Storage of Spatial Information by the Maintenance Mechanism of LTP

Eva Pastalkova,* Peter Serrano,* Deana Pinkhasova, Emma Wallace, André Antonio Fenton,† Todd Charlton Sacktor†

Analogous to learning and memory storage, long-term potentiation (LTP) is divided into induction and maintenance phases. Testing the hypothesis that the mechanism of LTP maintenance stores information requires reversing this mechanism in vivo and finding out whether long-term stored information is lost. This was not previously possible. Recently however, persistent phosphorylation by the atypical protein kinase C isoform, protein kinase Mzeta (PKMζ), has been found to maintain late LTP in hippocampal slices. Here we show that a cell-permeable PKMζ inhibitor, injected in the rat hippocampus, both reverses LTP maintenance in vivo and produces persistent loss of 1-day-old spatial information. Thus, the mechanism maintaining LTP sustains spatial memory.

The hippocampus encodes and initially stores experience-dependent spatial information (*1*, *2*). The physiological substrate of information storage in the hippocampus has been proposed to involve LTP, an activity-dependent, persistent increase in synaptic transmission (*3*–*8*). One approach to testing the role of LTP in behavior has been to inhibit the molecular mechanisms mediating plasticity. These mechanisms can be divided into two phases: induction, triggering the synaptic potentiation, and maintenance, sustaining the potentiation over time. The formation of long-term spatial memory can be prevented by inhibitors of molecules critical for inducing LTP, such as the *N*-methyl-D-aspartate receptor (NMDAR), protein kinases including Ca^{2+}/calmodulin–dependent protein kinase II (CaMKII), adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase (PKA), and conventional/novel isoforms of protein kinase C (c/nPKCs), as well as many other signaling molecules (*4*, *8*). These findings, however, do not distinguish between learning, the initial consolidation into long-term memory, and the persistence of memory storage; thus, they do not directly address the fundamental question of the role of LTP maintenance in the perpetuation of spatial information in the hippocampus. Addressing this question requires testing the hypothesis that inhibition of molecules maintaining LTP causes retrograde loss of information (*4*). This "maintenance hypothesis" has not been testable because inhibitors of NMDARs, CaMKII, PKA, or c/nPKC do not reverse late LTP maintenance (*9*). Indeed, NMDAR antagonists have been found to block the initial encoding, but not the maintenance, of memory (*10*). Thus, no agent specifically reversing established late LTP, critical for testing the maintenance hypothesis, has previously been available (*11*).

However, an unusual, persistently active kinase—the brain-specific, atypical PKC isoform, protein kinase Mzeta (PKMζ), is both necessary and sufficient for LTP maintenance (*9*, *11*–*14*). PKMζ introduced into CA1 pyramidal cells in hippocampal slices strongly potentiates postsynaptic α-amino-3-hydroxy-5-methylisoxazole-4-propionate receptor (AMPAR) responses (*9*, *14*), whereas inhibition of PKMζ reverses established LTP (*9*, *11*, *13*). PKMζ can be inactivated by applications of a cell-permeable synthetic peptide derived from the structure of the full-length PKCζ isoform (Fig. 1A, left) (*9*, *11*, *13*). This myristoylated ζ-pseudosubstrate inhibitory peptide (ZIP) potently and selectively inhibits PKMζ by reconstituting the autoinhibition of the absent PKCζ regulatory domain (Fig. 1A, left) (*9*, *11*, *13*). Bath application of ZIP to hippocampal slices both inhibits the synaptic potentiation produced by intracellular perfusion of PKMζ (*11*) and reverses established late LTP, without reversing early LTP or affecting baseline, nontetanized synaptic transmission (*9*, *11*, *13*). Thus, ZIP is the first tool available to test the maintenance hypothesis. Therefore, we addressed two related questions: Can PKMζ inhibition by ZIP reverse the late phase of LTP in vivo? And if so, does ZIP cause retrograde loss of spatial memory?

We stimulated the perforant path in the angular bundle and recorded stable responses of the field excitatory postsynaptic potential (fEPSP) slope (Fig. 1, A to D) and population spike (PS) amplitude (Fig. 1, E and F, and fig. S1) in the subgranular layer of the dentate gyrus (*15*). We then tetanized with high-frequency stimulation (HFS), using a protocol optimized for inducing strong 24-hour LTP (*16*, *17*). Twenty-two hours after the tetanization, intrahippocampal injection of ZIP (10 nmol in 1 μl saline) rapidly reversed the persistent potentiation of fEPSP slope (Fig. 1, A, right; and C; $P < 0.01$ between baseline and preinjection responses; $P < 0.01$ between preinjection and 2 hours postinjection; and $P = 0.55$ between baseline and postinjection) and PS amplitude (Fig. 1E and fig. S1). In interleaved experiments, saline injections had no effect on potentiation (Fig. 1B; $P = 0.71$ between responses preinjection and 2 hours postinjection). Two-way ANOVA confirmed that the effect of ZIP on potentiated responses was different from the effect of saline [interaction $F(2,18) = 10.3$; $P < 0.001$]. Confirming prior work in hippocampal slices (*9*, *11*, *13*), ZIP had minimal effects on baseline evoked responses (Fig. 1D; $P = 0.91$ between responses preinjection and 2 hours postinjection), which indicated that the circuitry of the hippocampus remains intact after ZIP injections. ZIP also had no effect on baseline synaptic responses when applied after 22 hours of recording, the same time as in our LTP experiments (fig. S2).

For LTP saturation to block hippocampus-dependent learning and memory retrieval, the proportion of stimulated synapses must be optimized (*6*, *7*), which indicates that the synaptic changes that might encode spatial information are widely distributed in the hippocampus (*18*, *19*). To determine the spatial extent of LTP reversal by ZIP within the hippocampus, we recorded LTP at multiple populations of neurons with an array of four recording electrodes, spaced at 0.5-mm intervals from the injection site in CA3 (Fig. 1F, top left). ZIP injection reversed LTP recorded at all four electrodes (Fig. 1E). Immunocytochemistry after injections of 10 nmol biotin-labeled ZIP showed the agent extended both transversely (Fig. 1F, bottom) and 3 to 4 mm longitudinally within the hippocampus, without diffusing substantially into other brain regions except along the cannula track (Fig. 1F, top right).

We next examined active place avoidance, a spatial behavior with the experimental advantages of rapid hippocampus-dependent acquisition and persistent hippocampus-dependent recall (*20*, *21*), which parallels the time course of LTP. The apparatus for the task consists of a slowly rotating platform, open to the room environment, within which a nonrotating 60° sector is a shock zone (Fig. 2A, top; and Supporting Online Material, movie S1). The rotation brings the animal into the shock zone, and the animal rapidly learns to avoid the shock by actively moving to the nonshock areas of the environment. After an initial 10-min exposure to the apparatus without shock (pretraining, Fig. 2A, bottom; 2B, left; C; and D; and movie S1, scene 1), rats were trained in eight 10-min sessions with the shock on, separated by 10-min rest intervals in their home cages (Fig. 2B, middle; and C; and movie S1, scene 2). The animals were tested 24 hours later. Retention of long-term stored spatial information can be measured by the increase in time between the

Departments of Physiology, Pharmacology, and Neurology, The Robert F. Furchgott Center for Neural and Behavioral Science, SUNY Downstate Medical Center, 450 Clarkson Avenue, Brooklyn, NY 11203, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: afenton@downstate.edu (A.A.F.); tsacktor@downstate.edu (T.C.S.)

placement of the animal into the apparatus and the initial entry into the shock zone (which slowly accrues during training). In addition, the retention of both short-term and long-term stored information can be tested by the decrease in time spent in the shock zone (which is expressed rapidly after a single training session).

If persistent PKMζ activity is necessary for spatial long-term memory storage, then inhibiting the kinase's activity a day after learning will cause retrograde amnesia. Twenty-two hours after the last training session, we injected either ZIP or saline into both hippocampi. Two hours later, long-term retention was tested on the apparatus without shock (i.e., extinction testing). The saline-injected animals demonstrated long-term spatial information storage by avoiding initial entry into the shock zone (Fig. 2B, above right; C, open circles; and movie S1, scene 3) and spending less time in the shock zone (Fig. 2D). In contrast, the ZIP-injected animals failed to demonstrate spatial information storage by not avoiding entry into the shock zone, actively exploring the entire apparatus as if naïve (Fig. 2B, below right; C, solid circles; and movie S1, scene 4; $P < 0.02$, ZIP compared with saline; $P = 0.13$, pretraining compared with retention after ZIP), and by spending time in the shock zone close to the level of chance (Fig. 2D).

We examined whether PKMζ inhibition disrupted recently acquired, as well as persistently stored, spatial information by taking advantage of the rapid learning measured by time spent in the shock zone. Immediately after testing long-term memory (LTM) retention, we reconditioned the animals with a single training trial and then retested without the shock (Fig. 2A, bottom, and D), to determine short-term memory (STM) retention by the decrease in time in the shock zone. Although the ZIP-injected animals showed near complete loss of LTM, the same animals could nonetheless recall the STM of the conditioned response (Fig. 2D; for ZIP, $P = 0.72$ between pretraining and LTM, and $P < 0.05$ between LTM and STM; for saline, $P < 0.01$ between pretraining and LTM, and between LTM and STM; $P < 0.05$ between ZIP and saline for LTM). ZIP also had no effect on STM without prior LTM training (fig. S3).

We determined the specificity of the effect of PKMζ inactivation on long-term memory retention. We first tested whether the loss of long-

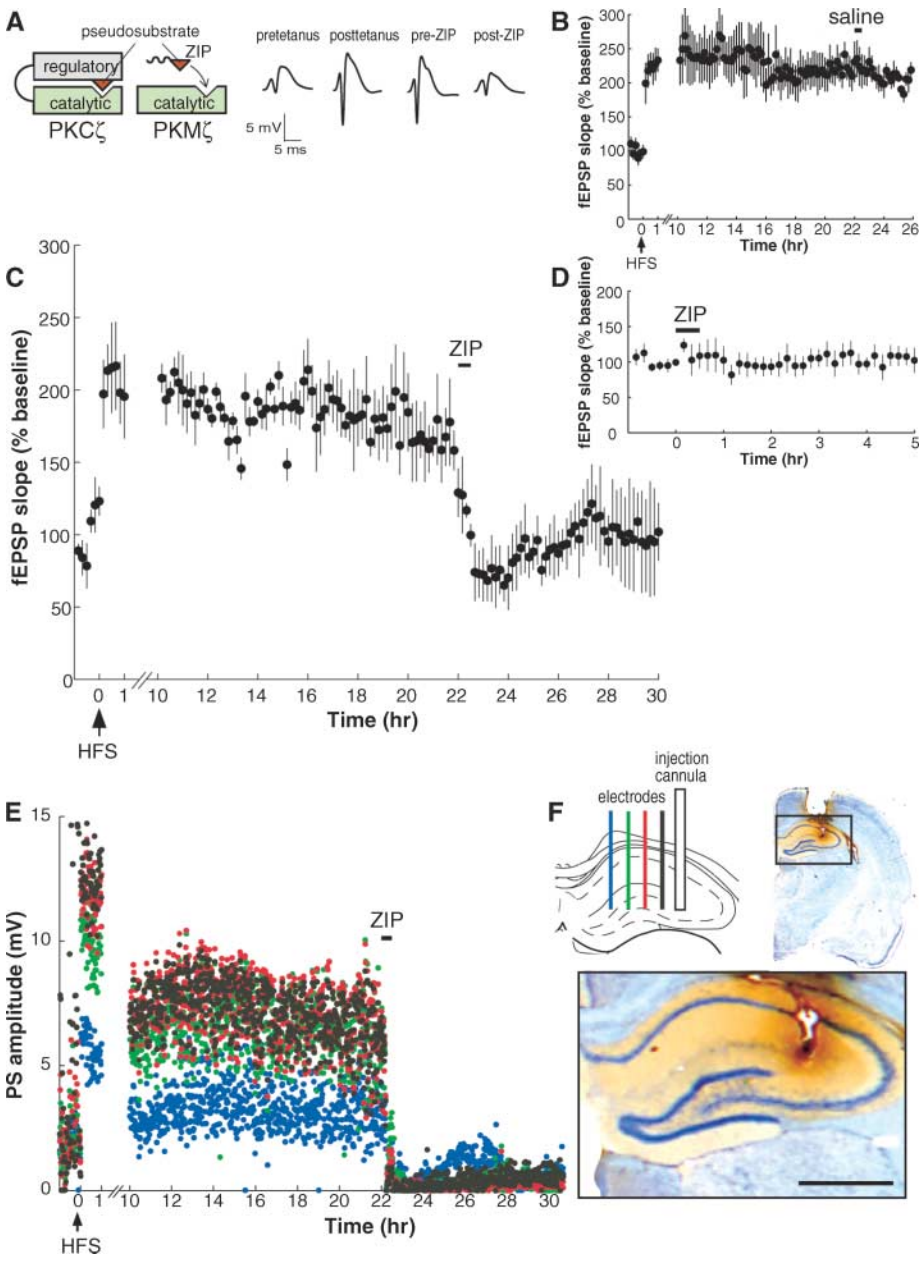

Fig. 1. PKMζ inhibition reverses the maintenance of late-phase LTP in vivo. (**A** to **D**) After recording stable baseline fEPSP responses for at least 1 hour, HFS inducing LTP was delivered. ZIP injection (10 nmol in 1 μl saline) 22 hours posttetanization reverses the persistent potentiation of fEPSP responses. (A, left) Schematic representation of PKCζ in its basal inactive state (left) and PKMζ (right), inhibited by ZIP. PKCζ consists of a catalytic domain (green) and a regulatory domain (gray). The regulatory domain contains a pseudosubstrate sequence (red triangle), which maintains the catalytic domain in an inactive state, until stimulated by second messengers. PKMζ, in contrast, is the independent catalytic domain of PKCζ, produced from a PKMζ mRNA (*27*, *28*), and, lacking a regulatory domain, is autonomously active. ZIP, consisting of the ζ pseudosubstrate sequence with a myristoyl moiety (wavy line) allowing for cell permeability, blocks the constitutive activity of PKMζ by reconstituting the inhibition of the missing regulatory domain. (A, right) Representative traces recorded 30 min pretetanus, 30 min posttetanus, ~2 hours pre-ZIP, and ~2 hours post-ZIP injection. (B) Saline injections 22 hours posttetanization have no effect on potentiation. (C) ZIP injections reverse potentiated responses to pretetanus levels. (D) ZIP injections have minimal effect on baseline responses. Means ± SEM; four rats were used in each experiment. (**E** and **F**) Representative PS amplitudes from four electrodes placed at 0.5-mm intervals from the cannula show ZIP reverses LTP up to 2 mm away from the injection site. (F, top left) Color-coded placement of electrodes and cannula. (F, top right) Immunocytochemistry 2 hours after injection of 10 nmol biotin-labeled ZIP shows the diffusion of the drug (brown) is largely restricted to the hippocampus. (F, bottom) The extent of drug diffusion within the hippocampus. Counterstain is cresyl violet; scale bar represents 4.7 mm (above right) and 1 mm (bottom).

term memory by ZIP was due to the agent's inhibitory effect on PKMζ activity by comparing ZIP with an inactive scrambled version of the myristoylated ZIP peptide (*11*, *13*). Whereas ZIP again disrupted long-term memory retention, the scrambled peptide did not (Fig. 3A; $P = 0.74$ between scrambled ZIP and saline; $P < 0.05$ between scrambled ZIP/saline and ZIP). We then examined the effect of staurosporine, a potent inhibitor of c/nPKC isoforms as well as other kinases, but an ineffective inhibitor of PKMζ (*9*). [Staurosporine blocks LTP induction but does not reverse LTP maintenance (*9*).] Although staurosporine is 10 times as potent in inhibition of the other PKC isoforms, CaMKII, and PKA than ZIP is on PKMζ (*9*), the general kinase inhibitor, injected at the same dose as we had injected ZIP, did not cause retrograde amnesia (Fig. 3B; $P = 0.56$ between staurosporine and vehicle). When injected before training, however, this staurosporine dose abolished place-avoidance learning [Fig. 3B, inset; $P < 0.01$, $F(1,7) = 15.2$ between staurosporine and vehicle; $P < 0.02$ for 24-hour retention].

PKMζ inactivation may disrupt information storage, in which case the effect of ZIP would be persistent, or information retrieval, in which case the effect would be transient. Because the delayed entrance into the shock zone is weakly expressed 1 week after the eight-trial training, we tested the decrease in number of entrances with the shock on, which is strongly retained for 1 week. Twenty-two hours after initial training, animals were injected with ZIP or saline and then returned to their home cages without testing (Fig. 3C, left). One week later, the saline-injected animals demonstrated spatial information storage by avoiding the shock zone (Fig. 3C, right; $P < 0.05$ between training trial 1 and retention at 1 week). In contrast, the animals that had been injected with ZIP showed no evidence of spatial information storage ($P = 0.26$ between training trial 1 and retention at 1 week; $P < 0.01$ between ZIP and saline at 1 week retention). In parallel experiments, no staining of biotin-labeled ZIP was detected in the hippocampus 1 week after its injection, which indicated elimination of the drug.

Immediately after testing the persistent loss of information, we examined whether ZIP persistently disrupted the ability to encode and store new long-term spatial information (Fig. 3C, right). The animals injected with ZIP or saline 1 week earlier showed equivalent performance during retraining from trial 3 onward and equivalent retention 24 hours later [$P = 0.92$ measured by time to first entry on extinction testing (Fig. 3C, inset), and $P = 0.86$ for number of entrances when the shock was turned back on]. Thus, although ZIP caused a persistent loss of previously stored information, once the agent was eliminated, it did not persistently impair relearning or long-term storage of newly acquired information. After the rats were killed, cellular staining with cresyl violet showed the structure of the hippocampi in the ZIP-injected animals was normal (fig. S4).

Finally, we tested whether PKMζ inhibition affected spatial memory that was more than 1 day old. Rats trained with two eight-trial sessions separated by 1 week retained spatial information for 30 days. Injections of ZIP 2 hours before testing abolished the retention of 1-month-old spatial memory (fig. S5).

PKMζ inactivation tested the maintenance hypothesis of LTP and showed that the persistence of synaptic potentiation and the persistence of spatial memory share a common molecular mechanism. PKMζ inhibition specifically disrupted the long-term retention of information because the ability to relearn, recall, and express the conditioned avoidance as a short-term memory was spared. This confirms and extends previous work on associative odor conditioning in *Drosophila*, in which inhibition of the fly PKMζ homolog prevented the formation of persistent, but not short-term, memory (*22*). Furthermore, we showed that the disruption of long-term retention was an effect on information storage, rather than retrieval, because the loss

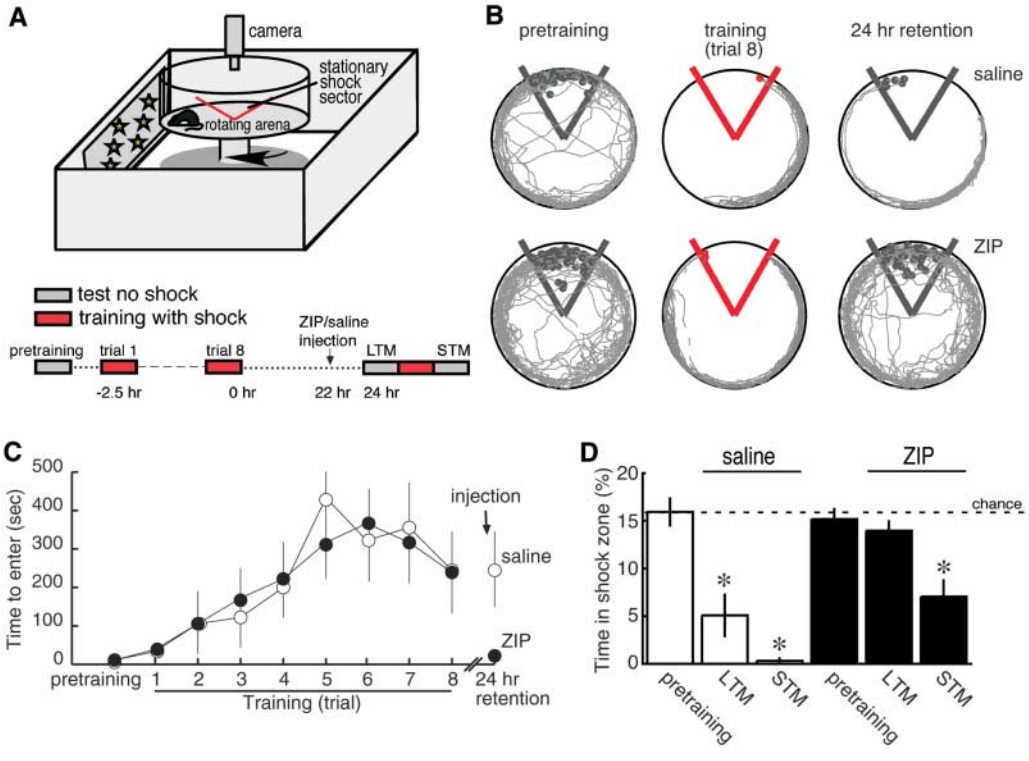

Fig. 2. PKMζ inhibition abolishes long-term retention of spatial information. (**A**) Above, the place avoidance training apparatus consists of a slowly rotating arena, within which a nonrotating 60° sector (delineated in red) is a shock zone. Visual cues are located on the walls of the room. Below, schematic of protocol for measuring effect of ZIP or saline on retention of place avoidance long-term memory (LTM) and short-term memory (STM). (**B**) Paths of individual animals in the apparatus during 10-min sessions. (Left) On first exposure during pretraining, the naïve animals explore the apparatus. Gray circles show locations where animals would have received shocks (delivered every 1.5 s) if the shock had been on. (Middle) By training trial 8, both animals show active place avoidance. Red circles show location of shocks. (Right) Retention testing 24 hours after training trial 8 (2 hours after bilateral intrahippocampal injections of saline or 10 nmol ZIP). The saline-injected animal (above) shows long-term memory retention; the ZIP-injected animal (below) explores the apparatus as if naïve. Gray circles show locations where animals would have received shocks if the shock had been on. (**C**) The time to the initial entrance into the shock zone during pretraining, training, and 24-hour retention testing sessions shows saline-injected animals (open circles) with place avoidance memory, and ZIP-injected animals with near complete loss of long-term stored information (solid circles) (means ± SEM of eight experiments). (**D**) The decrease of time spent in the shock zone demonstrates both long-term memory retention and further short-term memory retention in saline-injected animals. ZIP-injected animals show loss of long-term memory retention, but sparing of short-term memory retention. Dashed line represents time spent in shock zone if exploring the apparatus randomly. Six rats were used for each group.

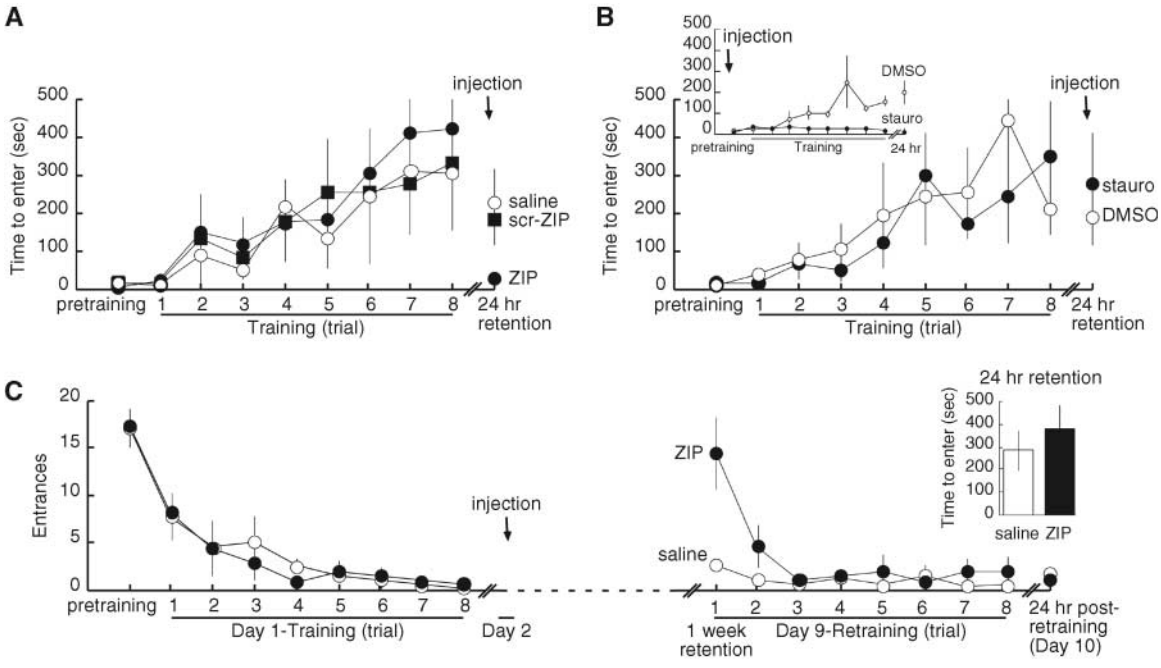

Fig. 3. Inhibition of PKMζ, but not other protein kinases, disrupts memory storage. (**A**) Animals show normal memory retention after injection of 10 nmol inactive scrambled ZIP (scr-ZIP). Five rats were used for each group. (**B**) Injections of 10 nmol staurosporine in 50% dimethylsulfoxide (DMSO) (stauro) does not affect long-term memory storage, compared with 50% DMSO alone. (Inset) Staurosporine (10 nmol) injected 20 min before training blocks place avoidance learning. Four rats were used for each group. (**C**) PKMζ inhibition disrupts memory storage. (Left) Twenty-two hours after training, ZIP or saline is injected without testing. (Right) One week later, ZIP-injected animals show no spatial information retention as measured by number of entrances into the shock zone, whereas saline-injected animals show place avoidance. Immediate retraining of ZIP-injected animals leads to normal acquisition and 24-hour recall of place avoidance, as measured first by time to initial entry into the shock zone, determined during 10 min with the shock off (inset), and then by the number of entrances during 10 min with the shock on. Six rats were used for each group.

persisted even after the elimination of the PKMζ inhibitor. Newly acquired long-term stored information could then be retained, which demonstrated that prior application of ZIP long before any new encoding did not permanently disrupt memory function. Information storage was specifically affected by PKMζ inhibition because staurosporine, a potent, broad-spectrum kinase inhibitor of CaMKII, PKA, and c/nPKCs, but not PKMζ, did not disrupt the long-term retention of stored information, whereas it strongly prevented the acquisition of new information.

The ability of ZIP to eliminate long-term stored information, while leaving recently acquired information intact, correlates well with the agent's ability to reverse late LTP, without disrupting the functional integrity of the hippocampal circuitry (Fig. 1D and fig. S2) or early LTP (*11*, *13*). PKMζ inhibition by ZIP thus contrasts with inactivation of the hippocampal circuit by tetrodotoxin, which disrupts learning and both short-term and long-term recall of place avoidance (*20*, *21*). In addition, the effect of hippocampal inactivation by tetrodotoxin is only transient and, thus, is on information retrieval (*21*, *23*), whereas the loss of long-term retention by ZIP is persistent and, thus, is on information storage. The minimal effect of ZIP on baseline synaptic transmission in the hippocampus suggests that information stored by PKMζ-mediated potentiation may have been sparsely encoded by a few synapses (*6*, *7*).

The ability of PKMζ inhibition to erase memory storage is distinct from an effect on memory reconsolidation. In reconsolidation studies, the combination of the injection of agents such as protein synthesis inhibitors and the reactivation of a memory from long-term stores results in a delayed loss of the memory observable on the following day (*24*, *25*). Here, ZIP produces rapid long-term memory loss detectable shortly after the agent's injection and within seconds of placing the animals into the test apparatus (Fig. 2C). Indeed, ZIP disrupts long-term memory retention without any retrieval close in time to the agent's injection (Fig. 3C). Thus, the persistent activity of PKMζ maintains the spatial memory trace. These findings may be pertinent to disorders of memory storage (*26*).

References and Notes

1. W. B. Scoville, B. Milner, *J. Neurol. Neurosurg. Psychiatry* **20**, 11 (1957).
2. J. O'Keefe, L. Nadel, *The Hippocampus as a Cognitive Map* (Clarendon Press, Oxford, 1978).
3. T. V. P. Bliss, T. Lomo, *J. Physiol.* **232**, 331 (1973).
4. S. J. Martin, P. D. Grimwood, R. G. Morris, *Annu. Rev. Neurosci.* **23**, 649 (2000).
5. O. Paulsen, R. G. Morris, *Nat. Neurosci.* **5**, 289 (2002).
6. V. H. Brun, K. Ytterbo, R. G. Morris, M. B. Moser, E. I. Moser, *J. Neurosci.* **21**, 356 (2001).
7. E. I. Moser, K. A. Krobert, M. B. Moser, R. G. Morris, *Science* **281**, 2038 (1998).
8. J. R. Sanes, J. W. Lichtman, *Nat. Neurosci.* **2**, 597 (1999).
9. D. S. Ling *et al.*, *Nat. Neurosci.* **5**, 295 (2002).
10. M. Day, R. Langston, R. G. Morris, *Nature* **424**, 205 (2003).
11. P. Serrano, Y. Yao, T. C. Sacktor, *J. Neurosci.* **25**, 1979 (2005).
12. T. C. Sacktor *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 8342 (1993).
13. S. Sajikumar, S. Navakkode, T. C. Sacktor, J. U. Frey, *J. Neurosci.* **25**, 5750 (2005).
14. D. S. Ling, L. S. Benardo, T. C. Sacktor, *Hippocampus* **16**, 443 (2006).
15. Materials and methods are available as supporting material on *Science* Online.
16. S. Frey, J. Bergado-Rosado, T. Seidenbecher, H. C. Pape, J. U. Frey, *J. Neurosci.* **21**, 3697 (2001).
17. T. Ahmed, S. Frey, J. U. Frey, *Neuroscience* **124**, 857 (2004).
18. M. B. Moser, E. I. Moser, *J. Neurosci.* **18**, 7535 (1998).
19. S. Kubik, A. A. Fenton, *J. Neurosci.* **25**, 9205 (2005).
20. J. M. Cimadevilla, A. A. Fenton, J. Bures, *Neurosci. Lett.* **285**, 53 (2000).
21. J. M. Cimadevilla, M. Wesierska, A. A. Fenton, J. Bures, *Proc. Natl. Acad. Sci. U.S.A.* **98**, 3531 (2001).
22. E. A. Drier *et al.*, *Nat. Neurosci.* **5**, 316 (2002).
23. K. Jezek, M. Wesierska, A. A. Fenton, *Physiol. Res.* **51**, (Suppl 1), S35 (2002).
24. K. Nader, G. E. Schafe, J. E. Le Doux, *Nature* **406**, 722 (2000).
25. C. M. Alberini, *Trends Neurosci.* **28**, 51 (2005).
26. J. F. Crary, C. Y. Shao, S. S. Mirra, A. I. Hernandez, T. C. Sacktor, *J. Neuropathol. Exp. Neurol.* **65**, 319 (2006).
27. A. I. Hernandez *et al.*, *J. Biol. Chem.* **278**, 40305 (2003).
28. I. A. Muslimov *et al.*, *J. Biol. Chem.* **279**, 52613 (2004).

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Supporting Online Material

www.sciencemag.org/cgi/content/full/313/5790/1141/DC1
Materials and Methods
Figs. S1 to S5
References
Movie S1

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