nature neuroscience

PKMζ maintains memories by regulating GluR2-dependent AMPA receptor trafficking

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The maintenance of long-term memory in hippocampus, neocortex and amygdala requires the persistent action of the atypical protein kinase C isoform, protein kinase Mζ (PKMζ). We found that inactivating PKMζ in the amygdala impaired fear memory in rats and that the extent of the impairment was positively correlated with a decrease in postsynaptic GluR2. Blocking the GluR2-dependent removal of postsynaptic AMPA receptors abolished the behavioral impairment caused by PKMζ inhibition and the associated decrease in postsynaptic GluR2 expression, which correlated with performance. Similarly, blocking this pathway for removal of GluR2-containing receptors from postsynaptic sites in amygdala slices prevented the reversal of long-term potentiation caused by inactivating PKMζ. Similar behavioral results were obtained in the hippocampus for unreinforced recognition memory of object location. Together, these findings indicate that PKMζ maintains long-term memory by regulating the trafficking of GluR2-containing AMPA receptors, the postsynaptic expression of which directly predicts memory retention.

Persistence of many kinds of long-term memory and long-term potentiation (LTP) requires the constitutive activity of the atypical protein kinase C isoform M ζ (PKMζ)[1]. It has been shown that PKMζ is involved in long-term memory maintenance in various behavioral tasks and brain regions, including allocentric spatial memory[1,2] and object location[3] in the hippocampus, conditioned taste aversion in the insular cortex[4] and auditory fear conditioning in the amygdala[2]. It is not understood how PKMζ maintains long-term memory. Recent results studying late LTP in hippocampal slices, however, indicate that PKMζ might regulate *N*-ethylmaleimide–sensitive factor (NSF)/glutamate subunit 2 (GluR2)-dependent AMPA receptor trafficking, thereby increasing the amount of postsynaptic AMPA receptors[5]. As the interaction of NSF with GluR2 seems to stabilize AMPA receptors in the postsynaptic membrane by preventing their internalization[6–8], PKMζ may maintain long-term memory by persistently inhibiting AMPA receptor removal from postsynaptic sites.

We decided to directly test the plausibility of this mechanism by blocking AMPA receptor synaptic removal and simultaneously inhibiting PKMζ. If PKMζ's ability to maintain memory is a result of the downregulation of AMPA receptor removal from postsynaptic sites, then blocking the removal process should abolish the long-term memory loss that is usually observed after inhibiting PKMζ. We found that PKMζ maintains long-term memories by blocking a GluR2-dependent pathway for removal of postsynaptic AMPA receptors to persistently promote increased levels of GluR2-containing AMPA receptors at postsynaptic sites.

RESULTS

PKMζ inhibition disrupts established auditory fear memory

We first confirmed the ability of the cell-permeable, myristoylated PKMζ pseudosubstrate inhibitory peptide (ZIP), the most selective PKMζ blocking compound, to impair the retention of previously established auditory fear memory[2]. We bilaterally infused either ZIP (20 nmol) or the scrambled inactive version of the peptide (Scr-ZIP) into the basal lateral amygdala (BLA) 1 d after auditory fear conditioning. Memory retention was tested the next day. As expected, ZIP infusions impaired conditioned fear memory (ZIP, 6.7 ± 4.4% freezing; Scr-ZIP, 58.2 ± 12.9% freezing; Mann-Whitney U test, $U = 0.00$, $P = 0.008$, $n = 5$) to the extent that the behavior of these rats was similar to that of untrained ones. Administering ZIP to another group of rats 7 d after training had the same effect on conditioned fear (ZIP, 6.7 ± 4.1% freezing; Scr-ZIP, 48.7 ± 11.0% freezing; Mann-Whitney U test, $U = 3.50$, $P = 0.031$, $n = 5$). We then retrained the Scr-ZIP and ZIP-infused rats, and both groups showed similar, normal conditioned-fear responses, indicating that ZIP infusions, which retrogradely disrupted memory retention, did not compromise the subsequent ability of the BLA to anterogradely acquire and maintain new fear memories (**Supplementary Fig. 1**).

We next examined whether PKMζ maintains long-term auditory fear memory by regulating GluR2-dependent AMPA receptor trafficking. We used a synthetic peptide derived from the GluR2 carboxy tail, $GluR2_{3Y}$, fused to the cell membrane transduction domain of the HIV 1 Tat protein for cell permeability[9]. $GluR2_{3Y}$ is the most selective compound that can block GluR2-dependent endocytosis without decreasing basal synaptic transmission or LTP in the BLA[10]. Biochemical analyses of the

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Received 23 November 2009; accepted 1 March 2010; published online 11 April 2010; doi:10.1038/nn.2531

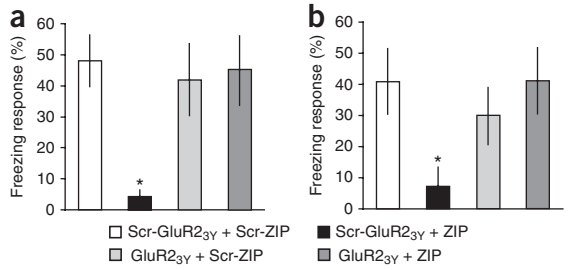

Figure 1 Blocking GluR2-dependent AMPA receptor synaptic removal prevents memory impairment induced by PKMζ inactivation. (**a**,**b**) GluR2$_{3Y}$ or Scr-GluR2$_{3Y}$ was infused into the amygdala 1 d after the training session. ZIP or Scr-ZIP was infused 1 h later. Memory was tested 1 d (**a**) or 10 d (**b**) after the infusions. Data represent the mean percentage of the freezing time during the tone. Error bars represent s.e.m. ZIP infusion abolished the freezing response (*$P < 0.001$), but infusions of both GluR2$_{3Y}$ and ZIP led to a performance that was similar to that exhibited by the inactive, scrambled peptide–infused controls, as determined by the Kruskal-Wallis analysis of ranks test.

surface expression of AMPA receptor subunits have shown that this effect of GluR2$_{3Y}$ is a result of abolishing the reduction of AMPA receptor levels without affecting their basal surface expression[10]. We therefore tested whether blocking GluR2-dependent internalization by infusing GluR2$_{3Y}$ (15 pmol) or its scrambled inactive version (Scr-GluR2$_{3Y}$) into the BLA 1 h before ZIP infusion prevents the loss of auditory fear memory that we observed. We tested memory retention at both 1 and 10 d after the peptide infusions, which were administered 1 d after training (**Fig. 1a**,**b**). The Kruskal-Wallis analysis by ranks indicated a significant difference among the groups at both 1 d ($H(3, N = 45) = 15.246$, $P = 0.002$) and 10 d ($H(3, N = 43) = 13.380$, $P = 0.004$). *Post hoc* comparisons revealed that the fear responses of rats infused with Scr-GluR2$_{3Y}$ and ZIP ($n = 12$) were significantly lower than the responses of the control group infused with both scrambled peptides ($n = 14$, 1 d, $P = 0.003$; 10 d, $P = 0.013$) and the group infused with both GluR2$_{3Y}$ and ZIP ($n = 11$, 1 d, $P = 0.017$; 10 d, $P = 0.022$). The GluR2$_{3Y}$ before ZIP group and the two scrambled peptide group were not different from each other (1 and 10 d, $P = 1.000$). GluR2$_{3Y}$ alone had no effect on memory retention, as there was no difference between the rats that received GluR2$_{3Y}$ and Scr-ZIP ($n = 8$) and the ones that received two scrambled peptides (1 and 10 d, $P = 1.000$). The fear responses observed after 1 d were not different from those observed after 10 d (Wilcoxon matched paired test, $T = 282.000$, $P = 0.423$). Fear memories, which may last the lifetime of an animal, can persist without any decrease in the fear response[11]. Taken together, these results indicate that blocking GluR2-dependent AMPA receptor synaptic removal with GluR2$_{3Y}$ prevents the memory impairment induced by ZIP. Moreover, the effect was not transient, but persisted for at least 10 d. The data suggest that the loss of memory caused by PKMζ inactivation may be a result of the loss of postsynaptic AMPA receptors through a GluR2-dependent pathway that is persistently inhibited by the activity of PKMζ after training.

Although rather unlikely, one alternative interpretation of these data is that a drug-drug interaction between GluR2$_{3Y}$ and ZIP decreased the efficacy of ZIP at inhibiting PKMζ. Ruling out this alternative, we found that the half-maximal inhibitory concentration of ZIP on PKMζ activity was not changed by GluR2$_{3Y}$ (**Supplementary Fig. 2**).

PKMζ inactivation decreases synaptic GluR2 in trained rats

To confirm that the effect of PKMζ inactivation by ZIP is indeed mediated by the removal of GluR2-dependent AMPA receptors from postsynaptic sites after training and that GluR2$_{3Y}$ prevents this removal, we replicated the experiment and then isolated subcellular fractions from the BLA 1 d after the memory test. We examined the levels of GluR2 (**Fig. 2a**) and GluR1 (**Fig. 2b**) in postsynaptic densities and in fractions containing extrasynaptic membranes by western blot after training and after the behavioral effect of training was disrupted by ZIP. Discriminating between surface and intracellular proteins by biotin-labeling and neutravidin pull-down assay revealed that the postsynaptic density fractions contained only surface AMPA receptors, whereas the extrasynaptic fractions contained both

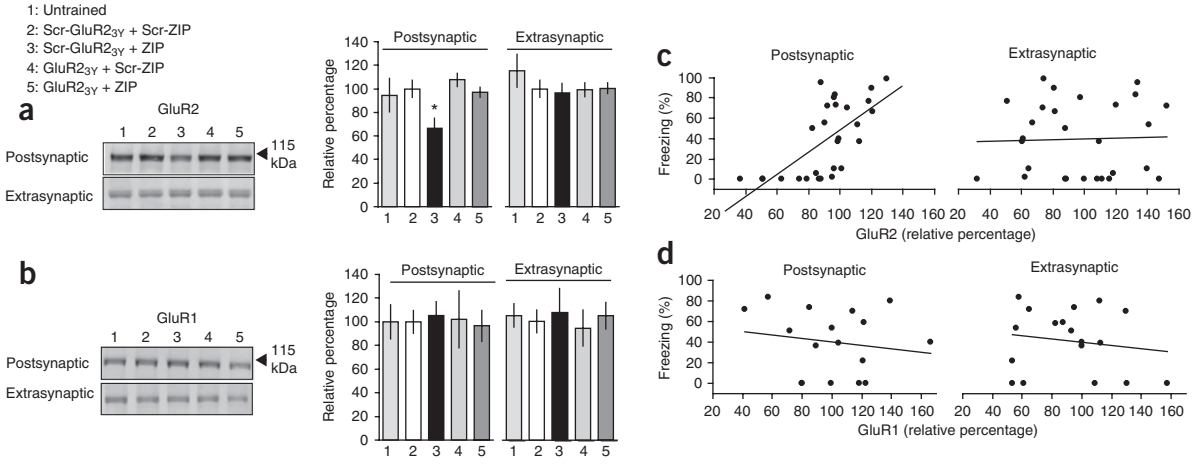

Figure 2 Postsynaptic GluR2 levels are decreased in fear-conditioned rats after PKMζ inactivation and the levels of postsynaptic GluR2 correlate with the magnitude of freezing during memory retention. (**a**,**b**) GluR2$_{3Y}$ or Scr-GluR2$_{3Y}$ was infused into the amygdala 1 d after the training session. ZIP or Scr-ZIP was infused 1 h later. Memory was tested 1 d after the infusions and rats were killed 1 d later for BLA extraction. Representative western blots (left) and quantification of GluR2 (**a**) and GluR1 (**b**) protein levels (right) in the indicated subcellular fractions from BLA are shown. The full-length western blots are shown in **Supplementary Figure 6**. Data were normalized to the Scr-GluR2$_{3Y}$ and Scr-ZIP group mean. Bars represent the group means, and error bars represent s.e.m. The levels of postsynaptic GluR2 in the group that received Scr-GluR2$_{3Y}$ and ZIP were significantly lower (*$P < 0.05$) than in the group infused with both scrambled control peptides and in the group that received both GluR2$_{3Y}$ and ZIP as determined by the *post hoc* Tukey's HSD test after significant one-way ANOVA. (**c**,**d**) Relationship between GluR2 (**c**) and GluR1 (**d**) levels in the postsynaptic or extrasynaptic membrane fractions and freezing levels during the test of trained animals. A significant correlation was observed only for the postsynaptic GluR2 fraction ($R = 0.65$, $P < 0.001$) by ANOVA overall goodness of fit.

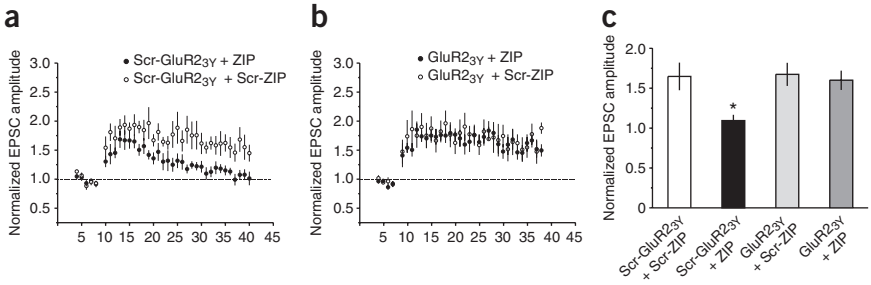

Figure 3 Blocking GluR2-dependent AMPA receptor synaptic removal prevents the LTP impairment induced by PKMζ inactivation in BLA slices. (**a**) Bath application of ZIP, but not Scr-ZIP, reversed LTP induced by 200 pulses at 2 Hz paired with postsynaptic depolarization to −5 mV. (**b**) The reversal of LTP caused by ZIP was prevented by intracellular perfusion of GluR2$_{3Y}$. (**c**) Averaged EPSC amplitude obtained at 40 min after LTP induction. The samples that received both Scr-GluR2$_{3Y}$ and ZIP were significantly different from all the other groups (*$P < 0.05$, *post hoc* Tukey's HSD test after significant one-way ANOVA).

surface and intracellular receptors (**Supplementary Fig. 3**), indicating that changes in receptor content in postsynaptic fractions represent changes in expression of surface receptors. A one-way ANOVA revealed a significant difference in the levels of postsynaptic GluR2 ($F_{3,24} = 7.522$, $P = 0.001$) and *post hoc* comparisons (Tukey's honestly significant differences (HSD) test) confirmed that GluR2 levels decreased in the postsynaptic membranes of fear-conditioned rats infused with Scr-GluR2$_{3Y}$ and ZIP ($n = 6$) compared with rats infused with Scr-GluR2$_{3Y}$ and Scr-ZIP ($n = 8$, $P = 0.002$). Consistent with our behavioral results, infusions of GluR2$_{3Y}$ prevented the decrease of postsynaptic GluR2, as the GluR2 levels of rats that received both GluR2$_{3Y}$ and ZIP ($n = 9$) were similar to the levels of rats that received both scrambled peptides ($P = 0.986$), but were different from the levels of rats infused with Scr-GluR2$_{3Y}$ and ZIP ($P = 0.004$). These results indicate that ZIP causes a decrease in postsynaptic levels of GluR2 in trained rats and blocking AMPA receptor synaptic removal prevents this decrease. GluR2$_{3Y}$ alone had no effect on the postsynaptic content of GluR2 (GluR2$_{3Y}$ + Scr-ZIP versus Scr-GluR2$_{3Y}$ + Scr-ZIP, $P = 0.995$, $n = 5$), consistent with the finding that the GluR2$_{3Y}$ peptide does not affect AMPA receptor basal surface expression[10,12]. There was no difference in extrasynaptic GluR2 levels between the treatment groups (one-way ANOVA, $F_{4,29} = 0.367$, $P = 0.829$; **Fig. 2a**). Finally, GluR1 levels did not differ among the groups at either postsynaptic ($F_{4,17} = 0.038$, $P = 0.996$) or extrasynaptic sites ($F_{4,19} = 0.103$, $P = 0.979$) (**Fig. 2b**).

Further examination of the relationship between behavior after training and postsynaptic GluR2 expression revealed a positive correlation ($R = 0.658$, $F = 19.905$, $P < 0.001$): stronger fear responses indicated stronger postsynaptic GluR2 expression (**Fig. 2c**). A correlation was not found between the fear response and the expression of GluR2 in the extrasynaptic membranes ($R = 0.031$, $F = 0.025$, $P = 0.875$) or of GluR1 (**Fig. 2d**) in either postsynaptic ($R = 0.166$, $F = 0.428$, $P = 0.522$) or extrasynaptic fractions ($R = 0.155$, $F = 0.393$, $P = 0.539$).

Notably, the total levels of postsynaptic GluR2 in the BLA between untrained and trained rats were indistinguishable (Scr-GluR2$_{3Y}$–Scr-ZIP untrained versus Scr-GluR2$_{3Y}$–Scr-ZIP trained, Mann-Whitney U test, $U = 35$, $P = 0.656$). One possible explanation for this finding is that decreases in GluR2 in some synapses in the BLA might have occurred following training, compensating for PKMζ-mediated increases in other synapses. Such counterbalanced changes between the mechanisms for increasing and decreasing synaptic strength have been observed after training in the hippocampus[13,14]. However, as ZIP injections into the BLA would reverse the mechanisms that lead to increases in postsynaptic GluR2, but not those mechanisms that lead to the compensatory decrease of postsynaptic GluR2 in other synapses, a detectable difference can be observed between trained animals infused with ZIP and those infused with the control peptide. Thus, injecting ZIP may reveal AMPAR trafficking in trained animals that is normally masked by compensatory responses. Consistent with this hypothesis, ZIP had a downward trend, but no significant effect on the level of postsynaptic GluR2 in the BLA of untrained rats ($P = 0.308$; **Supplementary Fig. 4**). Thus, ZIP has a selective effect on postsynaptic AMPARs of trained rats, suggesting that the number of synapses potentiated by PKMζ from prior experience in naive rats is relatively small and therefore below the level of detection. This is consistent with previous observations that ZIP has no substantial effect on baseline synaptic transmission[1,15].

A loss of AMPA receptors from postsynaptic sites also occurs in NMDA receptor–dependent long-term depression (LTD)[10,12]. Thus, although ZIP disrupts memory storage without overt behavioral cues, it is possible that an unintentional signal (or ZIP itself) induces LTD and may contribute to the memory loss. However, we found that blocking NMDA receptors with D(−)-2-amino-5-phosphonovaleric acid (AP5) before ZIP infusion did not diminish the disruption of memory by ZIP (**Supplementary Fig. 5**), suggesting that the removal of synaptic AMPA receptors by PKMζ inhibition is not dependent on NMDA receptor activation, as in LTD.

Loss of postsynaptic GluR2 mediates LTP disruption by ZIP

We asked whether ZIP could impair LTP in the BLA as it does in the hippocampus and, if so, whether GluR2$_{3Y}$ could prevent this impairment as it prevented ZIP-mediated amnesia in our behavioral experiments. Excitatory postsynaptic currents (EPSCs) were evoked by stimulating the auditory thalamic synaptic inputs in BLA slices. To ensure the GluR2$_{3Y}$ effect is the result of blocking postsynaptic endocytosis, we perfused GluR2$_{3Y}$ postsynaptically into amygdala neurons through a whole-cell recording patch pipette. Once stable EPSCs were obtained, ZIP or scrambled ZIP was applied to the bath and LTP was induced.

One-way ANOVA revealed a significant group effect ($F_{3,21} = 5.138$, $P = 0.007$; **Fig. 3**). This group effect was the result of application of Scr-GluR2$_{3Y}$ and ZIP ($n = 6$) impairing LTP ($n = 8$), as determined by *post hoc* comparisons (Tukey's HSD, versus Scr-GluR2$_{3Y}$ + Scr-ZIP, $n = 6$, $P = 0.019$; **Fig. 3a,c**), whereas application of GluR2$_{3Y}$ ($n = 6$) prevented the effect of ZIP ($P = 0.032$ versus Scr-GluR2$_{3Y}$ + ZIP) and led to a similar effect as applying both scrambled control peptides ($P = 0.993$) (**Fig. 3b,c**). EPSCs in the presence of GluR2$_{3Y}$ alone (GluR2$_{3Y}$ + Scr-ZIP, $n = 5$) were not significantly different from the responses recorded after application of both scrambled peptides ($P = 0.999$; **Fig. 3b,c**). The time course of the PKMζ-dependent phase that we observed was consistent with the rapid onset of the protein synthesis–dependent phase previously described for LTP in the amygdala[16]. Thus, GluR2-dependent postsynaptic removal of AMPA receptors mediates the effect of ZIP on LTP in amygdala slices.

PKMζ-mediated GluR2 trafficking sustains location memory

We then asked whether the mechanism by which PKMζ maintains long-term memory may represent a general principle that can be found in other brain regions and tasks. To maximize the difference

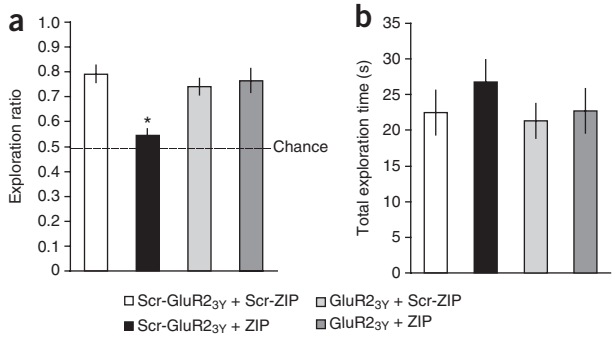

Figure 4 Blocking GluR2-dependent, postsynaptic AMPA receptor removal prevents the impairment of object location memory induced by PKMζ inactivation in the dorsal hippocampus. GluR2$_{3Y}$ or Scr-GluR2$_{3Y}$ was infused into the dorsal hippocampus 1 d after the training session. ZIP or Scr-ZIP was infused 1 h later. Memory was tested 1 d after the infusions. (**a**) Values represent the mean ratio of the time that the rats spent exploring a familiar object in a novel location over the time they spent exploring a familiar object in the same location as in the training session. ZIP infusions led to a total loss of memory for object location (*$P < 0.001$), as determined by *post hoc* Tukey's HSD test after significant one-way ANOVA, such that the rats explored both objects at the level of chance. GluR2$_{3Y}$ infusions before ZIP infusions prevented the memory loss. (**b**) Values represent the total exploration time. No significant differences were observed among the treatment groups. All error bars represent s.e.m.

between tasks and memory systems, we chose object location memory, which rats acquire in an emotionally neutral task by unrestricted and unguided exploration in the absence of any external reinforcement or punishment. Long-term memory for object location in this task engages brain areas that are different from those recruited in auditory fear conditioning[17] and depends on persistent PKMζ activity in the dorsal hippocampus 1 and 7 d after acquisition[3].

We bilaterally infused GluR2$_{3Y}$ (15 pmol) or its scrambled control peptide into dorsal hippocampus of rats 1 d after training and then infused ZIP (10 nmol) or its scrambled control peptide 1 h later. Memory for object location was assessed the next day. One-way ANOVA detected a significant difference among the groups in novel object-location exploration ($F_{3,22} = 9.484$, $P < 0.001$; **Fig. 4a**), whereas the overall time spent exploring was not significantly different among the groups ($F_{3,22} = 0.671$, $P = 0.579$; **Fig. 4b**), indicating that differences in novel object-location exploration were not the results of basic differences in exploratory activity. *Post hoc* comparisons (Tukey's HSD) determined that the group infused with Scr-GluR2$_{3Y}$ and ZIP ($n = 7$) explored the object at the novel location less than the group infused with both scrambled peptides ($P = 0.001$, $n = 7$) and the group infused with GluR2$_{3Y}$ and ZIP ($P = 0.001$, $n = 7$), which were not different from each other ($P = 0.952$). The group infused with GluR2$_{3Y}$ and Scr-ZIP ($n = 5$) was also not different from the control group infused with both scrambled peptides ($P = 0.797$), indicating that GluR2$_{3Y}$ alone had no effect on the normal performance. Comparing exploratory activity against what would have been expected by chance alone revealed that only the rats infused with Scr-GluR2$_{3Y}$ and ZIP explored both objects equally ($P = 0.123$, *t* test), whereas all other groups preferred the object at the new location to the object at the old location ($P < 0.001$). These data indicate that GluR2$_{3Y}$ prevents the impairment of object location memory induced by ZIP and suggest that the mechanism by which PKMζ maintains long-term memory is task and memory system independent.

DISCUSSION

Our results, across brain areas (amygdala and hippocampus), tasks (auditory fear memory and object location memory) and both in amygdala slices and *in vivo*, indicate that PKMζ maintains long-term memories by blocking a GluR2-dependent pathway that removes postsynaptic AMPA receptors to persistently promote increased levels of GluR2-containing AMPA receptors at postsynaptic sites. It is always possible, however, that other mechanisms may operate at later time periods after memory acquisition, especially in the hippocampus, where memory has been suggested to undergo a systems consolidation process that can last for several months after learning[18,19].

A previous study found that PKMζ causes synaptic potentiation in LTP by upregulating the NSF/GluR2-dependent trafficking pathway and focused on the initial trafficking of the receptor from extrasynaptic to synaptic sites[5]; however, the role of reducing postsynaptic GluR2/AMPA receptor clearance as the mechanism of maintaining these changes has not been examined. This latter role is consistent with observations that the interaction of NSF with GluR2 probably stabilizes surface AMPA receptors in the postsynaptic membrane by preventing the internalization of AMPA receptors (rather than membrane insertion)[6–8,20]. In addition, NSF seems to be involved in restricting GluR2-containing AMPARs from lysosomal degradation and maintaining them in a recycling pathway[21]. Our results are therefore compatible with the idea that PKMζ acts persistently through NSF/GluR2 interaction to prevent the removal of AMPA receptors from postsynaptic sites and to maintain the constitutive recycling of receptors. By blocking these mechanisms, ZIP could induce the removal of the receptors from postsynaptic sites and/or facilitate their degradation. Our biochemical results may therefore be consistent with the possibility that training induces counterbalanced PKMζ-maintained increases of GluR2-containing AMPA receptors in some synapses and non–PKMζ mediated decreases in GluR2 in others and/or a model in which training shifts the regulatory mechanisms of overall or subpopulations of postsynaptic GluR2 trafficking from a PKMζ-independent to a PKMζ-maintained mechanism. Such a shift of regulatory trafficking mechanisms would provide a way to maintain memories without affecting the receptor trafficking that maintains baseline synaptic transmission.

Our results further indicate that both memory maintenance and erasure are active molecular mechanisms, as both ZIP and GluR2$_{3Y}$ interfere with enzymatically driven processes. NSF/GluR2 interaction prevents the removal of postsynaptic AMPA receptors by disrupting the interaction of the carboxyl tail of GluR2 with proteins that are critical for receptor internalization such as PICK1 and AP2 (refs. 22,23). The binding motif comprised by GluR2$_{3Y}$, however, does not include the AP2, NSF or the PDZ/PICK1-binding motifs[12], indicating that the GluR2$_{3Y}$ peptide does not interfere with the interaction of GluR2 with these molecules. Instead, GluR2$_{3Y}$ comprises a cluster of tyrosine residues whose phosphorylation is a critical step in the postsynaptic removal of GluR2-containing receptors through intracellular internalization[12,24] and is required for insulin-stimulated AMPA receptor internalization and some forms of LTD, but not for baseline synaptic transmission[12]. Although the exact mechanism by which this binding motif regulates postsynaptic receptor removal is still poorly understood, our results suggest that, were it not for the persistent action of PKMζ, this mechanism would actively eliminate the postsynaptic pool of GluR2-containing receptors by which LTP maintenance and memory storage are expressed. Although enzymatically driven, the postsynaptic AMPA receptor removal mediating the memory erasure by ZIP does not appear to require the activation of NMDA receptors, as in LTD, because the amnestic effect of ZIP is not prevented by NMDA receptor blockade.

We also found that GluR2-containing AMPA receptors are crucial for the expression and maintenance of long-term memories. Several studies have shown that synaptic insertion of GluR1-containing receptors, believed to be GluR1/2 heteromers, is necessary for memory formation and LTP induction in the amygdala[10,25], hippocampus[13] and barrel cortex[26,27]. In the barrel cortex, however, GluR1-containing receptors are replaced by GluR2-containing receptors that lack GluR1, which are thought to be GluR2/3 heteromers[26,27], within 24 h of training. Our findings that disrupting memory by PKMζ inactivation decreased the levels of GluR2, but not GluR1, and that GluR2 levels predicted memory performance are therefore consistent with these results[26–28]. It is therefore possible that early phases of memory formation are characterized by the initial postsynaptic insertion of GluR1/2-containing receptors and that persistent postsynaptic increases of GluR2/3-containing receptors allow memory to be maintained in the long term[28]. A recent study found that GluR2 not only regulates synaptic function, but also promotes spine growth and maintenance in neuronal cultures through its interaction with N-cadherins[29]. Whether these morphological changes are sustained by the same persistent action of PKMζ that maintains postsynaptic GluR2 expression and the persistence of behavioral memory is an important question for future study.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

Note: Supplementary information is available on the Nature Neuroscience website.

ACKNOWLEDGMENTS

This work was supported by the Canadian Institutes of Health Research and the Natural Sciences and Engineering Research Council of Canada (K.N. and Y.T.W.), the E.W.R. Steacie Foundation (K.N.) and by US National Institutes of Health grants R01 MH53576 and MH57068 (T.C.S.). O.H. was supported by the German Research Society (Deutsche Forschungsgemeinschaft).

AUTHOR CONTRIBUTIONS

All of the authors contributed to the design of experiments, interpretation of results and editing of the manuscript. P.V.M. and O.H. conducted the behavioral studies. P.V.M. carried out the biochemical studies. D.C.W. conducted the electrophysiological studies. K.G. and O.H. performed the stereotaxic surgeries. P.V.M., O.H., T.C.S. and K.N. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Subjects. Male Sprague-Dawley and Long Evans rats (only for the object location recognition experiments) (275–350 g, Charles River) were housed in pairs in plastic Nalgene cages with environmental enrichment and maintained on a 12:12-h light dark cycle. All experiments were carried out during the light phase. Food and water were provided *ad libitum* throughout the experiment. All procedures complied with Canadian Council on Animal Care guidelines and were approved by McGill University's Animal Care Committee.

Surgery. Rats were anaesthetized with a mixture of xylazine (3.33 mg ml^{-1}), ketamine (55.55 mg ml^{-1}) and Domitor (0.27 mg ml^{-1}) by intraperitoneal injection of a volume of 1 ml per kg of body weight. Using a Kopf Stereotax, we implanted three jeweler screws into the skull and then placed two steel cannulae (22 gauge), aiming bilaterally at the BLA (anteroposterior, −3.0 mm; medial-lateral, ±5.1 mm; dorsoventral, −8.0 mm) or dorsal hippocampus (anteroposterior, −3.6 mm; medial-lateral, ± 3.1 mm; dorsoventral, −2.4 mm, with the cannula aimed 10° away from midline). Dental cement was applied to stabilize the implants. Obturators inserted in the guides prevented blocking. After surgery, an intramuscle injection of analgesic (buprenorphine, 0.324 mg per kg) was given. An intraperitoneal injection of Antisedan (7.5 mg per kg) suspended anesthesia. The rats were then allowed 7–10 d to recover from surgery. Rats were handled daily during the recovery period. At the end of the experiment, cannula placement was checked by examining 50-µm brain sections stained with formal-thionin under a light microscope.

Drug infusions. ZIP (Myr-SIYRRGARRWRKL-OH, Anaspec), Scr-ZIP (Myr-RLYRKRIWRSAGR-OH, Anaspec), $GluR2_{3Y}$ (TAT(47–57)-^{869}YKEGYNVYG^{877}), Scr-$GluR2_{3Y}$ (TAT(47–57)-AKEGANVAG) and AP5 (Sigma) were dissolved in 100 mM Tris-saline (pH 7.2). They were infused into the BLA (ZIP, 1 µl per hemisphere; $GluR2_{3Y}$ and AP5, 0.5 µl per hemisphere) or dorsal hippocampus (1 µl per hemisphere) via a microinjector (28 gauge) connected to a Hamilton syringe with plastic tubing at a rate of 0.25 µl per min. The injector remained connected for an additional min to allow for drug diffusion away from the tip of the cannula.

Auditory fear conditioning task. The training chambers (22 × 22 × 22 cm, Med Associates) were equipped with a stainless steel grid (bar radius = 2.5 mm, spread = 1 cm apart) and the floor was connected to a shocker. The walls were made of Plexiglass. Three key lights were mounted on the sidewalls. A fan provided a constant background noise. Behavior was recorded by a digital video camera mounted in front of the chamber door. To assess only the auditory component of the fear memory, the testing chambers were different from the training ones with respect to textual, visual and olfactory cues. The floor of the testing chambers was made of opaque plastic panels. The front and the back walls were pasted with wallpapers containing black and white alternating strips. Peppermint scent was sprayed onto the floor before the rat was placed in the box. A digital camera was mounted on the ceiling and videotaped the sessions for later analysis.

Rats were habituated to the training and testing chambers by placing them in each chamber for 20 min with a 5-h interval between them for 2 consecutive days. The order of chambers was reversed on the second day. Training was performed the day after habituation. Rats were placed into the box and, after 2 min, a tone (5 kHz, 75 dB) was presented for 30 s, which co-terminated with a foot shock (1.5 mA, 1 s). The rats remained in the box for another 30 s and were then returned to their home cages. Rats were tested for 6 min, during which three 30-s tones were presented, with an interval of 60 s between each. The first tone was presented 2 min after the rats were placed into the box. Memory was evaluated by averaging the freezing time during each tone. Because the rats did not freeze before the first tone, the freezing to the tone accurately represents auditory fear memory.

Object location recognition task. The experiment was carried out in a 40 × 40 × 60 cm open field arena (that is, a box made of white laminated wood). The walls were high enough to obscure external room cues. Sawdust covered the floor. Objects were secured in the floor by screws. A video camera above the box recorded behavior.

Rats were habituated over 3 consecutive days. On day 1, the co-housed pairs were placed into the open field and were able to explore it for 20 min together. No objects were present. On day 2, rats were placed individually into the open field and explored it, again without objects, for 10 min. On day 3, rats were placed individually into the open field in the absence of objects for 5 min. The sampling phase started the next day. Two sampling sessions were administered, the first in the morning and the second in the afternoon. Each session consisted of five 5-min trials per rat, separated by ~1 h. During each 5-min trial, rats were exposed to two identical copies of a junk object. The objects were made of different materials and had different shapes, colors and textures (for example, metal cup, ceramic incense burner, plastic baby bottle, etc.). The objects were placed in opposing corners during the ten sampling trials. The objects and their locations remained constant for each rat, but varied between rats and were counterbalanced. Object location knowledge was assessed in a 3-min probe trial. Rats were presented with the same objects that they encountered during sampling; however, one of the objects was moved to a new location. Object exploration was defined as the time when the rat's head was oriented toward the object within 45 degrees and was within 4 cm of the object. The exploration ratio observed per min was averaged across the first minute only[17]. The exploration ratio is defined as the relative time the animal spends exploring the novel stimulus (that is, the object at the novel location) relative to the total time the animal engaged in stimulus exploration: $t_{novel}/(t_{familiar} + t_{novel})$[17]. A ratio of 0.5 indicates the absence of location memory.

Subcellular fractionation. Rats were anesthetized as for surgeries, decapitated, and their brains were removed and frozen. The amygdala was dissected from the frozen brains with a neuro punch (1 mm, Fine Science Tools) and homogenized in ice-cold Tris-HCl buffer (30 mM, pH 7.4) containing 4 mM EDTA, 1 mM EGTA and a cocktail of protease inhibitors (Complete, Roche). Subcellular fractions were prepared as described[30]. Briefly, the amygdala homogenates were centrifuged twice at 4 °C at 500*g* for 5 min to remove nuclei and other debris. The two supernatants were pooled and centrifuged at 100,000*g* at 4 °C for 60 min. Pellets were resuspended in the same buffer containing 0.5% Triton X-100 and incubated at 4 °C for 20 min, layered over 1 M sucrose and centrifuged at 100,000*g* for 60 min. Finally, the Triton-soluble fraction that remained above the sucrose layer, which consists mainly of detergent-soluble membrane components and contains the extra-synaptic receptors, was collected and the Triton-insoluble material that sedimented through the sucrose layer, which is highly enriched in postsynaptic densities, was resuspended in the same buffer and stored at −80 °C. Total protein concentration was determined by the BCA protein assay kit (Pierce).

Western blotting. Western blotting was performed using 7.5% SDS-polyacrylamide gel electrophoresis. The proteins were transferred onto nitrocellulose membranes and incubated with polyclonal antibodies to GluR2 (0.5 µg ml^{-1}, Millipore), GluR1 (1 µg ml^{-1}, Millipore) or PSD-95 (0.6 µg ml^{-1}, Chemicon), followed by incubation with goat antibody to rabbit horseradish peroxidase–linked IgG. We used the ECL plus immunoblotting system (Amersham) for detection. Blots were scanned with a Storm Laser Scanner (Molecular Dynamics) and analyzed with ImageQuant software (ABI).

Slice electrophysiology. Rats were placed under deep anesthesia and decapitated. The brain was rapidly removed and placed in ice-cold slicing solution containing 87 mM NaCl, 2.5 mM KCl, 7 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 25 mM glucose and 75 mM sucrose, which was bubbled continuously with carbogen (95% O_2 and 5% CO_2) to adjust pH to 7.4. Coronal slices of 400-µm thickness containing the amygdala were produced using a vibrating blade microtome and recovered in an incubation chamber with carbogenated artificial cerebrospinal fluid (ACSF) containing 125 mM NaCl, 2.5 mM KCl, 1.0 mM $MgCl_2$, 2.0 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$ and 25 mM glucose for 45 min at 34 °C and were then returned to 20–22 °C for at least 30 min before recording. All experiments were carried out at 20–22 °C.

Recordings were carried out in a chamber perfused continuously by carbogenated ACSF containing bicuculline methiodide (10 µM) to block $GABA_A$ receptor–mediated inhibitory synaptic currents. Whole-cell patch-clamp recordings were performed using the 'blind' method from neurons in the dorsal part of the lateral amygdala. Recording pipettes were filled with pipette solution containing 122.5 mM cesium gluconate, 17.5 mM CsCl, 2 mM $MgCl_2$, 10 mM HEPES, 0.5 mM EGTA, 4 mM K-ATP and 5 mM QX-314 (pH 7.2, osmolarity 290–300 mOsm), with or without 100 µg ml^{-1} $GluR2_{3Y}$ or scrambled $GluR2_{3Y}$. The resistance of electrodes was typically 4–5 MΩ. After obtaining the whole-cell configuration, clamp current was used to identify the firing pattern of the cells.

After cell characterization, the membrane potential was held at −70 mV. EPSCs were evoked by stimulating the auditory thalamic synaptic inputs via a constant current pulse (0.05 ms) delivered through a tungsten bipolar electrode and recorded with a MultiClamp 700B amplifier (Axon Instruments). Synaptic responses were evoked at 0.05 Hz except during the induction of LTP. Once stable EPSCs were obtained, ZIP or scrambled ZIP was applied to the bath (5 μM, final concentration). After obtaining a stable EPSC baseline, LTP was induced by 200 pulses at 2 Hz while depolarizing the cell to −5 mV. The stimulation intensity of induction was the same as that used during baseline recording. LTP was induced within 10 min of the establishment of a whole-cell configuration to avoid washout of intracellular contents.

Slice biotinylation and neutravidin pull-down. Coronal brain slices of 300-μm thickness containing amygdala and hippocampus were prepared using a vibratome and maintained in ice-cold carbogenated ACSF containing 124 mM NaCl, 3 mM KCl, 1.0 mM $MgSO_4$, 2.0 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$ and 10 mM glucose. The biotinylation was performed as described previously[31] with some modifications. Briefly, slices were incubated with NHS-SS-Biotin (Pierce) for 45 min on ice. Excess biotin was removed by washes with NH_4Cl. Slices were then homogenized at 4 °C in Tris-HCl buffer (30 mM, pH 7.4) containing 4 mM EDTA, 1 mM EGTA and a cocktail of protease inhibitors (Complete, Roche), and subcellular fractions were prepared as described above. The subcellular fractions were solubilized in lysis buffer containing 150 mM NaCl, 20 mM HEPES (pH 7.4), 2 mM EDTA, 1% Triton X-100 and 0.1% SDS, sonicated and incubated in a shaker for 2 h at 4 °C. The total protein concentration was determined using the Pierce BCA kit. The samples were then incubated with Neutravidin beads for 10 h at 4 °C. Samples were centrifuged and the supernatant containing the unbound fraction was used to determine the intracellular content of receptors. The biotinylated proteins were eluted in equal volume as the unbound fraction with 2× loading buffer at 90 °C for 5 min. The amount of GluR1, GluR2 and PSD95 was determined by western blot.

Kinase activity. The reaction mixture (50 μl final volume) contained 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, PKMζ (0.015 pmol per min per assay phosphotransferase activity), 6 μM myelin basic protein as substrate, and varying concentrations of ZIP and $GluR2_{3Y}$ as indicated. The reaction, begun with the addition of 50 μM ATP (4 μCi [γ-^{32}P] per assay), continued for 10 min at 30 °C, which is in the linear range for time and enzyme concentration (data not shown). The reaction was stopped by addition of 25 μl of 100 mM ice-cold ATP and 100 mM EDTA, and 40 μl of the assay was spotted onto phosphocellulose paper and counted by liquid scintillation. PKMζ activity was measured as the difference between counts incorporated in the presence and absence of enzyme.

Statistical analysis. All data were tested for normality distribution fitting by the Shapiro-Wilk W test and for homogeneity of variance by Levene's test. When values were normally distributed and groups had identical variance, we used a one-way ANOVA, followed by Tukey's HSD *post hoc* test when appropriate. When data did not meet the above ANOVA assumptions, we used Kruskal-Wallis analysis of ranks for multiple comparison and Mann Whitney U test for two samples comparisons. For nonparametric two dependent samples comparisons, we used the Wilcoxon matched pairs test.

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doi:10.1038/nn.2531