Memory enhancement and formation by atypical PKM activity in *Drosophila melanogaster*

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Synaptic stimulation activates signal transduction pathways, producing persistently active protein kinases. PKMζ is a truncated, persistently active isoform of atypical protein kinase C-ζ (aPKCζ), which lacks the N-terminal pseudosubstrate regulatory domain. Using a Pavlovian olfactory learning task in *Drosophila*, we found that induction of the mouse aPKMζ (MaPKMζ) transgene enhanced memory. The enhancement required persistent kinase activity and was temporally specific, with optimal induction at 30 minutes after training. Induction also enhanced memory after massed training and corrected the memory defect of *radish* mutants, but did not improve memory produced by spaced training. The 'M' isoform of the *Drosophila* homolog of MaPKCζ (DaPKM) was present and active in fly heads. Chelerythrine, an inhibitor of PKMζ, and the induction of a dominant-negative MaPKMζ transgene inhibited memory without affecting learning. Finally, induction of DaPKM after training also enhanced memory. These results show that atypical PKM is sufficient to enhance memory in *Drosophila* and suggest that it is necessary for normal memory maintenance.

The study of PKC in memory formation has a long history[1–3]. However, most previous work was done before our current appreciation of the complexity of the PKC gene family. The PKC family can be divided into three classes based on their cofactor requirements[4,5]. Whereas all PKC proteins require phosphatidylserine for activation, the 'conventional' (cPKC) isotypes require diacylglycerol (DAG) and Ca^{2+} for full activity; 'novel' (nPKC) isotypes are Ca^{2+} independent but still require DAG, and the 'atypical' (aPKC) isotypes are both DAG and Ca^{2+} independent. Structurally, these kinases can be divided into an N-terminal regulatory domain, which contains a pseudosubstrate region as well as the binding sites for the required cofactors, and the C-terminal catalytic domain. Removal of the N-terminal regulatory domain produces a persistently active kinase, referred to as PKM[6,7]. Persistently active kinases have received attention as components of memory mechanisms[8,9].

The roles of PKC in hippocampal models of synaptic plasticity, long-term potentiation (LTP) and long-term depression (LTD) have been studied extensively[10]. PKC/M activities may have several roles in the mechanisms that initiate and sustain LTP[11]. However, western blot analyses with antibodies specific for each of the rat PKC isoforms demonstrate that the only one whose levels specifically increase and remain elevated during the maintenance phase of LTP is PKMζ, the truncated form of the atypical isozyme PKCζ (refs. 12,13). Expression analyses also show that the maintenance of LTD is associated with decreasing levels of PKMζ (ref. 14). Most interestingly, LTP maintenance is abolished by sustained application of low concentrations of the PKC inhibitor chelerythrine, whereas perfusion of PKMζ into CA1 pyramidal cells produces an increase in AMPA receptor-mediated synaptic transmission (D.S.F. Ling *et al.*[15], this issue).

Experiments in honeybees also indicate a role for PKC in memory formation. Biochemical analyses of extracts made from the antennal lobes of associatively trained bees show a sustained increase in cytosolic, Ca^{2+}-independent PKC activity[16]. This persistent increase correlates with long-lasting bee memory in four ways: it requires multiple training trials; it persists for up to three days; it is insensitive to a drug that blocks cPKC activity; and it is blocked by protein-synthesis inhibitors. Together with the LTP data, these studies point to an important role for a nonconventional PKC activity in the maintenance of memory.

In *Drosophila*, the best characterized assay for associative learning and memory is an odor-avoidance behavioral task[17]. This classical (Pavlovian) conditioning involves exposing the flies to two odors (the conditioned stimuli, or CS), one at a time, in succession. During one of these odor exposures (the CS^+), the flies are simultaneously subjected to electric shock (the unconditioned stimulus, or US), whereas exposure to the other odor (the CS^-) lacks this negative reinforcement. After training, the flies are placed at a 'choice point', where the odors come from opposite directions, and they decide which odor to avoid. By convention, learning is defined as the fly's performance when testing occurs immediately after train-

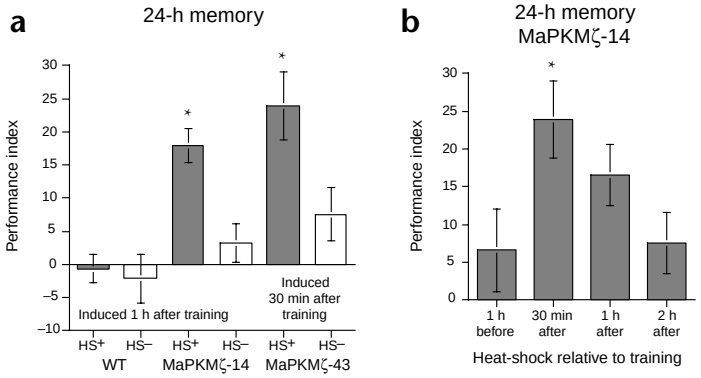
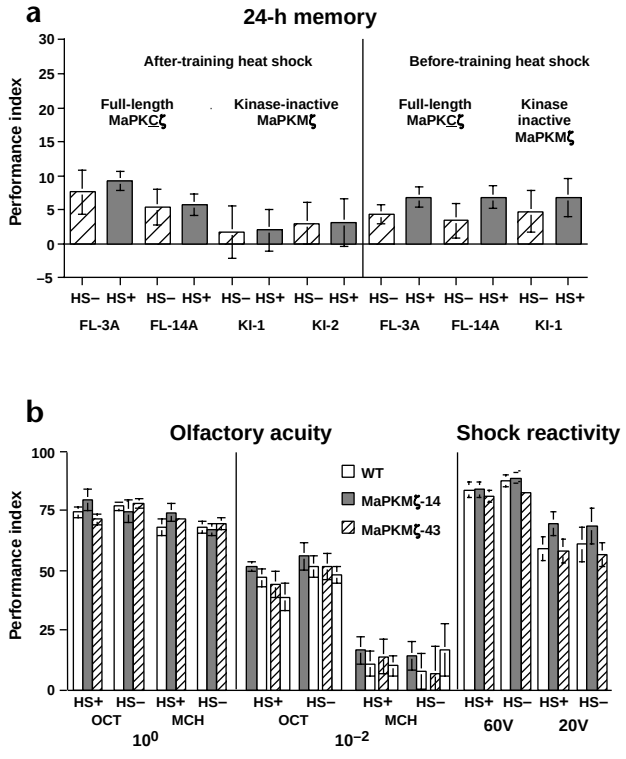


Fig. 1. Memory enhancement by MaPKMζ in *Drosophila*. (**a**) Memory enhancement by heat-shock induction after training of two independent lines bearing a *hsp70-MaPKM*ζ transgene. Flies were subjected to single-cycle training, allowed to recover at 25°C, and the *MaPKM*ζ transgene was induced with a 30-min, 32°C heat shock. Performance was measured 24 h later. The induction of line 14 and the wild-type (WT) control (Methods) began 1 h after training ended, and the induction of line 43 began 30 min after training. Both lines show clear induction effects (n = 8 for all groups). Error bars represent standard error of the mean and, unless noted, the asterisks indicate statistical significance ($p < 0.001$) calculated using ANOVA and Dunnet's test throughout this work. (**b**) Temporal specificity of the memory enhancement by MaPKMζ. MaPKMζ transgenic flies (line 14) were subjected to a 30-min, 32°C heat shock at various times before and after a single cycle of training, and tested for 24-h memory. Heat shock ended 1 h before training started, or began 30 min, 1 h or 2 h after training finished (n = 8 for all groups).

ing. A single training trial produces strong learning: a typical response is that >90% of the flies avoid the CS^+. Performance of wild-type flies from this single-cycle training decays over a roughly 24-hour period until flies once again distribute evenly between the two odors. Flies can also form long-lasting associative olfactory memories, but normally this requires repetitive training regimens[18].

We used this task in *Drosophila* to examine the role of atypical PKM in memory formation. We found that induction of the mouse aPKMζ (MaPKMζ) transgene enhanced memory, and corrected the memory defect of *radish* mutants. There is a single atypical PKC in *Drosophila* (http://www.fruitfly.org)[19], and we have shown that the truncated 'M' isoform, DaPKM, is preferentially expressed and active in fly heads. Both pharmacological and dominant-negative genetic intervention of DaPKC/M activity disrupted normal memory. Finally, induction of the predicted DaPKM also enhanced memory, further suggesting a general role of aPKM in memory processes.



RESULTS

MaPKMζ induction enhances memory in *Drosophila*

To investigate the role of PKC in learning and memory in *Drosophila*, we made transgenic lines of flies bearing heat shock-inducible, murine atypical PKC (MaPKC) isoforms (Methods). Considering that LTP experiments indicate that MaPKMζ levels increase after the presentation of the stimuli required for long-lasting potentiation, we tested whether inducing MaPKMζ after training affected olfactory memory. Induction by mild heat shock (32°C) after training strongly enhanced 24-hour memory (**Fig. 1a**). This enhancement was not due to transgene-independent heat-shock effects, because the wild-type flies did not show enhanced memory when exposed to heat shock. The transgenic flies were made in this wild-type strain, so the enhancement was not due to differences in genetic background. Finally, the memory enhancement did not result from an insertional mutation caused by the transgene, because two independent lines (MaPKMζ-14 and MaPKMζ-43) had similar effects.

Memory enhancement is temporally specific

We also tested whether we could enhance 24-hour memory after single-cycle training by inducing MaPKMζ with a strong heat shock (37°C) 3 hours before training, but this regimen had no effect (data not shown). Because transgene induction after behav-

Fig. 2. Memory enhancement requires persistent kinase activity and is not due to sensory enhancement. (**a**) Neither a kinase-inactive (KI) mutant of MaPKMζ, nor full-length (FL) MaPKCζ enhances 24-h memory. Two independent lines (FL-3A, FL-14A) of the full-length *hsp70-MaPKC*ζ transgene (Methods) and two independent lines (KI-1 and KI-2) of a *hsp70-KI-MaPKM*ζ transgene were assessed for memory enhancement. The KI-MaPKMζ mutant (K281W) disrupts kinase activity by altering the ATP-binding domain (see Results and Methods). Two different heat-shock schedules were used: a 30-min, 32°C induction 3 h before training, or a 30-min, 37°C heat shock given 30 min after the end of training. Neither heat-shock regimen produced induction-dependent enhancement of 24-h memory in any of these lines. (**b**) Induction of MaPKMζ does not affect peripheral behaviors. Flies were subjected to a 30-min, 32°C heat shock, allowed to recover for 1 h, then tested for shock reactivity. Olfactory acuity was tested by exposing the flies to a 30-min, 32°C heat shock, and assayed for odor acuity 24 h later. The legend inset in the olfactory acuity panel applies to both sets of histograms. In all measures, both the MaPKMζ lines (14 and 43) were indistinguishable from the wild-type controls. The most critical control is the 10^{-2} olfactory acuity test (see Results), and for this the transgenic flies were heat-shocked (or not), stored, and tested simultaneously with their respective wild-type controls. We include both sets of wild-type controls, adjacent to their transgenic counterparts, for direct comparison. A more complete description of the shock reactivity and olfactory acuity control assays can be found elsewhere[18,49].

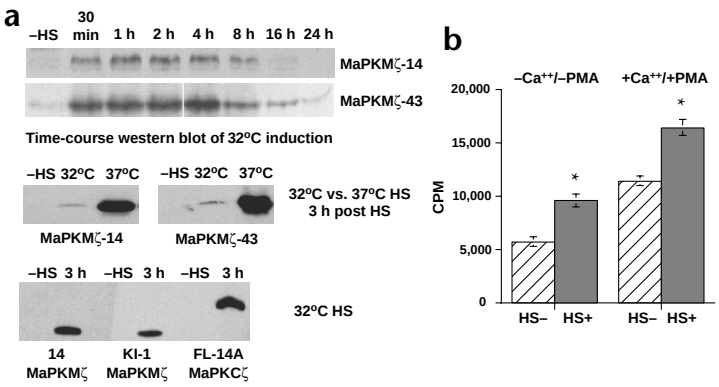


Fig. 3. Expression and biochemical analyses of transgenic lines. (**a**) Western blot analyses of MaPKMζ and MaPKCζ induction after heat shock. Flies were subjected to heat shock (32°C or 37°C) for 30 min. Top, time-course western blots from both MaPKMζ lines. At the indicated time points, flies were collected and head extracts were used for western blot analysis. Middle, direct comparison of the induction achieved at 32°C versus 37°C. In each case, the flies were heat-shocked for 30 min, and head extracts were made after 3 h of recovery time. Bottom, direct comparison of the expression of the MaPKMζ (line 14), the kinase-inactive (KI)-MaPKMζ (KI-1), and full-length (FL)-MaPKCζ (FL-14A). For each western blot, equivalent amounts of total protein (five heads per lane) were loaded in each lane. (**b**) Induction of MaPKMζ (HS+) results in an increase in atypical PKC activity in fly head extracts. MaPKMζ transgenic flies (line 43) were subjected to a 37°C heat shock for 30 min, allowed to recover at 25°C for 1 h, and frozen in liquid nitrogen. Control flies (HS–) remained at 25°C throughout. Total protein extracts were made from fly heads and assayed for PKC activity (Methods). An increase in kinase activity could be detected (–Ca^{2+}/–PMA: HS+ versus –HS, $p = 0.006$, $n = 4$; +Ca^{2+}/+PMA: HS+ versus –HS, $p = 0.001$, $n = 4$; Student's t-test) with a strong (37°C) but not with a mild (32°C) heat shock (data not shown). The induction levels are much greater at 37°C versus 32°C (**a**). We could not test whether the strong heat shock was better for memory enhancement because this regimen in itself is disruptive to memory formation[46].

ioral training enhanced memory, whereas induction before training did not, we next examined the temporal specificity of this MaPKMζ-dependent effect. We found that optimal enhancement occurs when heat-shock induction begins 30 minutes after training ends, and the effect is absent if heat shock occurs before, or is delayed until 2 hours after training (**Fig. 1b**).

Persistent MaPKMζ activity required

The memory enhancement was not observed when a kinase-inactive (KI) mutant of MaPKMζ (ref. 21) was induced either before or after training (**Fig. 2a**; two independent lines, KI-1 and KI-2, of KI-MaPKMζ; Methods). The enhancement was also not observed when full-length (FL)-MaPKCζ was induced before or after training (**Fig. 2a**; two independent lines, FL-3A and FL-14A; Methods). The failure of either the KI-MaPKMζ or the FL-MaPKCζ transgene to enhance memory was not due to lack of expression, because both are expressed at levels comparable to the MaPKMζ protein (**Fig. 3a**; KI-1 and FL-14A versus MaPKMζ-14). Together, these results indicate that the memory enhancement (**Fig. 1**) requires a persistently active aPKM isoform.

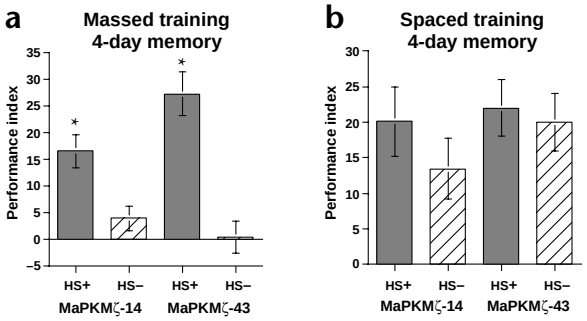


Fig. 4. MaPKMζ induction enhances 4-day memory after massed, but not spaced training. (**a**) MaPKMζ induction after massed training enhances 4-day memory. MaPKMζ flies were trained with 10 cycles of massed training (Methods), allowed to rest for 30 min, then subjected to a 30-min heat shock (32°C). Performance was measured at 4 days; $n = 8$ for all groups. Both lines (14 and 43) show significant induction-dependent improvement of 4-day memory. At 4 days, the memory induced by massed training has normally decayed so that the PI = 0 (ref. 18), as is the case for uninduced flies (–HS). (**b**) Four-day memory produced by spaced training is not improved by MaPKMζ induction. MaPKMζ flies were trained with 10 cycles of spaced training (Methods), allowed to rest for 30 min, and subjected to a 30-min, 32°C heat shock. Memory was measured 4 days after training. $n = 8$ for all groups. Neither line shows a significant effect of transgene induction on memory after spaced training. To rule out an effect of saturation, flies were also trained with a submaximal number of spaced trials (7), but transgene induction still had no effect (data not shown).

Biochemical detection of MaPKMζ induction

We detected inducible increases in MaPKMζ protein levels and kinase activity in extracts made from *Drosophila* heads (**Fig. 3**). Western blot analyses showed that both the mild and strong heat-shock regimens induced the MaPKMζ and MaPKCζ isoforms, and that these proteins persisted for ~18 hours after heat shock (**Fig. 3a**). The induced MaPKMζ protein was active, as we observed enhancement of Ca^{2+}/DAG-independent PKC activity in fly head extracts from induced but not from uninduced transgenic flies (**Fig. 3b**).

MaPKMζ does not affect peripheral behaviors

The memory enhancement occurred only when the transgene was induced after training; therefore it is not likely due to an effect of transgene expression on the perception of either the shock or the odors at the time of training. We found that there is no effect of MaPKMζ induction on shock reactivity, as the transgenic flies behaved indistinguishably from the wild-type strain, irrespective of heat shock (**Fig. 2b**). Thus, transgenic flies did not perceive shock better during training. Because the memory enhancement was induction-dependent (**Fig. 1a**), it cannot be attributed to small amounts of leaky expression during training. Although MaPKMζ had decayed to pre-heat-shock levels by the time of testing at 24 hours (**Fig. 3a**), it was possible that it enhanced olfactory responses at the time of testing. This was not the case because olfactory acuity at 24 hours after induction was normal (**Fig. 2b**). These data demonstrate that the MaPKMζ induction has no behavioral effect on either sensory modality, and indicate that the effect we observe is due to bona fide memory enhancement.

MaPKMζ enhances massed, but not spaced training

Drosophila can form associative olfactory memories lasting 24 hours and longer, but this normally requires repetitive training[19]. Multiple-trial training regimens have been established that produce both anesthesia-resistant memory (ARM) and long-term

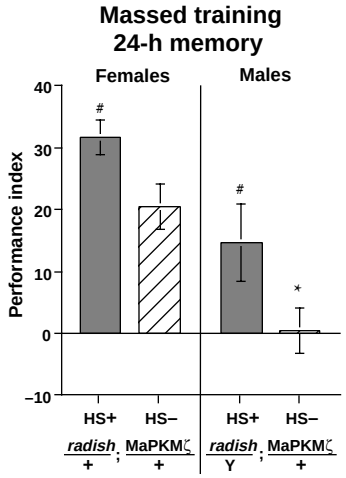


Fig. 5. MaPKMζ induction corrects the memory deficit of *radish* mutants. Homozygous *radish* females were mated to males homozygous for an autosomal MaPKMζ transgene. (Line 43 was used in this experiment.) Male and female progeny from this mating were trained and tested together, providing an internal control, and then counted separately to generate sex-specific PI values. The genotypes of the males and females are give below the histogram. Using Dunnet's test with uninduced females as the control group, the only group showing statistically different performance is the uninduced males (noted by the asterisk). Analysis of HS+ versus HS– groups using Student's *t*-test shows that both males and females display significant differences in performance by transgene induction (denoted by #).

memory (LTM)[18]. ARM can be produced by 10 cycles of 'massed' training (Methods) with no rest intervals between the individual training trials, and lasts 2–3 days. LTM results from repetitive training that contains rest intervals (15 min each; see Methods), and 10 cycles of this 'spaced' training generates LTM that lasts at least 7 days[18]. To test whether MaPKMζ could enhance ARM or LTM, we subjected flies to massed or spaced training regimens, induced the transgene for 30 minutes after training, and then measured 4-day memory.

MaPKMζ induction substantially increased 4-day memory after massed training (**Fig. 4a**; HS+ compared to –HS for each line) but did not improve 4-day memory after spaced training (**Fig. 4b**; HS+ compared to –HS for each line). These data indicate that MaPKMζ induction enhances massed training-induced, but not spaced training-induced memory.

Radish does not block memory enhancement

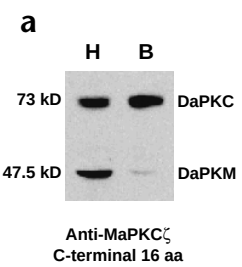
Previous work indicates that consolidated memory in *Drosophila* consists of two biochemically separable components: ARM and LTM. ARM is produced by either massed or spaced training, and it is insensitive to cycloheximide treatment. LTM is produced by spaced training and is blocked by cycloheximide treatment; thus it is considered to require acute protein synthesis. A previously identified *Drosophila* memory mutant, *radish*, is deficient in ARM, as this mutation blocks memory produced by massed training[18,21]. Spaced training of *radish* mutants does produce memory, but this memory can be completely blocked by treating the mutants with cycloheximide[18]. These results led to a two-pathway model of consolidated memory, one dependent on the *Radish* gene product (ARM) and the other dependent on activity-induced, acute protein synthesis (LTM)[18].

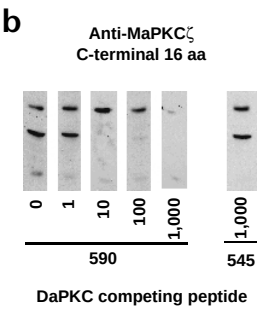
Because MaPKMζ induction enhanced memory after massed but not after spaced training, we tested the dependence of this effect on radish. The *radish* gene is on the X chromosome in *Drosophila*, and we crossed homozygous *radish* mutant females to males homozygous for an autosomal copy of the heat shock–inducible MaPKMζ transgene. The *radish* mutant is recessive, thus the heterozygous female progeny of this mating will have normal memory after massed training, whereas the hemizygous males will display the radish memory deficit in the absence of induction. We subjected the progeny to massed training, followed by the standard MaPKMζ induction after training, and then tested at 24 hours. Males and females were trained and tested en masse, and then separated and counted. The *radish* mutation did not block the memory effect of MaPKMζ induction (**Fig. 5**). The memory defect of *radish* males was apparent in the absence of heat-shock induction (HS–), but memory was clearly present in induced males (HS+). We also observed a lesser, but significant induction-dependent memory enhancement of the heterozygous *radish* females by MaPKMζ (**Fig. 5**; HS+ versus HS– females).

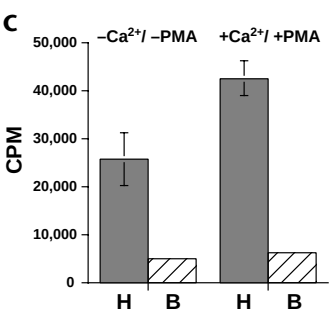
A *Drosophila* homolog of MaPKMζ

There is a single atypical PKC (DaPKC) gene in the *Drosophila* genome (http://www.fruitfly.org), and it is highly homologous to the MaPKCζ gene we have used. (The kinase domain shows 76% identity and 87% similarity[19,22].) A western blot of extracts made from wild-type fly heads and bodies showed that the antiserum used to detect MaPKMζ and MaPKCζ recognized two bands in fly

Fig. 6. A *Drosophila* homolog of MaPKMζ is present and active in *Drosophila* head extracts. (**a**) Antisera against MaPKC/Mζ detect DaPKC and DaPKM. Separate extracts from fly heads and bodies were analyzed on western blots using antisera directed against the C-terminal 16 amino acids of MaPKC/Mζ. The molecular weights of the two immunoreactive bands agree with those predicted for the DaPKC (73 kDa) and DaPKM (55 kDa) isoforms. The putative DaPKM is enriched in heads. (**b**) DaPKC and DaPKM immunoreactivity is competed with DaPKC-specific peptides. Western blots were done on head extracts. The antiserum was added along with 0, 1-, 10-, 100- or 1000-fold excess DaPKC-590 peptide. This peptide is derived from the C terminus of the DaPKC protein. The antiserum was made against the C terminus of MaPKCζ, which is homologous to the DaPKC protein. A peptide derived from an upstream region of DaPKC (545) does not compete the immunoreactivity even at 1,000-fold excess. **(c)** Atypical PKC activity is enriched in extracts made from *Drosophila* heads. Separate extracts were made from wild-type fly heads and bodies as in (**a**). We assayed them for aPKC activity as in Fig. 2b.







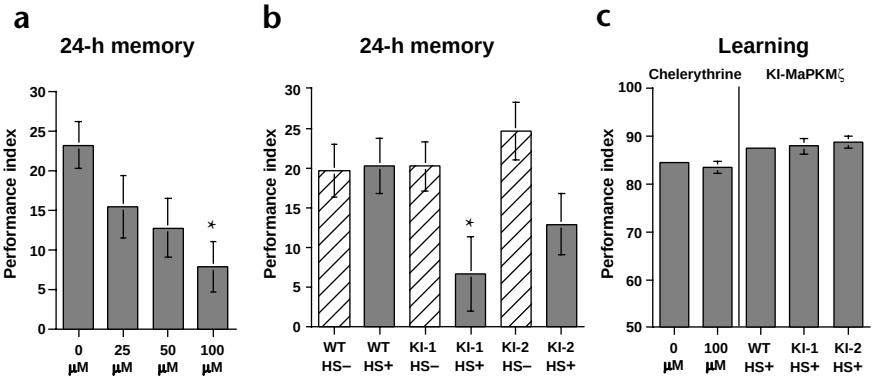


Fig. 7. Chelerythrine treatment or KI-MaPKMζ expression inhibits 24-h memory produced by massed training, but not learning, in *Drosophila*. (**a**) Chelerythrine inhibits 24-h memory after massed training in a dose-dependent manner. Wild-type flies were fed either sucrose (0 μM) or 25 μM, 50 μM or 100 μM chelerythrine in a sucrose solution (Methods). They were given three cycles of massed training and assessed for 24-h memory. $n = 8$ for all groups. (**b**) The KI-MaPKMζ mutant inhibits 24-h memory produced by massed training. Each line of the *hsp70-KI-MaPKM*ζ transgene (KI-1 and KI-2; Methods) was induced with a 30-min, 37°C heat shock, allowed to recover for 3 h at 25°C, and then given 10 cycles of massed training. Line KI-1 induction caused a significant reduction in 24-h memory, and induced KI-1 flies were also significantly different from induced control flies (WT, HS+). Line KI-2 induction also seems to reduce memory, but this line may not be as effective as KI-1. $n = 8$ for all groups. (**c**) Neither chelerythrine nor KI-MaPKMζ induction affects learning. After a 1-h starvation period, wild-type flies were fed either sucrose (0 μM) or 100 μM chelerythrine in a sucrose solution for 3 h (Methods). The KI-MaPKMζ was induced as in (**b**). In both cases, the flies were then given single-cycle training and tested immediately after training to assess learning. There was no difference between relevant groups: sucrose-fed flies were not different from chelerythrine-fed flies, and WT HS+ flies were not different from KI-1 HS+ or KI-2 HS+ flies. $n = 8$ for all groups.

extracts, the smaller of which was enriched in head extracts (**Fig. 6a**). This antiserum is directed against the C-terminal 16 amino acids of MaPKC/Mζ, which shares substantial homology with DaPKC. Antiserum from mice immunized with peptides derived from DaPKC recognized these same bands (data not shown). The molecular weights of these two bands indicate that they are probably the DaPKC (~73 kDa) and DaPKM (~55 kDa) isoforms.

We have not established the N-terminal sequence of the lower molecular weight band; however, it likely represents an endogenous DaPKM isoform. The immunoreactivity was competitively reduced by a peptide from the corresponding region of DaPKC, but not one outside of this epitope (**Fig. 6b**, 590 and 545, respectively) or by a peptide from another *Drosophila* protein (dCREB2, data not shown). In agreement with the western blot data, fly heads contained more Ca^{2+} and DAG-independent PKC activity than did bodies (**Fig. 6c**). The presence of the putative DaPKM correlates strongly with this enriched activity, suggesting that most, if not all, of the endogenous atypical kinase activity we measured in head extracts was due to this DaPKM isoform. These data indicate that flies possess both 'C' and 'M' forms of an atypical PKC that is highly homologous to MaPKC/M, and that the DaPKM is enriched in heads.

Inhibiting DaPKM blocks memory, but not learning

A P-element insertional mutant in DaPKC has been described; however, it is an embryonic lethal and thus is not suitable for examining a possible role in adult learning and memory formation[22]. To assess whether this gene's product is necessary for memory formation, we took two approaches. First, we monitored the effects on memory of feeding flies the PKC inhibitor chelerythrine[23]. This drug is reported to selectively inhibit PKMζ at low concentrations[15,24]; however, its specificity is controversial, and it inhibits other PKC isotypes at higher concentrations[25]. We also measured memory effects produced by inducing the kinase-inactive KI-MaPKMζ protein, which displays 'dominant-negative' activity[20] that is likely to be specific to the atypical PKCs, leaving cPKC and nPKC responses intact[26,27] (see Methods and Discussion).

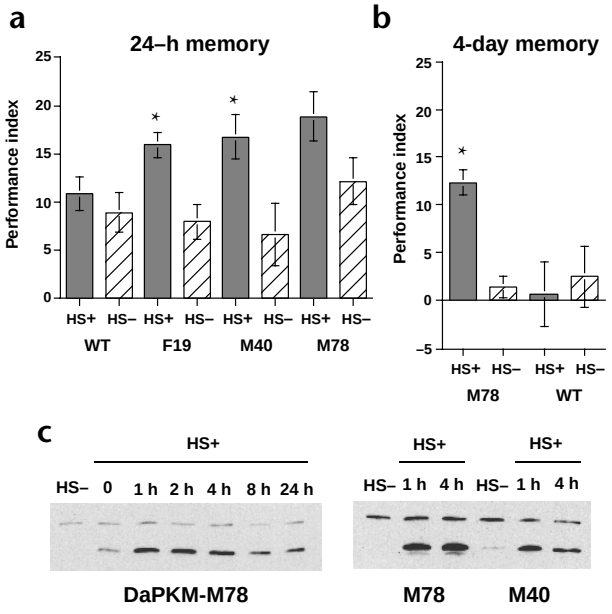


Fig. 8. DaPKM induction enhances memory. (**a**) DaPKM enhances 24-h memory after single-cycle training. Flies were given single-cycle training, allowed to recover at 25°C for 30 min, and the DaPKM transgene was induced by a 32°C heat shock lasting 30 min. Performance was measured 24 h later. Three independent transgenic lines were tested (F19, M40, and M78). $n = 8$ for all groups. The asterisks indicate significant differences. (**b**) DaPKM enhances 4-day memory after massed training. DaPKM-M78 flies were trained with 10 cycles of massed training (Methods), allowed to recover for 30 min at 25°C, and induced as in (**a**). Performance was measured at 4 days; $n = 8$ for all groups. Only line M78 was used for this analysis, and it shows significant induction-dependent improvement of 4-day memory. (**c**) Western blot analyses of DaPKM induction after heat-shock. Flies were subjected to heat shock (32°C) for 30 min. Left, time-course western blots of the DaPKM-M78 line. At the indicated time points, flies were collected and head extracts were used for western blot analysis. All times are relative to the end of the heat-shock treatment. Right, direct comparison of the induction of the M40 and M78 lines. In both panels, the upper band is a background band included as a load-control reference.

Feeding flies chelerythrine inhibited 24-hour memory formation in a dose-dependent manner (**Fig. 7a**), and induction of the KI-MaPKMζ inhibited 24-hour memory after massed training (**Fig. 7b**). The inhibitory effects of both chelerythrine and the KI-MaPKMζ were not likely due to effects on olfactory acuity or shock reactivity because learning was unaffected by either treatment (**Fig. 7c**).

DaPKM induction enhances memory

The memory enhancement produced by MaPKMζ could have been due to properties unique to this mammalian protein. The expression data showing that DaPKM was expressed and active in *Drosophila* heads, when combined with the chelerythrine and dominant-negative data, suggested that DaPKM is involved in normal memory processes in *Drosophila*. The extensive structural homology between MaPKMζ and DaPKM also argued against functional uniqueness. Our hypothesis of functional homology made a strong prediction: induction of DaPKM after training should also enhance memory.

Based on the approximate molecular weight of the DaPKM, we truncated the DaPKC gene within the hinge region separating the regulatory from the catalytic domains such that our putative *DaPKM* gene begins at methionine 223 (Methods). Induction of the *DaPKM* transgene after training enhanced 24-hour memory after single-cycle training (**Fig. 8a**). We then used one of these lines to show that 4-day memory after massed training was also enhanced (**Fig. 8b**). As with the *MaPKM*ζ transgenes, the DaPKM lines showed rapid heat-shock induction (**Fig. 8c**). These results confirm those obtained with MaPKMζ, and thus indicate that aPKM is fundamental in the mechanisms underlying memory across species.

Discussion

Atypical PKM and normal memory

Our results provide strong evidence that atypical PKM activity is sufficient to enhance memory in *Drosophila*. Ideally, we could have addressed necessity by assessing potential memory deficits of flies bearing null mutations in the *DaPKC/M* gene. However, the lethality of such mutants precluded these analyses, and no special alleles existed that might have preserved the gene's vital function while disrupting its role in memory. In an attempt to circumvent these problems, we used both pharmacological and dominant-negative interventions. We found that chelerythrine inhibited normal memory in a dose-dependent manner (**Fig. 7a**), and induction of a predicted dominant-negative atypical PKM produced the same memory deficit (**Fig. 7b**).

The evidence that DaPKC/M activity is necessary for normal memory rests solely on the specificity of our inhibiting reagents. Chelerythrine inhibits PKCs[23] and is reported to be more potent against purified atypical PKM activity relative to full-length PKCs or other kinases at low concentrations *in vitro*[15]. However, controversy exists over chelerythrine's specificity even at low doses, and the selectivity is unlikely to hold at higher concentrations (refs. 15,24,27). Our crude method of administering this drug (feeding; Methods) prevents concentration assessment in the behaving flies. The dominant-negative PKCζ blocks the insulin-stimulated and PKCζ-dependent translocation of the GLUT4 glucose transporter in rat adipocytes, whereas dominant-negative versions of conventional or novel PKCs do not block this translocation[27]. Constitutively active aPKC completely rescues the effect of the dominant negative, consistent with the effect of dominant-negative aPKC being subtype-specific[27]. Also, dominant-negative PKCζ blocks the TNF-α-stimulated and PKCζ-dependent activation of IKKβ while leaving the phorbol ester-stimulated, conventional PKC-dependent activation of IKKβ intact[26].

Despite these results, we cannot be certain of the specificity of either chelerythrine or KI-MaPKMζ in our behavioral assay. Each treatment could cause broader effects. However, it is important to note that neither of these inhibitory interventions disrupted learning (**Fig. 7c**). Screens in *Drosophila* have identified many learning mutants that disrupt several signaling pathways[29,30] (e.g., cAMP-PKA, integrin-mediated, and 14-3-3 protein-dependent processes; see refs. 29,30 for original citations). Considering that learning remains normal, it is unlikely that either intervention produced very broad signaling defects. With this in mind, the observation that each intervention inhibited memory without disrupting learning indicates that DaPKC/M is a component of an endogenous memory mechanism. Establishing a role for DaPKC/M in this mechanism will require the ability to separate PKC subtype-specific signaling *in vivo*, using these or other reagents, in both chronic and acute manners.

Phase specificity of MaPKMζ-enhanced memory

We found that heat-shock induction of MaPKMζ did not enhance long-term memory, because it did not improve memory after spaced training (**Fig. 4b**). One explanation for this is that spaced training induces endogenous maintenance mechanisms, and thus occludes the effect of inducing the *MaPKM*ζ transgene. Thus, memory after single-cycle or massed training may be prolonged by transgene induction because these training regimens do not normally induce prolonged atypical PKM activity. Work in honeybees shows that single-cycle training produces neither persistent PKC activity nor long-lasting memory, but multiple-cycle training produces both[16]. The memory enhancement we observed when inducing MaPKMζ may simply bypass the endogenous requirements (normally provided by spaced training) for prolonged activation of aPKM.

The MaPKMζ-induced enhancement of massed, but not spaced training prompted us to examine the involvement of the *radish* gene product in this process. If radish were required for the enhancement, the radish mutation would have blocked the MaPKMζ-induced effect, and this was clearly not the case (**Fig. 5**). Although MaPKMζ induction phenotypically rescues the memory defect of radish, it does not do so because radish encodes for the *Drosophila* aPKM. *DaPKM* is on the second chromosome and radish is on the X, and no *Drosophila PKC* gene maps to the genetically defined radish locus. There are two principal possibilities explaining how MaPKMζ-induced memory enhancement bypasses the defect of radish mutants: (i) MaPKMζ is downstream of radish or (ii) MaPKMζ activates a pathway that is parallel to and independent of radish. We favor the first interpretation because we can either enhance or disrupt memory after massed training, as well as partially rescue the radish phenotype.

The temporal specificity of the MaPKMζ-dependent memory enhancement implies restrictions on its biochemical mechanism(s); enhancement requires that prior activity-dependent mechanisms be in place, and MaPKMζ has a narrow post-training interval in which to act. If these kinetic restrictions do exist, the rapid induction achievable with the heat-shock promoter was essential for the detection of memory enhancement in our experiments.

Atypical PKM and synaptic plasticity

There are two general interpretations of our data: PKMζ acts to increase either (i) the magnitude or (i) the duration of the synap-

tic potentiation that underlies the behavior. In the first model, PKMζ enhances the synaptic machinery induced by training, making a 'stronger' synaptic connection that decays more slowly. In the second model, PKMζ acts solely to maintain the synapses previously modified by experience, with no effect on the induction of the potentiation. If one considers the behavioral measurements of learning (testing done immediately after training) and memory (testing done after a longer time) with induction and maintenance, respectively, our chelerythrine and dominant-negative data argue for a role in maintenance. Neither of these treatments affected learning (**Fig. 7c**), but each inhibited memory (**Fig. 7a** and **b**). We also did not detect an enhancement of learning by prior induction of PKMζ (data not shown), nor was there an improvement of 3-hour memory if PKMζ was induced 30 minutes after training (data not shown). Although the magnitude and duration models may be artificially exclusive, taken together our data are most consistent with a role of PKMζ in the maintenance of experience-dependent synaptic plasticity.

The stability of a synapse varies in response to different regimens of stimuli. Long-lasting changes normally require multiple stimuli and depend on new protein synthesis. Recent experiments support the existence of a synaptic marking system that enables neurons to tag recently active synapses, thus maintaining synaptic specificity during the cell-wide process of protein synthesis–dependent long-term memory formation[31–33]. A synapse that would normally be stable for only a short period of time can be potentiated for a much longer period of time. However, to do so it must be activated within 2–4 hours of stimulation that produces long-term changes at a second and separate synapse within the same neuron[31,33,34]. Although we have no direct evidence for a role of PKMζ in this process, the similarity between the temporal windows for the proposed synaptic tag and the memory enhancement we observe suggest a mechanistic relationship between them.

DaPKC is part of a multiprotein complex important for both cell polarity and the asymmetrical cell divisions of early *Drosophila* neurogenesis[22,35–37]. These processes show strong structural and functional parallels with the first asymmetrical cell division of *Caenorhabditis elegans* embryogenesis[38,39]. The *Drosophila* homologs of *C. elegans* proteins important for this process, Par-3 (Bazooka) and Par-6 (DmPar-6), interact with each other and with DaPKC to direct a specific and interdependent subcellular localization of the complex. During early *Drosophila* embryogenesis, Bazooka, DmPar-6, and DaPKC are localized to the zonula adherens, a cell junction structure. Mutation in any one of these genes disrupts the ability of the remaining two proteins to localize to this structure properly, and this disrupts cell polarity[22,35,40]. This mutual dependence for localization is also apparent during neurogenesis, and causes the inappropriate segregation of cell determinants[35–37]. This multiprotein complex is critical in mammalian cell polarity and in organizing junctions between epithelial cells[41–45]. The mouse homologs of Bazooka and Par-6 are expressed in various regions of the CNS, and their subcellular localization within CA1 hippocampal neurons is consistent with a role in synaptic plasticity[44]. Bazooka and DmPar-6 are expressed in *Drosophila* heads (E.A.D., unpublished observations), as are DaPKC and DaPKM (**Fig. 6a**). It remains unclear how DaPKM activity is regulated during memory mechanisms; however, the subcellular localization affected by the Bazooka–DmPar-6–DaPKC complex provides hypotheses with attractive physical properties.

We have shown that atypical PKM is sufficient to enhance memory in *Drosophila*, and the chelerythrine and dominant-negative data suggest that it is also necessary for normal memory. Strikingly corroborative results have also been obtained for the role of PKMζ in the maintenance phase of LTP[15]. These investigators found that injection of MaPKMζ into CA1 pyramidal cells is sufficient to potentiate evoked excitatory postsynaptic currents. The potentiation occludes LTP and is reversed by chelerythrine. They also found that the introduction of the KI-MaPKMζ into a CA1 cell abolishes its ability to support LTP. The non-NMDA receptor antagonist CNQX blocks this potentiation, indicating that it occurs via AMPA receptors. When these physiological results, obtained in rat hippocampal slice preparations, are combined with our *Drosophila* behavioral data, they point to a central role of atypical PKM in the mechanism of memory maintenance. Understanding the regulation of atypical PKMζ, as well as what it in turn regulates, may be critical to unraveling this process.

Methods

Fly stocks and maintenance. The background stock (2202u) used as wild-type flies in all the experiments is *w* (isoCJ1), which is an isogenic line derived from a w^{1118} line backcrossed repeatedly to a Canton-S wild-type strain[46,47]. To minimize differences in genetic background, 2202u also served as the recipient strain for all of the transgenic lines used in these experiments. Fly stocks used for behavioral analyses were maintained as described[48].

Pavlovian learning and memory in *Drosophila*. To assess learning and memory in *Drosophila*, we used an olfactory-avoidance classical (Pavlovian) conditioning protocol[17]. This protocol has since been modified to facilitate automated and repetitive training regimens[18]. We used 3-octanol (OCT) and 4-methylcyclohexanol (MCH) as odors in these experiments. Detailed descriptions of single-cycle, massed and spaced training, as well as testing, and the tests for olfactory acuity and shock reactivity can be found elsewhere[18,48,49]. The performance index (PI) was calculated by subtracting the number of flies making the incorrect choice from those making the correct one, dividing by the total number of flies, and multiplying by 100. To avoid odor-avoidance biases, we calculate the PI of each single *n* by taking an average performance of two groups of flies, one group trained with the CS+ being OCT, the other with the CS+ being MCH.

Transgenes. The MaPKMζ DNA construct was produced using PCR amplification using the full-length, MaPKCζ cDNA (American Type Culture Collection (ATCC) no. 63247) as the template. PCR primers (upstream primer: 5′-CTAGCGAATTCAACATGAAGCTGCTGGTC-CATAAACG-3′; downstream primer: 5′-CTAGCTCTAGATCACACG-GACTCCTCAGC-3′) were used to produce the truncated MaPKMζ gene. The upstream primer contained an *Eco*RI restriction site just 5′ of a consensus Kozak sequence and an ATG start codon. The last 20 nucleotides of the upstream primer correspond to 20 nucleotides in the hinge region of the MaPKCζ gene. The second codon in this truncated gene corresponds to amino acid 165 in the MaPKCζ protein. The downstream primer is antisense to the last 20 nucleotides in the MaPKCζ open reading frame, and contains a *Xba*I restriction enzyme site immediately after the translation stop codon. The PCR product produced using these primers was cut with *Eco*RI and *Xba*I, then subcloned into a heat-shock P-element vector, using the same restriction sites, and sequenced. The kinase-inactive KI-MaPKMζ was produced in the same manner except that the K281W-MaPKCζ mutant DNA was used as the template for PCR amplification. The full-length MaPKCζ gene was subcloned into the same P-element vector using the *Eco*RI sites located at both ends of the cDNA and in the heat-shock vector. Transgenic flies were made using standard techniques. Based on homology with mammalian PKCζs, we define the N terminus of DaPKC as beginning with residues M-P-S. Using this reference point, the DaPKM transgene begins at Met223 within the hinge region of DaPKC.

Heat-shock induction. All fly stocks were maintained at 25°C before and after heat-shock. We performed heat-shock inductions in 15-ml plastic tubes (~100 flies/tube) by partially submerging the tubes in a water bath at the appropriate temperature (32°C or 37°C) for 30 min.

Biochemical assay. MaPKMζ transgenic flies maintained at 25°C were heat-shocked at 37°C for 30 min, then allowed to recover at 25°C for 1 h and frozen in liquid nitrogen. Uninduced controls and wild-type flies remained at 25°C throughout. After freezing, fly heads were separated from bodies and ~100 µl of heads were homogenized in 1 ml extraction buffer (20 mM Hepes pH 7.4, 0.2 M sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and Complete protease inhibitor cocktail (Roche, Indianapolis, Indiana)), and centrifuged at 176,500 *g* for 10 min. The homogenates were diluted to 1 mg/ml protein and adjusted to 2.5 mM EGTA. PKC activity was assayed by measuring the incorporation of ^{32}P from γ-^{32}P-ATP into peptide-ε (Peninsula, Belmont, California). Each 50-µl reaction contained 50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 25 µM peptide-ε, 20 µg of protein homogenate and 20 mM γ-^{32}P-ATP (2 µCi). The protein was incubated at 26°C for 1 min in the reaction mixture lacking ATP. The reaction was started by the addition of γ-^{32}P-ATP, incubated at 26°C for 2 min and stopped on ice with 20 µl of 75 mM H_3PO_4. A 20-µl aliquot from each reaction was spotted onto a 2-cm circle of P81 chromatography paper (Gibco, Gaithersburg, Maryland) and washed three times in 75 mM H_3PO_4, dried, and the radioactivity measured by scintillation counting. The effect of Ca^{2+} and phorbol esters in the reaction was measured in the presence of 200 µM $CaCl_2$ and 400 µM phorbol myristate acetate (PMA). Background activity was estimated from mock reactions lacking peptide-ε. PKC activity increased linearly with time up to 4 min. The values reported are averages from quadruplicate assays.

Western blots. We monitored the heat-shock induction time course using fly-head extracts (approximately five heads per lane were loaded) for the western analyses. A rabbit polyclonal antibody against MaPKMζ (Sigma, St. Louis, Missouri) was used as the primary antibody, and we detected the protein using chemiluminescence.

Chelerythrine feeding. We used a 4% (wt/vol) sucrose solution as a vehicle for the chelerythrine feeding. Onto Whatman 3MM filter paper cut to fit and placed in the bottom of standard fly culture vials, we applied 120 µl of 0 µM (sucrose alone), or 25 µM, 50 µM or 100 µM chelerythrine. Flies were starved in empty vials (~100 flies/vial) for 1 h, and then transferred to the feeding vials for 3 h to allow sufficient feeding. Flies were then trained and tested as described in the text and figures. Feeding was monitored by placing 1/50 volume of green food coloring into the solutions, which could then be seen in the abdomens of flies after eating. There was no noticeable difference in consumption between any of the solutions.

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Competing interests statement

The authors declare that they have no competing financial interests.

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