown, but currently under investigation.

Multiple roles in learning and memory have been described for protein kinase A in Drosophila (Skoulakis et al., 1993; Li et al., 1996) and other invertebrates (Muller and Carew, 1998; Muller, 2000), but not yet for a protein without enzymatic activity like DRK. However, GRB2 is

required for long term fear memory in the rat, which requires the RhoGAP, RhoA and Rho-Kinase-dependent pathway (Lamprecht et al., 2002). Whether an equivalent pathway is employed for DRK-dependent 90-minute memory formation in Drosophila warrants further investigation. However, a Drosophila p190-RhoGAP is expressed in the MBs and is essential for repression of axonal retraction and is negatively regulated by the integrin-MYOSPHEROID and SRC64 (Billuart et al., 2001). The latter, suggests a potential link with cytoskeletal dynamics in congruence with the vertebrate data (Lamprecht et al., 2002), but potential roles for p190-RhoGAP and MYOSPHEROID in MB-mediated learning and memory have not been evaluated yet.

The distribution for most RAS/RAF/MAPK cascade member proteins in the adult MBs is currently unknown. However, roles for some signaling pathway members have been described for physiological and structural plasticity at the larval neuromuscular junction (Guo et al., 1997; Koh et al., 2002; Hoeffer et al., 2003), but not for behavioral neuroplasticity. An exception is dNF1, a negative RAS regulating GTPase-activating protein (GAP), loss of which also results in olfactory learning and memory deficits (Guo et al., 2000). The protein is known to engage RAS and signaling via MAPK is involved in regulation of circadian behaviors (Williams et al., 2001). However, other reports suggest that the function of dNF1 in learning is to directly or indirectly regulate the RUTABAGA adelylyl cyclase or PKA activity (Guo et al., 2000; Ho et al., 2007) and to engage RAS only for long term memory formation (Ho et al., 2007). Because our data suggest that RAF in the MBs is involved in the DRK-mediated signal that contributes to learning but not memory formation, it is tempting to speculate that dNF1-regulated species of RAS mediate the latter, independently of RAF. However, a potential conflict with involvement of dNF1 in these pathways is presented by its apparent absence in the MBs (Walker et al., 2006). Therefore, it is presently unclear whether DRK-mediated signaling requires dNF1 activity in MBs for normal learning and memory.

Interestingly, our data demonstrate that *drk* mutant heterozygotes and animals with abrogated DRK in the MBs learn inefficiently, but do nevertheless learn to the same level as controls upon overtraining. Thus, learning ability is not compromised in the mutant heterozygotes, or c772/ $drk^{\Delta P24}$; $drkR$-2/+ animals which retain even less protein in their MBs (Figure 3 A, B and C). This pairing-specific learning deficit may reflect the proposed engagement of the molecule in multiple processes and therefore explain the demonstrated dosage sensitive phenotypes. Upon extended training, DRK molecules liberated from signaling complexes formed during the initial pairings may become re-engaged, thus overcoming the limitation of reduced protein and yielding near normal performances. Similar association between pairings and the dynamics of enzymatic activities relative to behavioral output have been described for associative conditioning in invertebrates (Muller and Carew, 1998; Muller, 2000; Friedrich et al., 2004). Significantly, a similar under-training protocol was necessary to uncover fear conditioning defects in mice conditionally lacking Kinase Suppressor of Ras1 (KSR), a scaffolding protein that could compartmentalize RAS/RAF/MAPK signaling within hippocampal neurons (Shalin et al., 2006). Thus, it is possible that DRK is involved in signaling that regulates the rate of learning per CS/US. A similar phenotype was observed by manipulation of the RDL GABA$_A$ receptor in the MBs. Over-expression of this receptor resulted in a pairing-specific decrease in learning efficiency, which also reached control levels with overtraining. The learning rate was increased respectively upon RNAi mediated abrogation of the protein in the same neurons (Liu et al., 2007).

Collectively, these observation lead to the interesting hypothesis that signaling pathways which regulate learning efficiency operate within the MBs in addition to molecules and pathways regulating learning ability. Mutations in the former may be manifested as small, perhaps not significant changes in learning exaggerated upon reduced training. In contrast, we predict that mutations in the latter are likely to result in larger overall learning defects, which could not be

eliminated by overtraining. Because a lot of studies and screens for olfactory learning mutants have been conducted using intensive training protocols of at least 12 CS/US pairings, it is possible that mutations affecting learning efficiency and not learning ability have been missed. Interestingly, RUT adenylyl cyclase null mutants exhibit large learning deficits (Han et al., 1992; Skoulakis and Grammenoudi, 2006) and although they improve their performance upon overtraining, they do not reach control levels (Sideri and Skoulakis unpublished), suggesting a compromise in learning altogether. Ongoing, careful re-evaluation of extant learning and memory mutants is likely to reveal additional members for these categories and support this hypothesis. Finally, the nature of the signals that DRK mediates and the identities and roles of additional molecules engaged in mediating it remain currently unknown. Nevertheless, our results suggest that they will likely be involved in learning efficiency and provide a platform and several testable hypotheses to elucidate them in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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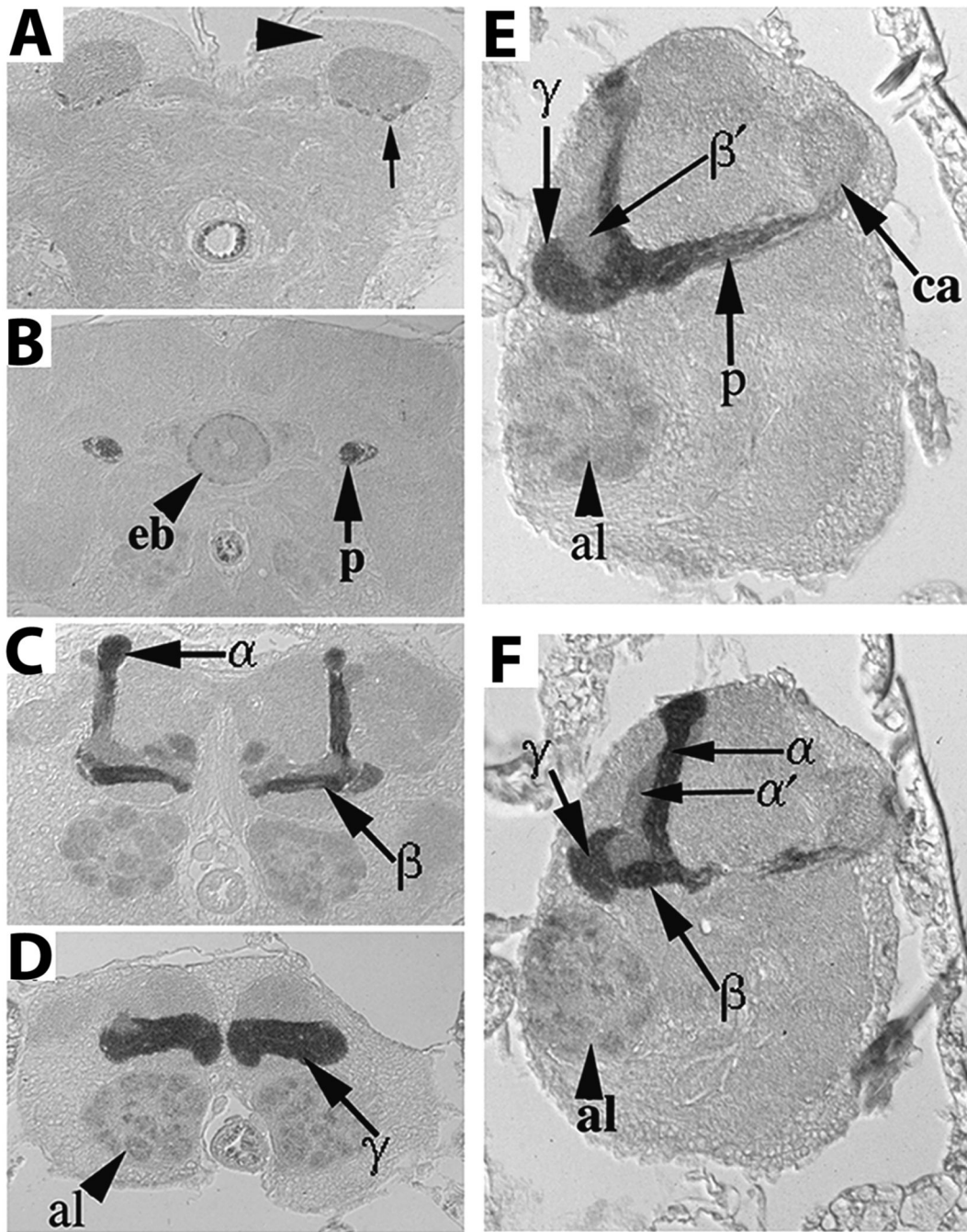
Figure 1. Distribution of DRK in the adult brain

Immunohistochemical detection of DRK in 5μm frontal (A-D) and sagittal (E-F) paraffin sections through the adult brain. Dorsal is up in all photographs. (A) DRK is absent from MB cell bodies (large arrowhead), while low levels appear in the calyces (ca) barely above general neuropil staining. Arrow points to four positively staining axonal fascicles. (B) DRK accumulates within the pedunculus (p), and R4 neurons of the ellipsoid body (eb arrowhead). (C) Preferential DRK distribution within the α, β and γ (D) lobes. Low level of the protein is detectable in glomeruli of the antennal lobe (al, arrowhead in D). (E and F) Saggital sections demonstrate the differential distribution of DRK within the pedunculus, α, β and γ lobes as indicated by the arrows. Anterior is to the left.
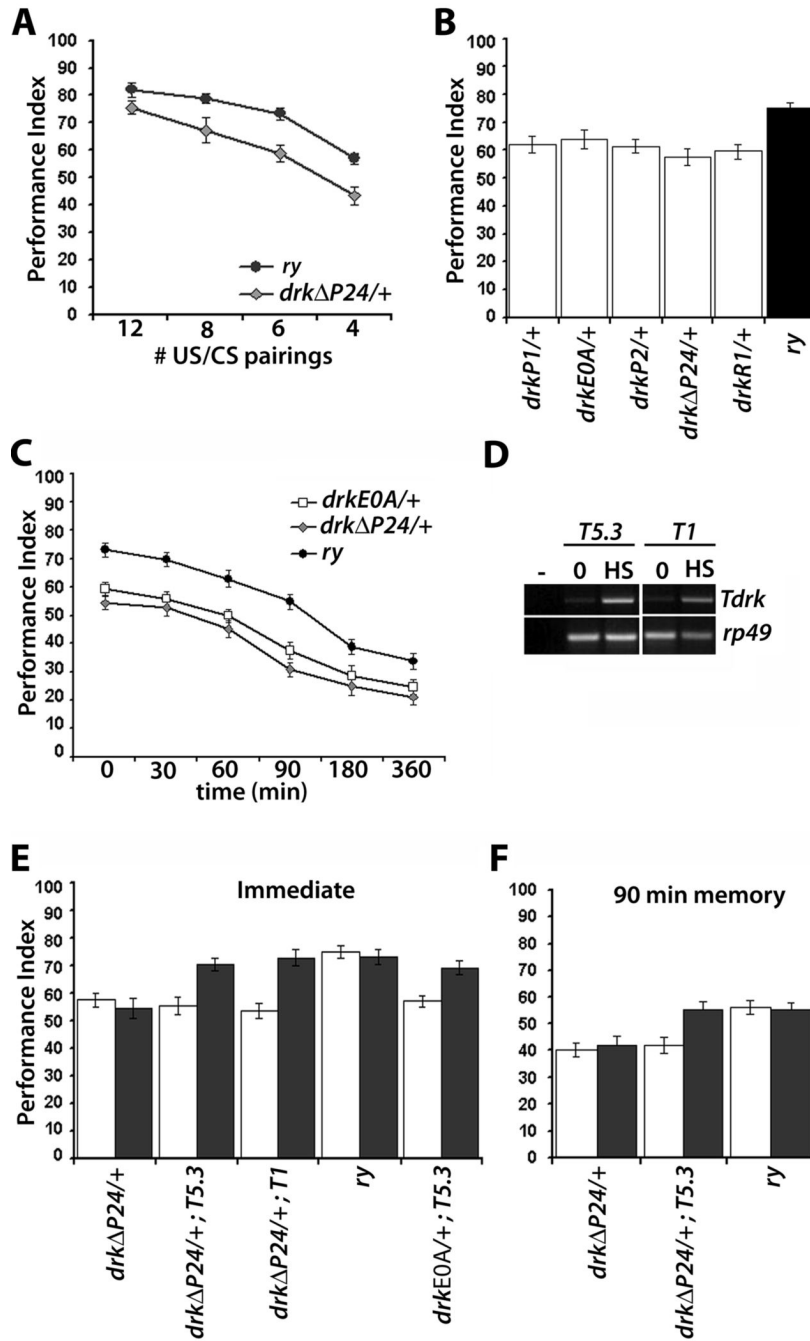
Figure 2. Learning and memory deficits of *drk* mutant heterozygotes

Mean Performance Indices and their Standard Errors of the Mean (PI ± SEM) are shown for all experiments.

(A) Performance immediately after conditioned odor avoidance after training with the indicated number of pairings. n=10 for all points. The performance of ry^{506} flies was significantly different from that of and $drk^{\Delta P24}/+$ after 6 and 8 ($p<0.001$- Student's t-test), but not after 12 pairings ($p<0.02$).

(B) Learning after training with 6 CS/US pairings of *drk* mutant alleles and controls. n≥12. ANOVA indicated significant effects of genotype ($F_{(5, 83)}=13.956$, $p<0.001$) and subsequently confirmed for each allele against the control with Dunnett's tests.

(C) Memory of 6 CS/US training for two representative *drk* alleles. n≥ 12 for each time point. Time 0 represents learning. Two way ANOVA revealed significant effects of genotype ($F_{(2, 218)}$= 11.627, p<0.001) and time ($F_{(5, 218)}$ = 18.732, p<0.001).

Subsequent planned comparisons revealed significant differences (p<0.001) between the *drk^{ΔP24}* and *drk^{EoA}* heterozygotes and controls at all time intervals, except at 360 minutes where the differences were significant at the p<0.05 level (actual values p=0.016 and p=0.028 for *drk^{ΔP24}* and *drk^{EoA}* respectively).

(D) RT-PCR from heterozygous *drk* deletion mutants carrying either the *drkT5.3* or the *drkT1* transgenes subjected to three heat shock inductions, or left untreated (0) prior to RNA isolation. Amplification of *rp49* transcripts served as a semi-quantitative control. The empty lane displays PCR amplification products from non reverse transcribed RNA.

(E) Learning of *drk* mutant heterozygotes bearing the *drkT5.3*, *drkT1* transgenes and controls after induction (black bars), or without induction (open bars). n ≥ 10. ANOVA indicated significant effects of treatment ($F_{(1, 104)}$ =15.376, p<0.001) and genotype ($F_{(9, 104)}$=21.327, p<0.001). Subsequent Tukey-Kramer test (α=0.001) indicated significant differences between induced and uninduced *drk^{ΔP24}/+; T5.3, drk^{ΔP24}/+; T1* and *drk^{EoA}/+; T5.3*, but not between heat shocked and not shocked *ry^{506}, drk^{ΔP24}/+*. In addition, the performance of induced *drk^{ΔP24}/+; T5.3, drk^{ΔP24}/+; T1* and *drk^{EoA}/+; T5.3* was different from that of controls under the same conditions.

(F) Conditional rescue of the 90 minute memory deficit of *drk* deletion heterozygotes. All strains were treated prior to conditioning as described above, trained, stored at the training temperature (24-25°C) and tested 90 minutes post-training. Group mean PIs ± SEM are shown for n ≥ 10. ANOVA indicated significant effects of treatment ($F_{(1, 64)}$ =21.368, p<0.001) and genotype ($F_{(5, 64)}$ =14.521, p<0.001). Subsequent Dunnett's tests confirmed significant differences in performance of heat shocked *ry* and *drk^{ΔP24}/+* and non heat shocked *drk^{ΔP24}/+; T5.3* (p<0.001). However the performance of heat shocked *drk^{ΔP24}/+; T5.3* was statistically indistinguishable from that of similarly treated *ry* and untreated *ry* flies.
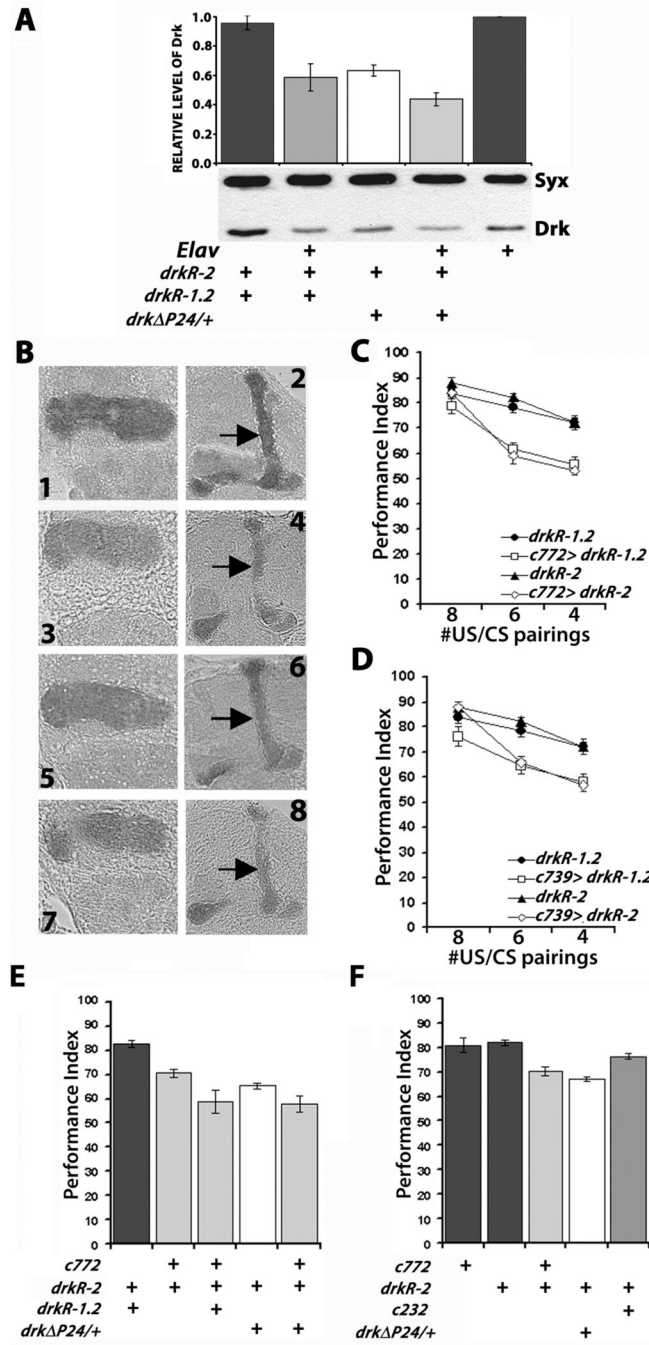
Figure 3. RNA-interference-mediated abrogation of DRK within the MBs phenocopies the *drk* mutant phenotype

(A) Accumulation of DRK in head lysates from controls, $drk^{ΔP24}/+$ and animals driving pan-neuronal expression of *drkR-1.2* and *drkR-2* simultaneously with *Elav*. A representative semi-quantitative western blot of such lysates from the indicated genotypes challenged with a-DRK and a-SYNTAXIN as loading control is shown below, while quantification of five independent such experiments is shown above. Means ± SEM are shown. The level of DRK normalized over the level of SYNTAXIN in control strains was arbitrarily set to 1 (Philip et al., 2001). DRK in extracts from heterozygous mutants and *elav* driven transgenes was statistically significant from that in controls (p<0.001, planned comparisons). No difference was observed

between *drkR-1.2/+*; *drkR-2/+* animals controlling for potential effects of the insertions on DRK levels or *elav/+* controls (p>0.3).

(B) Reduction of DRK levels in the MBs upon RNA-i transgene expression illustrated in 6 μm paraffin sections challenged with a-DRK antibody. 1,2: *drkR-2/+* control animals. 3,4: *drk^{ΔP24}/+*; *drkR-2/+* mutant heterozygotes. 5,6: c772/+; *drkR-2/+*. 7,8: *drk^{ΔP24}/c772*; *drkR-2/ +*. Sections from all genotypes were processed in parallel and each slide contained control animals and all experimental genotypes. Representative images at the levels of the γ-lobe (1, 3, 5, 7) and α/β lobes (2, 4, 6, 8) are shown. Arrows point to the α lobes on the respective genotypes where the reduction in DRK is most obvious.

(C) Abrogation of DRK in α/β and γ MB lobes with c772 impairs learning and phenocopies the *drk* mutant phenotype. Group mean PI ± SEMs are shown, n≥10. ANOVA showed significant effects of genotype ($F_{(3,122)}$=16.006, p<0.0001) and number of CS/US pairings ($F_{(2,122)}$=19.185, p<0.0001). The performance after 8 CS/US was not significantly different in experimental animals with their respective un-driven heterozygous transgene controls (p>0.53). However, with reduced pairings the effects were significant (planned comparisons, p<0.0001)

(D) Abrogation of DRK in α/β MB lobes with c739 impairs learning and phenocopies the *drk* mutant phenotype. Group mean Performance Indices and their Standard Errors of the Mean (PI ± SEM) are shown, n≥7. ANOVA showed significant effects of genotype ($F_{(3,88)}$=13.282, p<0.0001) and number of pairings ($F_{(2,88)}$=19.168, p<0.0001). The performance after 8 CS/ US was not significantly different in experimental animals with their respective undriven heterozygous transgene controls (p>0.56). However, with reduced pairings the effects were significant (planned comparisons, p<0.0001)

(E) The learning impairment is dependent on the amount of DRK protein within the MBs. PI ± SEMs are shown, n≥9. ANOVA indicated significant effects of genotype $F_{(4,56)}$=14.878, p<0.0001. Subsequent contrast analysis showed significant differences in the performances of *drkR-1.2/+*; *drkR-2/+* control animals (black bars) and c772/+; *drkR-2/+* (p<0.0005). The performance of c772/+; *drkR-2/+* was also significantly different from that of *drk^{ΔP24}/c772*; *drkR-2/+* (p<0.001), and from *drkR-1.2/c772*; *drkR-2/+* (p<0.05).

(F) Abrogation of DRK only within the MBs impairs learning. PI ± SEMs are shown, n≥9. ANOVA revealed significant effects of genotype $F_{(4,57)}$=17.480, p<0.0001. Planned comparisons did not reveal any differences between *drkR-1.2/+*; *drkR-2/+* controls 2/+, 772/+ and *drkR-2/c232* (p>0.06). The differences between controls and c772/+; *drkR-2/+* and *drk^{ΔP24}/+*; *drkR-2/+* remained significant p<0.0001).
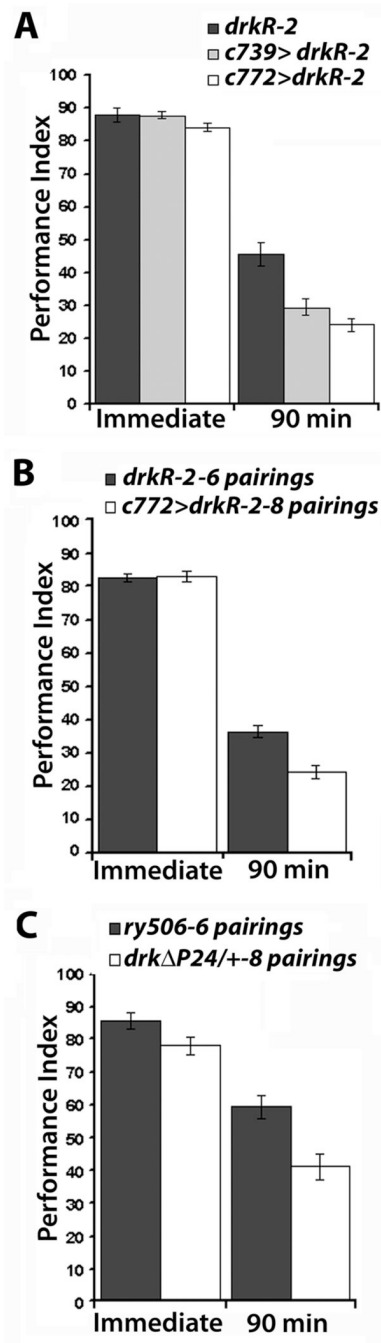
Figure 4. Differential effects of DRK reduction on 90-minute memory

Mean Performance Indices and Standard Errors of the Mean (PI ± SEM) are shown for the indicated genotypes.

(A) Abrogating DRK in the MBs impairs 90-minute memory. Initial training with 8 CS/US produced equal immediate performance between control animals and animals expressing drkRNA-i ($F_{(2,15)}$=1.761, p>0.2), n=5. ANOVA for 90-minute memory of the training revealed significant effects of genotype ($F_{(2,50)}$=13.490 p<0.0001). n≥15.

(B) Training RNA-i transgene expressing animals with increased number of pairings does not improve the memory impairment, revealing a specific effect of DRK abrogation in the MBs on memory. Learning was equal between under-trained controls (6 pairings) and *drkR-2*

expressing animals trained with 8 CS/US. ($F_{(2,12)}$=0.029, p>0.86, n=6). In contrast, 90-minute memory remained impaired in RNAi-expressing flies compared to under-trained controls ($F_{(1,46)}$=18.057, p<0.0001, n≥20).

(C) The 90-minute memory impairment remained unaltered by more intensive training of $drk^{\Delta P24}$/+ heterozygotes indicating a memory specific impairment. ANOVA did not reveal differences between under-trained controls and $drk^{\Delta P24}$/+ heterozygotes trained with 8 pairings. ($F_{(1,12)}$=2.327, p>0.16, n=6). 90-minute memory was significantly impaired in $drk^{\Delta P24}$/+ flies compared to under-trained controls ($F_{(1,16)}$=5.978, p<0.05, n≥ 7).
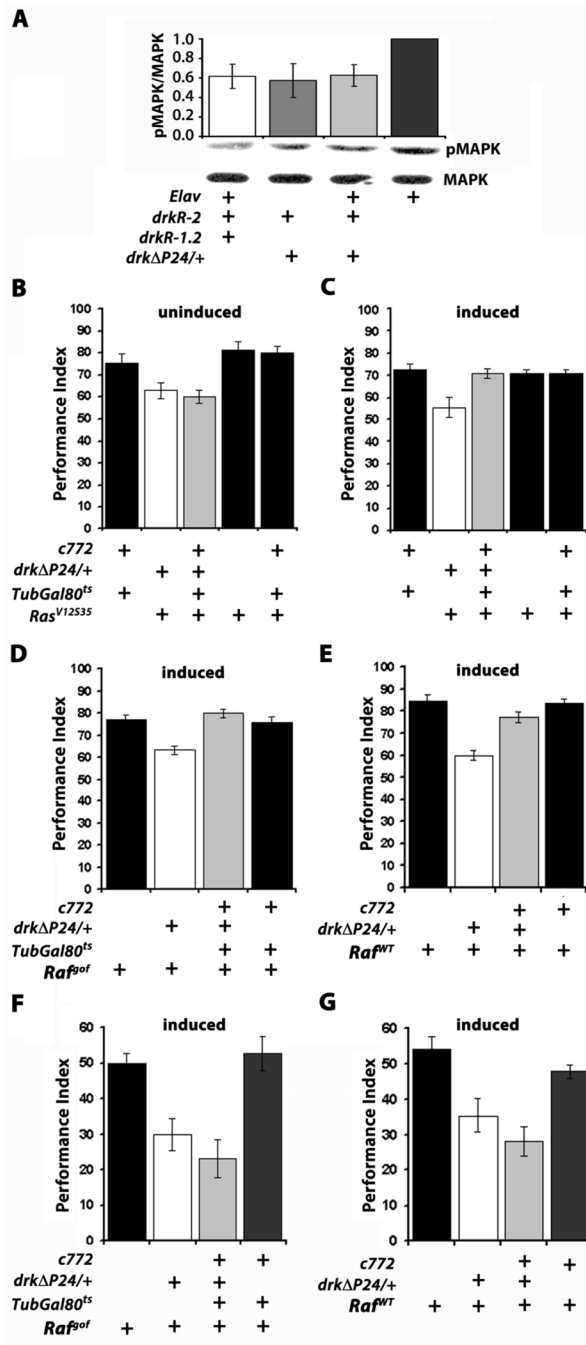
Figure 5. Rescue of the learning, but not the 90-minute memory deficit of $drk^{\Delta P24}$ heterozygotes by conditional expression of *Ras* and *Raf* transgenes in the MBs

(A) MAPK phosphorylation is impaired upon abrogation of DRK in the adult nervous system. A representative semi-quantitative western blot is shown below the quantitative data from four independent blots. The ratio of pMAPK/MAPK in the control *Elav*/+ strain was arbitrarily set to 1. The Mean ± its standard error are shown. ANOVA indicated significant differences between genotypes ($F_{(3,19)}$=3.853, p<0.05). Compared to control, phosphorylated MAPK was significantly reduced in *Elav*/+; *drkR-1.2*/+; *drkR-2*/+ (p<0.01) and $drk^{\Delta P24}$/+; *drkR-2*/+ (p=0.01) while the difference from *Elav*/+; $drk^{\Delta P24}$/+; *drkR-2*/+ was significant at p=0.02 (Student's t-test)

(B) Performance after 6 CS/US training in flies carrying a Ras^{V12S35} effector loop mutant transgene in a mutant background (gray bar) kept inactive prior to training (uninduced), did not perform better than mutant animals without it (open bars). Mean Performance Indices and Standard Errors of the Mean (PI ± SEM) are shown as in all experiments below. ANOVA indicated genotype effects ($F_{(4,30)}$=5.991, p<0.0001, n= 6). However, subsequent contrast analysis demonstrated that the differences were among control groups (black bars) and flies carrying the $drk^{\Delta P24}$ mutation (p<0.001) and the differences between the later two groups were not significant (p<0.57).

(C) Expression of the Ras^{V12S35} transgene in the MBs (induced) by inactivation of the Gal80^{ts} rescued the learning deficit of $drk^{\Delta P24}$ animals (open bar) as indicated by ANOVA ($F_{(4,42)}$= 6.407, p<0.0001, n≥ 8). Contrast analysis demonstrated highly significant differences (p<0.0003) in the performance of animals carrying the $drk^{\Delta P24}$ mutation, but expressing the transgene (gray bar) and those that do not (open bar), while the performance of the former was not different than that of controls (p<0.84).

(D) Induction of constitutively active RAF (Raf^{gof}) in the adult MBs rescues learning after 6 CS/US training. Performance only after induction of the transgene is shown, as uninduced transgene did not have any effect on the mutant phenotype as shown above for the Ras^{V12S35} transgene. ANOVA revealed significant effect of genotype ($F_{(3,57)}$= 8.441, p<0.0001, n≥10) and treatment ($F_{(1,87)}$=34.02, p<0.0001). Contrast analysis indicated significant differences between $drk^{\Delta P24}$/+; Raf^{gof}/+ animals and flies expressing the transgene (p<0.0002), indicating full rescue of the defect. No differences were observed between flies expressing the transgene and control groups (p<0.14).

(E) Partial rescue of $drk^{\Delta P24}$ heterozygote learning deficits upon conditional accumulation of wild-type RAF protein in the MBs. Performances only after transgene induction is shown. ANOVA revealed differences among RAF accumulating strains ($F_{(3,35)}$=20.816, p<0.0001, n≥7). Subsequent contrast analysis showed significant differences (p<0.0001) between drk mutant heterozygotes not expressing the Raf^{wt} transgene and those expressing it under c772. However, the performance of Raf^{wt}/+; $drk^{\Delta P24}$/+ (gray bar) was different (p<0.05) from that of Raf^{wt}/+and Raf^{wt}/+; c772/+ animals.

(F) The $drk^{\Delta P24}$/+ 90-minute memory impairment is not reversed upon conditional expression of constitutively active RAF in the MBs. Only the performance of animals after induction of the transgenes and 6 CS/US training is shown. ANOVA suggested significant differences ($F_{(3,37)}$=10.449, p<0.0001, n≥8). Subsequent Dunnett's tests using the performance of Raf^{gof}/+as control indicated significant differences with $drk^{\Delta P24}$/+; Raf^{gof}/+ (p< 0.005) and c772/$drk^{\Delta P24}$; Raf^{gof}/ TubGAL80^{ts} (p<0.0001) suggesting a lack of rescue. (The difference with c772/+; Raf^{gof}/ TubGAL80^{ts} was not significant, p>0.7)

(G) The $drk^{\Delta P24}$/+ 90-minute memory impairment is not reversed upon expression of wild type RAF in the MBs after 6 CS/US training. ANOVA indicated significant differences ($F_{(3,27)}$=9.926, p<0.0002, n≥7) and Dunnett's tests indicated that compared to the performance of Raf^{wt}/+, that of Raf^{wt}/+; $drk^{\Delta P24}$/+ was significantly different (p<0.007) and importantly, so was that of Raf^{wt}/+; c772/ $drk^{\Delta P24}$ (p<0.0002) indicating lack of rescue. Performances after transgene induction are shown.
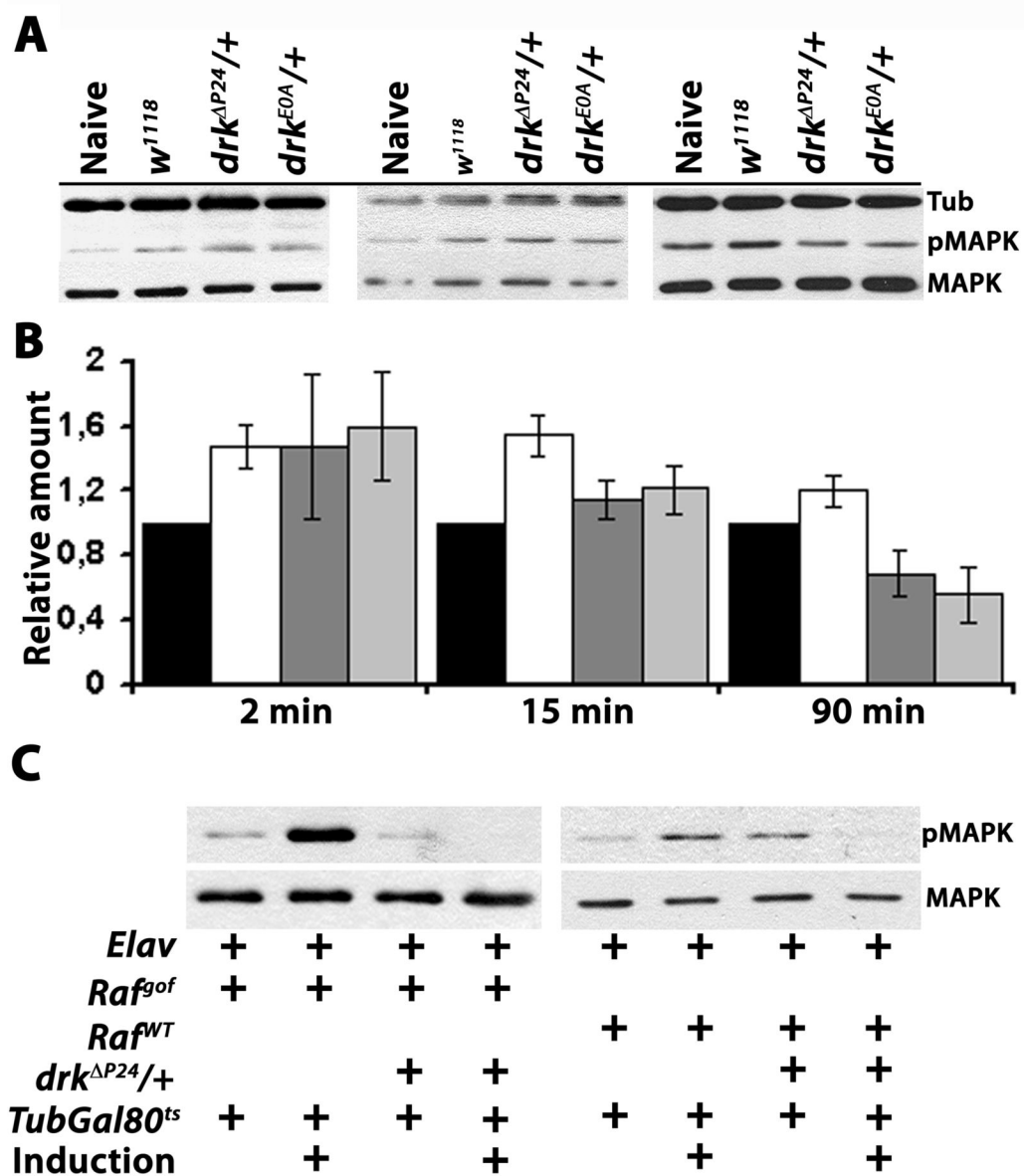
Figure 6. Impaired MAPK activation in *drk* mutant heterozygotes

(A) Representative western blots probing the level of phosphorylated MAPK (pMAPK) in comparison to total levels of the protein (MAPK) following associative conditioning as detailed in Materials and Methods. The level of Tubulin in each of the indicated control and mutant head lysates was used to normalize protein levels in each lane and for quantification. Lysate from a single head equivalent was loaded per lane. Naïve denotes lysates from w^{1118} animals that underwent all manipulations in parallel except for associative learning. Blots from animals allowed to rest 2 minutes, 15 minutes or 90 minutes post-training (as indicated in the graph below n B), followed by lysate preparation are shown left to right.

(B) The mean and its standard error from at least 3 independent determinations of the relative amount of pMAPK to that of MAPK in the samples is shown. The levels of pMAPK and MAPK were determined relative to Tubulin to normalize loading differences. This ratio for naïve animals was fixed to 1. ANOVA did not indicate significant differences among the samples obtained 2 minutes after training ($F_{(3,19)}$=1.350, p<0.29, n=4). In contrast, differences among

the samples obtained 15 minutes post-training were significant ($F_{(3,15)}$=3.998, p<0.03, n=3). Subsequent pairwise t-tests showed significant difference from naive only for wild type flies (p<0.009), but not for $drk^{\Delta P24}$/+ or drk^{E0A}/+ (p<0.45 and p<0.29 respectively). Contrast analysis showed that w^{1118} pMAPK levels were significantly different from $drk^{\Delta P24}$/+ and drk^{E0A}/+ (p<0.02). 90 minutes post-training, ANOVA indicated significant effects of genotype ($F_{(3,17)}$= 7.638 p<0.003, n=4). Pairwise t-tests showed that wild type pMAPK levels were not different from that in naïve animals (p<0.16) indicating that MAPK phosphorylation returned to naive levels. In contrast, pMAPK levels in $drk^{\Delta P24}$/+ and drk^{E0A}/+ were different (lower) that in naïve flies (p<0.04 and p<0.01 respectively). Contrast analysis also showed that pMAPK levels in w^{1118} were significantly higher than those in $drk^{\Delta P24}$/+ and drk^{E0A}/+ (p<0.004). (C) Western blots probing the level of pMAPK in single dissected brains of naïve flies carrying Raf^{gof} (left side), or Raf^{WT} (right side) transgenes in control (w^{1118}), or $drk^{\Delta P24}$/+ mutant background, with of without transgene induction by incubation at 30°C as indicated by the crosses. The pMAPK level is elevated upon Raf^{gof} and to a lesser degree Raf^{WT} expression in w^{1118}, but not when the flies are heterozygous for the $drk^{\Delta P24}$ null allele. Quantification of the levels revealed highly significant differences between controls and $drk^{\Delta P24}$/+ upon Raf^{gof} ($F_{(1, 6)}$= 354, p<0.001, n=3) and upon Raf^{WT} ($F_{(1,6)}$= 7.85, p<0.04, n=3) expression.

Table 1

Task-relevant Sensory Behaviors

Genotype	Benzaldehyde	Octanol	Electric Shock
$drk^{P1}/+$	68.27 ±2.95	54.82 ±3.27	76.23 ±1.94
$drk^{E0A}/+$	66.22 ±3.16	58.26 ±3.07	78.74 ±2.55
$drk^{P2}/+$	71.09 ±3.36	57.94 ±2.78	73.93 ±2.36
$drk^{AP24}/+$	69.43 ±2.76	60.12 ±2.97	75.43 ±1.94
$drk^{R1}/+$	64.98 ±3.87	58.29 ±3.28	74.29 ±2.86
+; T5.3	64.65 ±2.85	52.82 ±4.06	72.92 ±2.74
$drk^{E0A}/+$; T5.3	62.28 ±3.73	59.46 ±4.16	74.05 ±2.56
$drk^{AP24}/+$; T5.3	70.14 ±3.65	59.36 ±3.76	73.24 ±2.32
$drk^{AP24}/+$; T1	69.32 ±3.46	61.08 ±4.17	76.48 ±2.05
ry^{506}	65.71 ±2.89	57.32 ±2.98	74.61 ±2.25
UAS-drkR-2/+	73.40 ±5.47	67.30 ±7.60	77.98 ±3.38
UAS-drkR-2/c772	68.27 ±7.51	59.91 ±3.36	82.21 ±3.45
UAS-drkR-2/c739	70.30 ±7.42	66.19 ±2.52	83.20 ±3.23
UAS-drkR-2/c232	74.67 ±8.65	68.88 ±6.30	77.88 ±4.19
UAS-drkR-1.2/+	63.71 ±3.51	50.32 ±1.80	83.78 ±3.20
UAS-drkR-1.2/c772	72.36 ±6.85	44.62 ±4.54	85.66 ±2.87
UAS-drkR-1.2/c739	56.88 ±7.69	50.86 ±3.47	81.96 ±1.08
UAS-drkR-1.2/+;drkR-2/+	83.45 ±5.18	60.17 ±5.22	77.25 ±2.64
UAS-drkR-1.2/c772;drkR-2/+	84.46 ±1.20	55.17 ±5.17	75.27 ±3.97
$UAS-Ras^{V12S35}/+$	72.35 ±3.29	57.29 ±2.80	76.87 ±2.93
$drk^{AP24},UAS-Ras^{V12S35}/+$	68.84 ±3.47	62.54 ±4.13	75.58 ±3.26
$UAS-Ras^{V12S35}/c772$	64.24 ±4.69	59.63 ±3.88	78.35 ±3.89
$c772/+; TubGAL80^{ts}/+$	70.36 ±3.82	65.62 ±2.96	81.29 ±2.53
$drk^{AP24},UAS-Ras^{V12S35}/c772;TubGAL80^{ts}/+$	72.67 ±3.27	64.82 ±4.81	78.43 ±3.16
$drk^{AP24},UAS-Ras^{V12S35}/+;TubGAL80^{ts}/+$	69.64 ±4.72	66.40 ±3.95	73.62 ±3.68
$UAS-Raf^{gof}/+$	64.34 ±3.94	79.02 ±6.11	84.26 ±3.65
$drk^{AP24}/+;UAS-Raf^{gof}/+$	61.50 ±3.64	81.71 ±5.38	76.48 ±4.44
$drk^{AP24}/c772;UAS-Raf^{gof}/TubGAL80^{ts}$	69.02 ±5.68	87.28 ±5.46	78.98 ±6.02
$drk^{AP24}/c739,TubGAL80^{ts};UASRaf^{gof}/+$	64.75 ±7.71	82.20 ±5.55	89.29 ±3.89
$c772;UAS-Raf^{gof}/TubGAL80^{ts}$	62.09 ±6.07	75.05 ±4.55	90.76 ±3.76
$c739,TubGAL80^{ts};UAS-Raf^{gof}/+$	58.02 ±4.02	85.60 ±5.44	78.20 ±3.72
$UAS-Raf^{wt}/+$	81.52 ±3.37	50.29 ±3.31	81.89 ±4.19
$UAS-Raf^{wt}/+;drk^{AP24}/+$	85.41 ±1.92	54.82 ±7.08	88.82 ±2.83
$UAS-Raf^{wt}/+;c772/+$	84.28 ±5.79	47.75 ±6.91	88.59 ±4.86
$UAS-Raf^{wt}/+;drk^{AP24}/c772$	84.24 ±2.09	55.41 ±5.17	77.60 ±6.18

Avoidance of the aversive odor stimuli (CS) and electric shock (US) is shown for all relevant strains. The performance of strains with ry^{506} genetic background (upper group) was compared to that of their genetic control (ry^{506}) and ANOVA did not indicate differences (n≥7 for all). Similarly, the performance of strains expressing the RNA-i mediating transgenes in the second group was compared with that of their heterozygous transgene controls and significant differences were not uncovered (n≥6). Avoidance of strains in the third group was compared to that of their proper control *UAS-*$Ras^{V12S35}/+$ but differences were not significant (n≥ 6). The performance of strains in the fourth group was also compared to that their proper control

UAS-Raf ^{gof}/+ or *UAS-Raf* ^{wt}/+ respectively and significant differences were not revealed (n≥7). All strains were not tested simultaneously, although all strains within a group were. Thus, statistical analyses for performance differences were performed exclusively within each group.