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Enhancement of Consolidated Long-Term Memory by Overexpression of Protein Kinase M ζ in the Neocortex

Reut Shema, *et al.*

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of plasmablasts by swIg$^+$ but not IgM$^+$ (Fig. 4G) memory cells after challenge. Thus, IgM$^+$ memory cells were not intrinsically hyporesponsive in immune hosts but were functionally inhibited by antigen-specific immunogloulins produced either before challenge by plasma cells or after challenge by memory cell–derived plasmablasts. Inhibitory FcγRIIb (*23*) was probably not involved, because IgM$^+$ and swIg$^+$ memory cells expressed equal amounts of this receptor (fig. S2A).

PE- and allophycocyanin-specific naïve B cells accounted for about 1:5000 and 1:25,000 of all B cells in mice. These high frequencies are likely related to the presence of multiple epitopes on these large multimeric proteins (*24*). It will be of interest to use the enrichment approach to enumerate naïve B cells specific for monomeric antigens, although antigen mulitmerization may be required (*25*).

Naïve PE-specific B cells generated IgM$^+$ memory cells after immunization with PE and the adjuvants CFA (Fig. 2K), lipopolysaccharide, or alum (fig. S4), which suggested that this is a general feature of the primary immune response. These memory cells had few mutations in their IgM molecules, which indicated inefficient selection in germinal centers. It is possible that these poorly mutated IgM molecules had a high enough natural affinity for PE to trigger memory cell differentiation before extensive somatic mutation could occur.

The remarkable stability of the IgM$^+$ memory cells compared with swIg$^+$ memory cells was not related to selective enrichment of IgM$^+$ cells (fig. S5A), migration of swIg$^+$ cells to bone marrow (fig. S5B), or homeostatic proliferation (fig. S5C). The instability of swIg$^+$ memory cells may be related to inhibitory signals through TACI (fig. S2A) (*21*) or deleterious off-target mutations induced by AID (*26*). Despite being shorter-lived and outnumbered by IgM$^+$ memory cells, swIg$^+$ memory cells dominated the secondary response because of a capacity to be activated in the presence of high-affinity neutralizing serum immunoglobulin. However, even swIg$^+$ memory cells could not produce germinal center cells, perhaps because their plasmablast progeny secreted enough immunoglobulin to clear the antigen very quickly. The failure to be activated efficiently in the face of immunoglobulin from swIg$^+$ memory cells or plasma cells suggests that IgM$^+$ memory cells do not contribute to the secondary response until these molecules decline. Serum immunoglobulin induced by certain subunit vaccines has been reported to decrease over time in humans (*27*), which suggests that IgM$^+$ cells could become the reservoirs of humoral immune memory for these vaccines. Because of their lower affinity and ability to produce germinal center cells, IgM$^+$ memory cells may also be useful for responding to antigenic variants produced by mutating pathogens.

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Enhancement of Consolidated Long-Term Memory by Overexpression of Protein Kinase Mζ in the Neocortex

Reut Shema,[1] Sharon Haramati,[1] Shiri Ron,[1] Shoshi Hazvi,[1] Alon Chen,[1] Todd Charlton Sacktor,[2] Yadin Dudai[1]*

Memories are more easily disrupted than improved. Many agents can impair memories during encoding and consolidation. In contrast, the armamentarium of potential memory enhancers is so far rather modest. Moreover, the effect of the latter appears to be limited to enhancing new memories during encoding and the initial period of cellular consolidation, which can last from a few minutes to hours after learning. Here, we report that overexpression in the rat neocortex of the protein kinase C isozyme protein kinase Mζ (PKMζ) enhances long-term memory, whereas a dominant negative PKMζ disrupts memory, even long after memory has been formed.

Amnesic agents impair fresh memories during encoding and consolidation, and some can block reactivated long-term memories at reconsolidation (*1*). Furthermore, selective brain lesions result in extensive loss of remote memories (*2*). Some agents have been proposed as potential memory enhancers, but their beneficial effect seems to be limited to the encoding and immediate consolidation period (*3*–*7*). The importance of memory enhancement for treating cognitive decline calls for an intensive exploration of the effect of manipulating components of the memory storage machinery on memory performance.

Protein kinase Mζ (PKMζ) is a persistently active, atypical protein kinase C isoform that is critical for maintaining the storage of long-term memory long after its initial consolidation (*8*, *9*). We have previously reported that inhibition of PKMζ in the insular cortex (IC) of the behaving rat by the pseudosubstrate zeta inhibitory peptide (ZIP) leads to erasure of long-term memory of conditioned taste aversion (CTA), an associative type of memory, up to at least 3 months after encoding, without affecting the ability of the rat to relearn the same association and without

[1]Department of Neurobiology, Weizmann Institute of Science, Rehovot 76100, Israel. [2]Departments of Physiology, Pharmacology, and Neurology, The Robert F. Furchgott Center for Neural and Behavioral Science, SUNY Downstate Medical Center, Brooklyn, NY 11203, USA.

*To whom correspondence should be addressed. E-mail: yadin.dudai@weizmann.ac.il

impairing recognition memory (*10*, *11*). The training itself leads to a persistent increase in the level of the endogenous PKMζ protein in the IC (fig. S1). We wanted to investigate the effect on CTA memory of targeted modulation of the level of PKMζ in the IC. We therefore designed and generated lentiviruses expressing PKMζ ($LV_{PKMζ}$) or a dominant negative (DN) version of PKMζ mutated at the active domain (LV_{DN}, PKMζ-K281W) (Fig. 1A), and microinfused them, or control lentiviruses (LV_{GFP}) (GFP, green fluorescent protein), bilaterally into the IC (Fig. 1, B and C). Under the conditions used, 43 ± 9% of the neurons (identified by NeuN staining, $n = 6$ hemispheres) at the core of the injected volume expressed the GFP reporter. Overexpression of the PKMζ protein was evident (Fig. 1D).

Rats infected in the IC with LV_{DN} 6 days after single-trial CTA training and tested for CTA memory 7 days later displayed a marked reduction in memory (Fig. 2A). In parallel in vitro experiments, the DN inhibited PKMζ activity tested on an exogenous kinase substrate (fig. S2). These data support our previous conclusion that

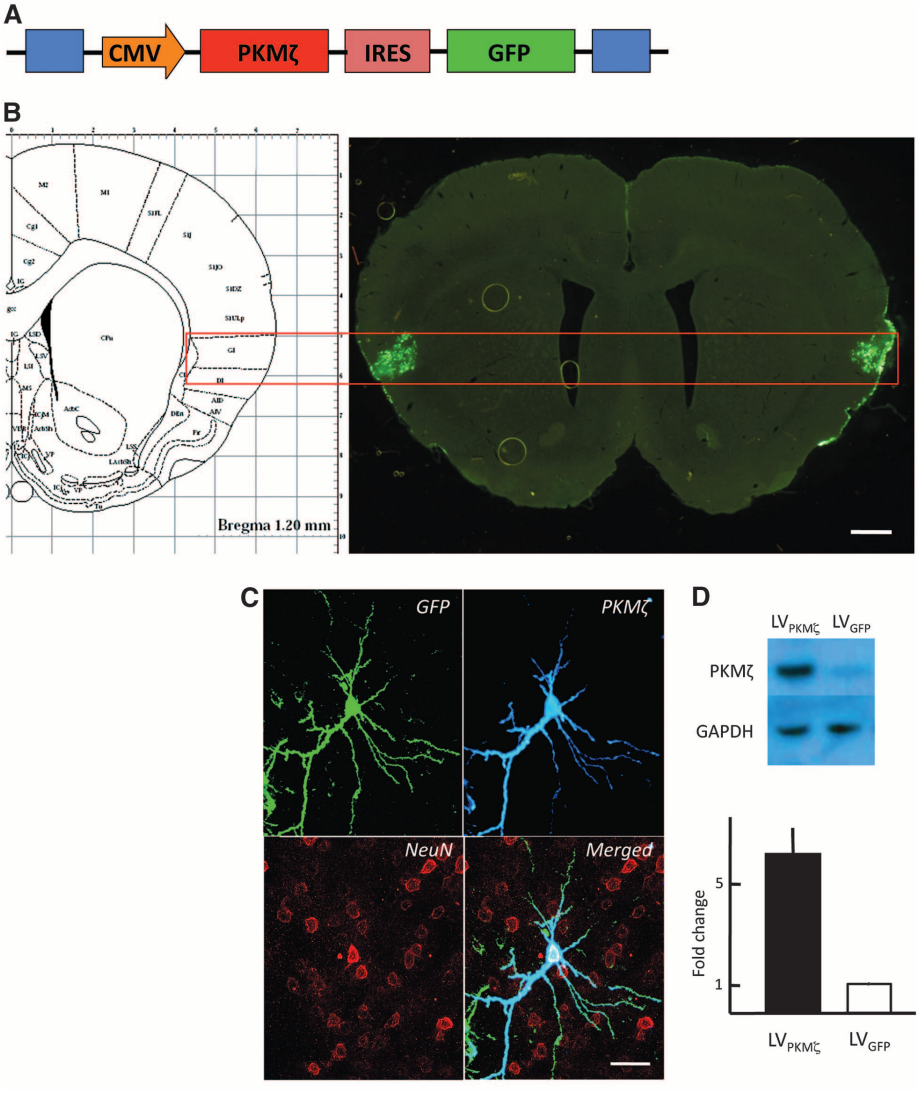

Fig. 1. Overexpression of PKMζ in the IC. (**A**) Schematic map of the lentiviral construct designed to overexpress PKMζ and GFP. The PKMζ gene (or the DN mutation, PKMζ-K281W) is under the control of a cytomegalovirus (CMV) promoter, followed by an internal ribosome entry site (IRES) and a GFP reporter. (**B**) A representative picture of a coronal section of the rat brain (bregma = 1.2 mm, 25 μm width section), depicting GFP-infected cells in the bilateral IC. Scale bar, 100 μm. (**C**) A neuron stained with GFP (green), PKMζ (blue), and NeuN (red) and the merged picture of all the above. To allow clear single-neuron presentation, the picture was taken toward the periphery of the infected area. Scale bar, 20 μm. (**D**) Upper panel: Western blot with an antibody specific for the catalytic domain of PKMζ, depicting PKMζ expression in the IC infused with the lentiviral vector containing the PKMζ gene ($LV_{PKMζ}$), compared to that infused with GFP alone (LV_{GFP}). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Lower panel: Quantification of the increase in PKMζ expression as a consequence of infection with $LV_{PKMζ}$.

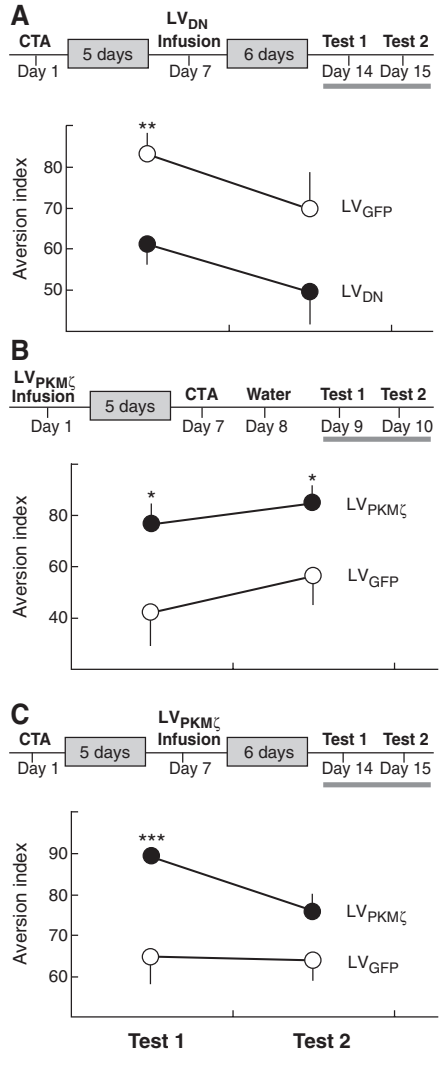

Fig. 2. Expression of a DN mutation of PKMζ blocks, whereas overexpression of PKMζ enhances, long-term memory in the IC of the behaving rat. (**A**) The IC of rats was infused bilaterally with LV_{DN} (Fig. 1) 6 days after CTA training, followed by testing 7 days later (two subsequent tests, a day apart, were used to quantify experimental extinction). Memory was significantly disrupted by DN PKMζ overexpression. Here and below, test days are underlined in the protocol flowchart. LV_{GFP}, $n = 9$; LV_{DN}, $n = 10$. (**B**) $LV_{PKMζ}$ was infused bilaterally into the IC 6 days before CTA training, using a weak US to circumvent the ceiling effect of the conventional CTA training and hence permit detection of potential memory enhancement (see methods in the SOM). Memory was tested starting 2 days after training. Memory was significantly enhanced, and no extinction was evident. LV_{GFP}, $n = 7$; $LV_{PKMζ}$, $n = 9$. (**C**) $LV_{PKMζ}$ was infused bilaterally into the IC 6 days after CTA training, and memory was tested starting a week later. Memory was significantly enhanced by overexpression of PKMζ, and extinction is evident. LV_{GFP}, $n = 23$; $LV_{PKMζ}$, $n = 28$. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. In (C), the statistics are for experimental versus control on test 1 and experimental on test 1 versus test 2.

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the effect of ZIP on CTA memory in the IC is due to the inhibition of PKMζ (*10*, *11*).

Whereas infection of the IC with LV_{DN} disrupted long-term CTA memory, overexpression as a consequence of infection with $LV_{PKM\zeta}$ significantly enhanced long-term memory. Memory enhancement was positively correlated with the extent of $LV_{PKM\zeta}$ infection in the IC (fig. S3). It was evident both when the infection was performed before CTA training (Fig. 2B) and when performed at day 7 after CTA training (Fig. 2C), a time point when the long-term CTA trace in the IC is already consolidated (*12*). Because the strong unconditioned stimulus (US, LiCl) used in conventional CTA training results in a ceiling effect that could mask potential memory enhancement, in the $LV_{PKM\zeta}$ experiments we used a diluted LiCl solution as the US (*13*). This US produced little CTA memory when tested at day 7 after training [aversion index (AI) = 64.1 ± 4.7, n = 7 rats], which is significantly lower than that observed in the PKMζ-overexpressing group tested a week later (Fig. 2C; AI = 89.1 ± 2.5, $P < 0.001$). Hence overexpression of PKMζ did not retard the normal fading of memory but rather enhanced the already faded memory. To exclude the possibility that the overexpression shifted the cortex into a persistently aversive state, in which continual expression of the aversion would mimic stronger CTA memory, we tested the effect of microinfusion of $LV_{PKM\zeta}$ into the IC on aversion to a saccharin conditioned stimulus (CS) in the absence of CTA training. No significant effect on aversion was detected when infection was performed in the IC of naïve rats before they had ever encountered saccharin (Fig. 3A) or in the IC of rats that had previously been exposed to saccharin (Fig. 3B). We also did not detect differences in the volume of liquid consumed in the instrumental situation of the CTA conditioning protocol by the rats infected in the IC with $LV_{PKM\zeta}$ (total liquid consumed on the first exposure to saccharin, $LV_{PKM\zeta}$ = 8.29 ± 0.58 ml, LV_{GFP} = 8.36 ± 0.69 ml, n = 18 and 15, respectively). We thus conclude that overexpression of PKMζ in the IC has no significant effect on the sensorimotor or motivational faculties required to express CTA.

Does PKMζ overexpression affect only the most recently acquired memory? The answer is no. When trained consecutively on two CSs with clearly distinguishable taste qualities, saccharin and NaCl (*11*), overexpression enhanced the long-term memory of both (Fig. 4).

Our data show that whereas inhibition of PKMζ in the IC by competition with a DN mutation of the enzyme disrupts long-term CTA memory, overexpression of PKMζ in the IC enhances memory, including memory formed long before the enzyme was overexpressed. Recently, a model was proposed to account for the erasure of memory by the inhibition of PKMζ, according to which disruption of persistent activity of the enzyme disrupts trafficking of GluA2-containing AMPA receptors into the postsynaptic membrane and hence annuls the use-dependent increase in synaptic efficacy (*9*, *14*). The inhibition of memory by the DN thus makes sense. But how does overexpression of PKMζ increase memory already formed?

Considering the functional systems level; that is, what it is that the brain now does differently, three types of possibilities come to mind. One is that overexpression of PKMζ enhances global, item-invariant operations, such as attention, incentive valence, or sensory or motor operations, all of which are required for memory expression but are not memory per se. Nevertheless, as noted above, there was no significant effect of infection with $LV_{PKM\zeta}$ in the IC on sensorimotor and motivational attributes of either naïve or CS-exposed rats, as manifested in their normal approach to and consumption of saccharin and in their total liquid consumption. Rats whose IC was microinfused with $LV_{PKM\zeta}$ 6 days before training, as in Fig. 2B, displayed a normal immediate reaction to LiCl, augmenting the conclusion that the treatment and overexpression of PKMζ did not significantly alter sensorimotor, including visceral, responses relevant to CTA [supporting online material (SOM)]. Furthermore, in rats whose IC was infected with $LV_{PKM\zeta}$ on day 7 after conditioning, the enhanced long-term memory was still capable of experimental extinction, similar to a normal CTA trace obtained after the conventional, one-trial training (*15*) (Fig. 2C), implying no persistent shift in the reaction to the CS. All in all, the aforementioned data favor an effect on mnemonic functions. We cannot, however, completely exclude the possibility that overexpression of PKMζ culminates in alterations of fine-tuning or implicit properties of the system that might amplify memory performance, yet are undetected in the nonmnemonic tests. A second possibility is that overexpression of PKMζ results in the enhancement of global, item-invariant memory operations, of the type postulated to take part in memory retrieval, such as retrieval and search mode (*16*, *17*). Finally, a third possibility is that overexpression of PKMζ results in strengthening of item-variant memory operations (*17*), such as specific associations of specific items. The encoding of CTA in the IC was reported to be distributed, and specific associations are estimated to engage plastic changes in about 25% of the neurons (*18*), suggesting that the level

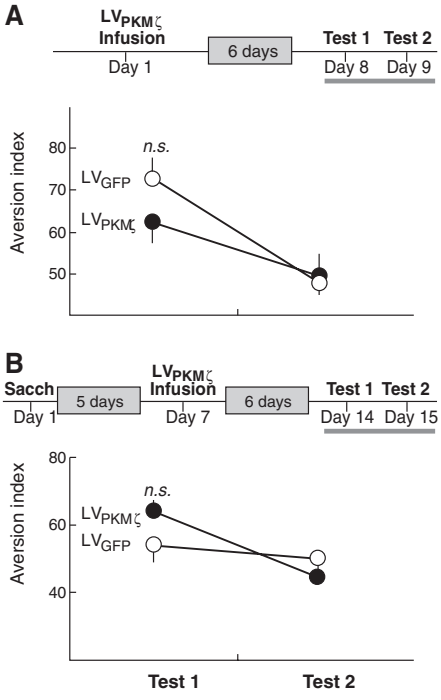

Fig. 3. Overexpression of PKMζ in the IC does not alter innate preferences for saccharin. (**A**) The IC of rats was microinfused bilaterally with $LV_{PKM\zeta}$ or LV_{GFP}, followed by a multiple choice test (see methods in the SOM) for the preference for saccharin versus water on days 8 and 9. There was no significant difference between the groups in their preference for saccharin (see methods in the SOM). LV_{GFP}, n = 16; $LV_{PKM\zeta}$, n = 17. (**B**) Rats were first exposed to saccharin, followed 6 days later by infusions into the IC of either $LV_{PKM\zeta}$ or LV_{GFP}. Preference for saccharin was tested 7 and 8 days later. Again, there was no significant difference between the groups in their preference for saccharin. LV_{GFP}, n = 25; $LV_{PKM\zeta}$, n = 26.

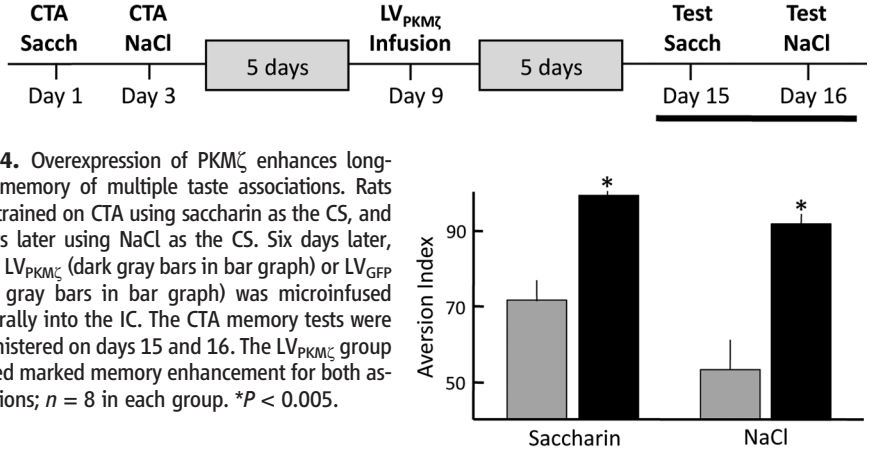

Fig. 4. Overexpression of PKMζ enhances long-term memory of multiple taste associations. Rats were trained on CTA using saccharin as the CS, and 2 days later using NaCl as the CS. Six days later, either $LV_{PKM\zeta}$ (dark gray bars in bar graph) or LV_{GFP} (light gray bars in bar graph) was microinfused bilaterally into the IC. The CTA memory tests were administered on days 15 and 16. The $LV_{PKM\zeta}$ group showed marked memory enhancement for both associations; n = 8 in each group. *$P < 0.005$.

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of overexpression that we obtained in the IC in the present study could affect multiple behaviorally relevant representations. The possibility that we indeed achieved strengthening of item-variant memory operations is perhaps the most exciting, but at this point in time we cannot dissociate item-invariant from item-variant effects, particularly given that overexpression appears to enhance more than one stored association.

As to the hardware implementation level of consolidated memory enhancement by PKMζ, it is implausible that this is due to flooding the cortex indiscriminately with the overexpressed enzyme, because this could lead to nonselective change in synaptic weights or even to saturation of use-dependent plasticity. Indeed, saturating amounts of exogenously applied PKMζ, when perfused directly into neurons, appear to produce potentiation of all of the cell's synapses (*19*). However, PKMζ synthesized in neurons is captured at recently active synapses, by a process of synaptic tagging, enabling these specific synapses to maintain a persistent state of enhanced efficacy (*20*). Thus, it is plausible to assume that the overexpressed kinase may augment the endogenous increase (fig. S1). We did observe differential accumulation of overexpressed PKMζ in dendritic spines in the IC in vivo (fig. S4), which is consistent with but does not prove the hypothesis. Selectivity resulting in strengthening memory might hence be achieved if the new enzyme molecules synthesized by the overexpressed gene are preferentially attracted to tagged synapses. The enhancement of memory long after encoding also raises the possibility that synaptic tagging and capture (*21*, *22*) can mold memory over much longer periods of time than previously supposed, a mechanism that might be useful in situations such as the integration of new episodic items into long-term memory schemata (*23*) or, more generally, memory summation over prolonged periods of time (*24*).

The observation that overexpression of PKMζ enhances memories long after they had been formed renders it plausible to consider PKMζ a potential target not only for memory blockers (which might be useful, for example, in treating post-traumatic stress) but also for novel types of memory enhancers in the treatment of amnesia and cognitive decline.

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Supporting Online Material

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Reut Shema, Sharon Haramati, Shiri Ron, Shoshi Hazvi, Alon Chen, Todd Charlton Sacktor, Yadin Dudai*

*To whom correspondence should be addressed. E-mail: yadin.dudai@weizmann.ac.il

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1. Materials and methods

Animals: Male Wistar rats (60 days, 250–350 g) were caged individually at 22°C ± 2°C in a 12 h light–dark cycle. Water and food were available ad libitum unless otherwise indicated. All experiments were approved by the Weizmann Institute of Science Institutional Animal Care and Use Committee (IACUC).

Behavioral procedures: All the tests (here and below) were conducted with the experimenter blind to the identity of the groups. The behavioral procedures were performed in the home cages unless otherwise indicated. Conditioned taste aversion (CTA) was induced and tested as previously described (*S1, S2*). Briefly, unless otherwise indicated, rats were deprived of water for 24 h, and then trained over 3 subsequent days to obtain their daily water ration within 10 min from two pipettes, each containing 10 ml of tap water. On day 5, water was replaced with the tastant solution (saccharin 0.1% or NaCl 0.45%, w/v, the conditioned stimulus, CS). This was followed 40 min later by an i.p. injection of LiCl (the unconditioned stimulus, US). In conventional CTA training, the intensity of the US leads to a ceiling effect that could mask memory enhancement. To overcome this, different intensities of the US were used: 0.05 M, 2% body weight, for the enhancement experiments in which saccharin was the CS; 0.075 M, 2% body weight, for the enhancement experiments in which the CS was NaCl and for the DN experiments. Testing was performed at the times indicated in the text, by presenting the rats with an array of 6 pipettes, 3 each with 5 ml of the relevant taste and 3 each with 5 ml water. In this test situation, the rats explore, sample, and subsequently consume or avoid the contents of several pipettes, none of which contains by itself sufficient liquid to satiate their thirst. The *Aversion Index* (AI) was defined as ([water consumed] / [water + taste consumed] × 100) (*S2*). For determination of the effect of the LiCl US on behavior, rats were injected i.p. with the same dose of LiCl as in the experiments involving microinfusion of $LV_{PKM\zeta}$ or with saline as control, placed individually in transparent cages, and observed. Three behavioral responses were monitored starting immediately at intervals of 15 min up to 240 min after the injection: lying-on-the-belly (*LOB*), characterized by little or no movement and a prone, flaccid posture with the chin on the floor of the cage; *grooming*; and *rearing*, expressed as lifting the forepaws simultaneously off the floor in the absence of grooming (*S3*). The behavioral events were scored as 0 or 1 at each time bin and the values summated to yield the *US malaise score*.

Western blot analysis of endogenous PKMζ expression: PKMζ levels in the insular cortex (IC) were tested 3 days and 1 week after 2 CTA trainings, employing a ζ–specific catalytic domain antibody (*S4*), as previously described (*S5*). In brief, the bilateral IC was removed from each rat, the tissue pooled and homogenized at 4°C in 150 µl homogenization buffer (50 mM Tris-HCl [pH 7.5]/1 mM EDTA/1 mM EGTA/5 mM 2-mercaptoethanol/0.02 mg/ml aprotinin/0.4 mM PMSF/0.05 mg/ml leupeptin), by 15 strokes in a Teflon-glass tissue grinder, and centrifuged at 3000 x g for 5 min to remove unhomogenized material and nuclei (P1). The supernatant (S1) was centrifuged again at 100,000 x g for 30 min to produce a supernatant (S2, cytosolic fraction) and pellet (P2, particulate fraction). Total protein in the cytosolic fraction was determined by Bradford assay or by Pierce BCA protein assay kit. The cytosolic fractions were subjected to gel electrophoresis on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel; separated proteins were transferred onto

nitrocellulose membranes, which were cut at ~40 kD based on molecular weight markers. The upper part of the membrane containing the PKMζ protein (~55 kD) was probed with the ζ-specific catalytic domain rabbit antiserum against PKMζ (1:4000), and then horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (1:10,000). The lower part was probed with anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, ~35 kD, 1:4000) for aliquot loading control, and HRP-conjugated anti-mouse as secondary antibody (1:2500). The immunostaining was visualized using EZ-ECL Chemiluminescence detection kit. Films were scanned and analyzed using ImageJ program.

Design, construction and validation of lentiviral vectors for site-specific overexpression of PKMζ: Vector plasmids were constructed for the production of lentiviruses expressing PKMζ or DN mutation of PKMζ (PKMζ-K281W, amino acid sequence number as in PKCζ), as previously described (*S6, S7*). The pSinRep5-pPKMζ-Ires-ZsGreen vector plasmids were from the Sacktor laboratory, SUNY Downstate Medical Center, Brooklyn, NY. PKMζ was PCR amplified from this vector and subcloned into the lentiviral expression vector (pCSC-SP-PW-IRES/GFP), using PstI and AgeI restriction sites introduced with the PCR primers. The DN was produced in the same manner except that the K281W mutation was introduced (*S8*). All vectors contained the enhanced green fluorescence protein (GFP) coding sequence, allowing identification of infected cells.

Production of lentiviral vectors: Recombinant lentiviruses were produced by transient transfection in HEK293T cells (*S9*), using pCSC-SP-PW-PKMζ-IRES/GFP (to yield $LV_{PKM\zeta}$), pCSC-SP-PW-DN-IRES/GFP (to yield LV_{DN}), or pCSC-SP-PW-IRES/GFP (to yield LV_{GFP}). Briefly, infectious lentiviruses were harvested at 48 and 72 h post-transfection, filtered through 0.45-μm-pore cellulose acetate filters and concentrated by ultracentrifugation. Aliquots were kept at −70°C. Viral titers were determined by infection of 293T cells and GFP visualization.

In vitro validation of lentiviral vectors: To determine the efficiency of the PKMζ lentiviruses to overexpress PKMζ, we infected HEK293T cells with the PKMζ-expressing lentiviruses and then analyzed the lysates of the infected cells by Western blot using anti-PKMζ antiserum. The cells were harvested 48 h postinfection in a lysis buffer (RIPA buffer/0.8 mM PMSF/10 μg/ml leupeptin/5 μg/ml aprotinin/1 mM DTT). The lysates were subjected to gel electrophoresis on 10% SDS-polyacrylamide gel; separated proteins were transferred onto nitrocellulose membranes and probed with rabbit antibody against PKMζ (1:2000). Membranes were incubated with HRP-conjugated anti-rabbit secondary antibody. Thereafter, the blots were stripped and re-probed with anti-β-actin, using HRP-conjugated anti-mouse as secondary antibody. The immunostaining was visualized using EZ-ECL Chemiluminescence detection kit.

Intracerebral injections of lentiviruses: Rats were anesthetized with 80 mg/kg sodium pentobarbitone i.p., and injected subcutaneously with Kamacain at the infusion area. They were then restrained in a stereotaxic apparatus (Kopf), and the lentiviral vectors (2.5 μl to each side) were delivered bilaterally using a 5 μl Hamilton syringe connected to a motorized nanoinjector (Leica), at a rate of 0.17 μl-min^{-1} (approximately 2.5 μl within 15 min). The slow infusion allowed the diffusion of LVs into the cortex while minimizing tissue damage. The coordinates of the IC injection

site relative to bregma were: AP +1.2 mm, L ± 5.4 mm, V 6.4 mm. At the end of the experiment, all rats were anesthetized and perfused with 2.5% paraformaldehyde and 5% sucrose in phosphate-buffered saline (PBS), pH 7.4.

Microinfusion lesion score: To estimate the damage to IC tissue due to microinfusion and infection, equivalent sections of the IC that were removed 8 days post-infusion were Nissl stained, and the treatment effect on the tissue determined by calculating the percent decrease in cells around the microinfusion site, compared to the equivalent area in non-microinfused rats (percent decrease from 5510 ± 109 cells).

Immunohistochemistry: The brains were removed and post-fixed in 1% paraformaldehyde and 30% sucrose in PBS, frozen and sectioned coronally at 30 μm using a sliding microtome and stored in PBS and 0.01% sodium azide at 4°C until used. Brain slices were blocked for 1 h with PBS containing 0.2% Triton and 20% normal horse serum in PBS to prevent non-specific binding and incubated overnight at room temperature with goat biotinylated anti-GFP (1:100) in PBS, followed by green streptavidin (1:200, in PBS). The slices were then washed and mounted on superfrost/plus slides, and screened using a fluorescent microscope for GFP expression at the injection sites. Representative images were captured. Western blots were also performed, as described above, to compare PKMζ expression in the IC of rats that received $LV_{PKM\zeta}$ versus PKMζ expression in the IC of rats infected with LV_{GFP}.

Kinase assays: To assure equal amounts of expression of PKMζ and DN, Sindbis virus vectors were used to express fusion proteins of GFP-PKMζ and GFP-DN, according to methods described in the Invitrogen Sindbis Expression Manual. PKMζ or DN was ligated into a plasmid vector (pSinRep5) that is under the control of the Sindbis subgenomic promoter using a PCR-based strategy. An insert corresponding to amino acids 184-592 of PKCζ or DN mutation was obtained using a plasmid containing the mouse cDNA as a template. The forward primer (5'-AAATCTAGAACATGGCATACCCATACGACGTCCCAGACTACGCTATGGAT TCTGTCATGCCTTCC-3') was flanked by an XbaI restriction site followed by a sequence corresponding to GFP. The reverse primer (5'-AATTCTAGATCACACGGACTCC-TCAGC-3') was flanked by an XbaI restriction site. Baby Hamster Kidney (BHK) cells were electroporated with RNA transcribed in vitro from pSinRep5 vectors encoding GFP-PKMζ or GFP-DN and from the helper plasmid DHBB. BHK cells were plated at 4 X 10^5 per 35 mm^2 wells in Dulbecco's Modified Eagle's Medium/F12 (50:50) with 5% fetal bovine serum. After 24 hr, the cells were infected with 5-10 plaque forming units/cell of Sindis virus expressing GFP-DN, equivalent amount of GFP-PKMζ, or the two together. After 24 hr, the cells were trypsinized, collected by centrifugation (3000 g X 3 min), washed with PBS, and sonicated in 50 μl water for 15 sec on ice. The cell debris was removed by centrifugation (12,000 g X 5 min) and the supernatant used for the kinase reaction. The reaction mixture (50 μl) contained: 10 μl supernatant containing GFP-DN, GFP-PKMζ (0.15 pmol/min/μl), or GFP-DN + GFP-PKMζ, 50 mM Tris-HCl (pH 7.5), 10 mM Mg_2Cl_2, 1 mM DTT, and 1 μM epsilon peptide substrate (AnaSpec). The reaction was begun with 50 μM ATP and 1 μCi [γ-^{32}P]ATP. The reaction was for 10 min at 30°C, which was linear with respect to enzyme concentration (data not shown). The reaction was stopped by 100 mM cold ATP and 100 mM EDTA, and 45 μl of the

reaction mixture was spotted onto phosphocellulose paper, washed 5 min X 3 in 5% phosphoric acid. The radioactivity incorporated into substrate was counted by liquid scintillation. The background activity was measured as counts incorporated by extracts of BHK cells without transfection.

Statistics: t-Test (two-tail unpaired unless otherwise indicated) was used for comparison of two groups. One-way ANOVA and repeated-measures ANOVA were used for comparisons of more than two groups and in cases of repeated tests, respectively, with an α level of 0.05.

2. Supporting text

US malaise score (see *Methods*) was 66.3 ± 5.9 for the $LV_{PKM\zeta}$ group and 60.6 ± 3.3 for the LV_{GFP} control group (n = 6 and 7 rats, respectively; *n.s.*).
Microinfusion lesion score (see Methods) was LV_{GFP}, 5.9 ± 1.0%; $LV_{PKM\zeta}$, 6.7 ± 1.9%; LV_{DN}, 7.9 ± 2.6% (n = 17, 11, and 8 infusions, respectively; *n.s*).

3. Supporting figures

Fig. S1. Endogenous PKMζ expression in the insular cortex increases after CTA training. Rats were subjected to two trials of CTA training using saccharin as the CS on subsequent days. A control group was exposed to water only. The rats were decapitated either 3 days or 1 week later, the IC removed and homogenized, and aliquots subjected to Western blotting using an antibody against the catalytic domain of PKMζ to quantify the amount of the kinase. **A.** Representative aliquots from two animals from each group. GAPDH was used as the protein-loading control. **B.** Quantification of the amount of PKMζ (dark gray bars), as compared to control (light gray bars), 3 days and one week after training. *, $P < 0.05$.

Fig. S2. The PKMζ dominant negative mutation (DN) suppresses PKMζ activity. GFP-DN (DN), an equivalent amount of GFP-PKMζ (PKMζ), and the two together were expressed in BHK cells, the kinases extracted, and the activity toward an exogenous substrate assayed. DN has little activity, compared to PKMζ, and PKMζ and DN together has less activity than PKMζ alone (mean ± SEM, n = 6). One way ANOVA showed a significant difference among groups [$F(2,17) = 534.8$, $p < 0.0001$]. Post-hoc comparisons using the Tukey HSD test indicated that all three groups are significantly different from each other ($P < 0.0001$).

Fig. S3. Correlation between CTA memory enhancement and the extent of $LV_{PKM\zeta}$ infection in the IC. Efficiency of infection is expressed on a scale referring to the number of GFP-expressing cells in 0.5 mm^2 in the IC, scored blindly on a scale of 1-5 (1, 10-25 cells; 2, 25-45; 3, 45-75; 4, 75-150; 5, >150 cells).

Fig. S4. Selective overexpression of PKMζ in dendritic spines in the IC in vivo. Arrows indicate spines that contain GFP but not PKMζ. Overall, 65 ± 9% of the GFP-labeled spines in samples of $LV_{PKM\zeta}$-infected neurons in the IC contained PKMζ. Scale bar = 1.5 μm.

Fig. S1

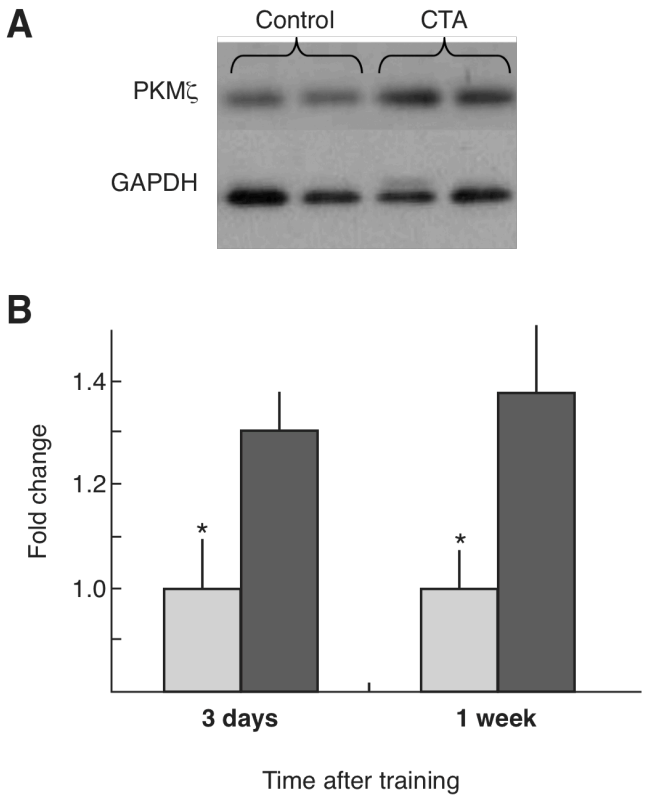
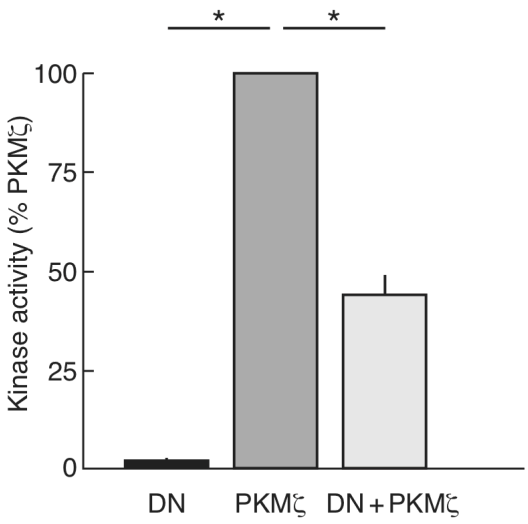

A
Control
CTA
PKMζ
GAPDH
B
Fold change
1.4
1.2
1.0
*
*
3 days
1 week
Time after training

Fig. S2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*
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75
50
25
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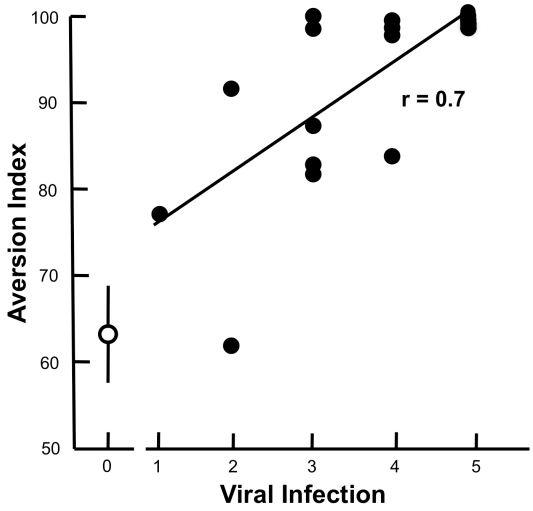
Kinase activity (% PKMζ)
DN
PKMζ
DN + PKMζ

Fig. S3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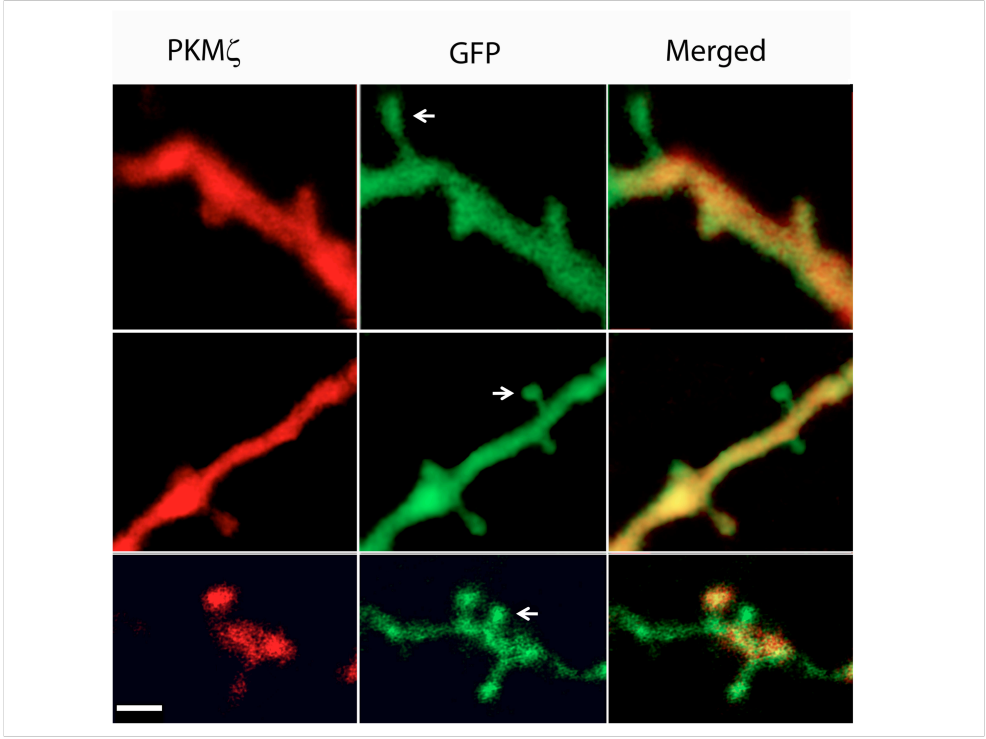
2
3
4
5
Viral Infection

Fig. S4



4. Supporting references and notes

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