Behavioral/Cognitive

Memory Destabilization and Reconsolidation Dynamically Regulate the PKMζ Maintenance Mechanism

Matteo Bernabo,[1] Josue Haubrich,[2] Karine Gamache,[2] and Karim Nader[2]
[1]Integrated Program in Neuroscience, McGill University, Montreal, Quebec H3A 2B4, Canada, and [2]Department of Psychology, McGill University, Montreal, Quebec H3A 1B1, Canada

Useful memory must balance between stability and malleability. This puts effective memory storage at odds with plasticity processes, such as reconsolidation. What becomes of memory maintenance processes during synaptic plasticity is unknown. Here we examined the fate of the memory maintenance protein PKMζ during memory destabilization and reconsolidation in male rats. We found that NMDAR activation and proteasome activity induced a transient reduction in PKMζ protein following retrieval. During reconsolidation, new PKMζ was synthesized to re-store the memory. Failure to synthesize new PKMζ during reconsolidation impaired memory but uninterrupted PKMζ translation was not necessary for maintenance itself. Finally, NMDAR activation was necessary to render memories vulnerable to the amnesic effect of PKMζ-antisense. These findings outline a transient disruption and renewal of the PKMζ memory maintenance mechanism during plasticity. We argue that dynamic changes in PKMζ protein levels can serve as an exemplary model of the molecular changes underlying memory destabilization and reconsolidation.

Key words: **amygdala; destabilization; maintenance; memory; PKMζ; reconsolidation**

Significance Statement

Maintenance of long-term memory relies on the persistent activity of PKMζ. However, after retrieval, memories can become transiently destabilized and must be reconsolidated within a few hours to persist. During this period of plasticity, what happens to maintenance processes, such as those involving PKMζ, is unknown. Here we describe dynamic changes to PKMζ expression during both destabilization and reconsolidation of auditory fear memory in the amygdala. We show that destabilization induces a NMDAR- and proteasome-dependent loss of synaptic PKMζ and that reconsolidation requires synthesis of new PKMζ. This work provides clear evidence that memory destabilization disrupts ongoing synaptic maintenance processes which are restored during reconsolidation.

Introduction

Long-term potentiation is stabilized by active molecular mechanisms that maintain long-term memory in animals (Sacktor et al., 1993; Osten et al., 1996; Migues et al., 2010). Long-term memories can also become transiently labile following retrieval through a process known as memory destabilization. What triggers the switch from stability to plasticity is becoming increasingly clear with several initiating mechanisms identified (Ben Mamou et al., 2006; Suzuki et al., 2008; J. L. Lee and Flavell, 2014; Merlo et al., 2015). What remains unknown is how the pro-stability processes are affected by destabilization and how they are recapitulated during reconsolidation.

Memory destabilization requires the activation of NMDARs (Ben Mamou et al., 2006; Zhang et al., 2018). Activation of these receptors leads to the activation of CaMKII, which promotes protein degradation, another crucial component of memory destabilization in both vertebrates and invertebrates (S. H. Lee et al., 2008, 2012; Cai et al., 2012; Jarome et al., 2016). Following a brief period of plasticity, the memory must be reconsolidated to ensure long-term stability. Importantly, infusion of protein synthesis inhibitors, such as anisomycin (Nader et al., 2000) or rapamycin (Jobim et al., 2012) after retrieval impairs long-term memory, suggesting that new protein synthesis is crucial to reconsolidation. Yet which proteins must be degraded and synthesized remain largely unknown.

One critical component of post-retrieval memory plasticity is the transient reorganization of AMPARs at the synapse. During destabilization, GluA2-containing AMPARs are internalized and

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Correspondence should be addressed to Matteo Bernabo at matteo.bernabo@mail.mcgill.ca.

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replaced with calcium-permeable AMPARs (i.e., those lacking GluA2 subunits) (Hong et al., 2013). During reconsolidation, the calcium-permeable AMPARs are internalized and the GluA2-AMPARs are reinserted into the membrane. While the movement of these receptors has been defined, what triggers their rearrangement after retrieval is not clear. One clue lies in the regulation of GluA2-AMPARs by the protein PKMζ during memory maintenance (Migues et al., 2010).

PKMζ is an atypical protein kinase C (PKC) isoform, whose mRNA is transcribed from an internal promoter of the *Prkcz* gene (Hernandez et al., 2003). It contains the catalytic and hinge regions of the PKCζ protein but crucially lacks the regulatory domain of that protein (Sacktor et al., 1993). While there is still ongoing debate (A. M. Lee et al., 2013; Volk et al., 2013; Tsokas et al., 2016), research has primarily examined its potential role as a key regulator of long-term memory maintenance (Sacktor et al., 1993; Hardt et al., 2010; Shema et al., 2011; Hsieh et al., 2017). PKMζ seems to maintain long-term memories by regulating the movement of GluA2-AMPARs after LTP formation. It prevents endocytosis of GluA2-AMPARs (Migues et al., 2010) and limits their lateral diffusion (Yu et al., 2017). Given the role of PKMζ in GluA2-AMPAR trafficking, it is likely implicated in the AMPAR exchange that is central to memory destabilization and reconsolidation.

The aim of this work was to investigate changes in PKMζ expression during memory destabilization and reconsolidation. We hypothesized that memory reactivation induces a transient disruption of PKMζ expression and that reconsolidation restores the expression of this protein. We therefore tracked changes in PKMζ protein levels throughout this process and determined whether its synthesis is critical to reconsolidation. We found that destabilization induces an NMDAR- and proteasome-dependent reduction in PKMζ and that reconsolidation requires *de novo* protein synthesis to increase PKMζ and stabilize the memory.

Materials and Methods

Animals

Male Sprague Dawley rats (275-300 g) were obtained from Charles River. Rats were housed in pairs and maintained on a 12 h light/dark cycle (lights on at 7:00 A.M., lights off at 7:00 P.M.). Rats received food and water *ad libitum*. All methods and procedures were approved by McGill University's Animal Care Committee and conformed to Canadian Council on Animal Care's guidelines.

Surgery

Rats were given an intraperitoneal injection of anesthetic cocktail (1 ml/kg) containing ketamine (50 mg/ml), xylazine (3 mg/ml), and dexdomitor (0.175 mg/ml). Before surgery, rats also received carprofen analgesic (5 mg/ml; 1 ml/kg) subcutaneously. Rats were bilaterally implanted with 22-gauge guide cannulas targeted to the Please change to: basolateral amygdala (BLA) (from bregma: AP −3.0 mm; ML 5.1 mm; DV −8.0 mm). Cannulas were secured to the skull with dental cement and three jeweler's screws. To ensure the interior of the cannula remained clear of debris, metal dummies were inserted and remained in place except during infusions. Following surgery, rats were given an intraperitoneal injection of anesthetic reversal containing 0.5 mg/ml of antisedan. Following surgery, rats were monitored and individually handled for at least 7 d before the start of behavioral experiments.

Infusions

DL-2-Amino-5-phosphonopentanoic acid (APV, Sigma Millipore, A5282) was dissolved in saline to reach a final concentration of 5 μg/μl, pH 7.0. Clasto-Lactacystin β-lactone (β-lac, Abcam, ab141412) was first dissolved in 2% DMSO-HCl and brought to a final concentration of 32 ng/μl in saline, pH 7.4.

Antisense oligodeoxynucleotides (ODNs) were obtained from Integrated DNA Technologies at 2 nmol/μl dissolved in TE buffer-PBS, pH 7.5, for both PKMζ-antisense and scrambled controls. The sequence for single-stranded PKMζ-antisense was C*T*C*TTGGGAAGGC AT*G*A*C and the sequence for the scrambled control was A*A*C*AATGGGTCGTCT*C*G*G where each asterisk represents a phosphorothioate linkage from 5′ to 3′. These sequences followed previous work showing selective impairment of PKMζ synthesis after PKMζ-antisense administration but not the scrambled ODN (Tsokas et al., 2016). The PKMζ-antisense ODN is the complementary sequence to start site of PKMζ mRNA, whereas the scrambled control had no complementarity to a known mRNA sequence.

All infusions were performed bilaterally into the BLA at a rate of 0.25 μl/min with a total volume of 0.5 μl/side. Intracranial infusions used 23-gauge injectors (Plastics One) connected to 20-gauge polyethylene tubing (Braintree Scientific) which were connected to 26-gauge Hamilton syringes (model 1701N). Injectors extended 1.5 mm beyond the guide cannula and remained in place for an additional 1 min following the infusion to ensure proper drug diffusion. Rats were handled by the experimenter during infusions and returned to their home cage following each infusion. For experiments where infusions occurred before retrieval, each rat was given a sham infusion before each day of habituation to habituate rats to the infusion experience. Sham infusions followed the same procedure, but no solutions were injected.

Fear conditioning

Each day, rats were transported to a nearby holding room 30 min before the start of the experiment. Experiments used two different Coulbourn Habitest (model I-I10-24A) chambers referred to here as Context A and Context B. Context A had white, curved, plastic walls, and a plastic, white floor. Context B had square, checkered walls, stainless-steel grid floors, and a vanilla scent was sprayed in the chamber before each rat entered. Additionally, chambers for Context A were housed in a room of bright ambient lighting, whereas Context B was in a different room with very low lighting.

In each experiment (except Experiment 2), rats were habituated and trained as follows. On days 1 and 2, rats were placed in Context A for 20 min to habituate to the context. On day 3, rats were placed in Context B for training. During training, rats were allowed to habituate to the context for 2 min followed by a 30 s tone (4 kHz, 75 dB) which coterminated with a 1 s, 1.0 mA shock. Rats remained in the context for an additional 30 s before being removed. Following habituation and training:

Experiment 1. On day 4, one group of rats was placed back in Context A. After 2 min in the box, these rats were exposed to one unpaired tone (30 s, 4 kHz, 75 dB) and remained in the context for an additional 30 s (i.e., a retrieval test). Rats were returned to their home cage following this reactivation session and killed 1 h later. The other group of rats did not undergo reactivation and remained in their home cage during this period. These nonreactivated rats were killed at the same time as the reactivated group. Rats' brains were flash frozen and collected for Western blot analysis.

Experiment 2. Rats underwent the same procedure as Experiment 1, except that during the training session on day 3, rats did not experience any shock, only a 30 s tone.

Experiment 3. On day 4, rats received an intracranial infusion of APV (5 μg/μl; 0.5 μl per side) or saline vehicle to the BLA. After 30 min, rats underwent a retrieval test (as in Experiment 1). Rats were returned to their home cage following the retrieval test and killed 1 h later. Their brains were flash frozen and collected for Western blot analysis.

Experiment 4. On day 4, rats received an intracranial infusion of APV (5 μg/μl; 0.5 μl per side) or vehicle to the BLA and returned to their home cage. Rats were killed 90 min later. Their brains were flash frozen and collected for Western blot analysis.

Experiment 5. On day 4, rats underwent a retrieval test (as in Experiment 1). Immediately after retrieval, rats were infused intracranially with β-lac (32 ng/μl; 0.5 μl per side) or vehicle in the BLA. Rats were returned to their home cage following the infusion and killed 1 h after retrieval. Their brains were flash frozen and collected for Western blot analysis.

Experiment 6. On day 4, rats were infused intracranially with β-lac (32 ng/μl; 0.5 μl per side) or vehicle in the BLA. Rats were returned to their home cage following the infusion and killed 50 min after infusion. Their brains were flash frozen and collected for Western blot analysis.

Experiment 7. On day 4, rats underwent a retrieval test (as in Experiment 1). After retrieval, rats were returned to their home cage; and one group was killed 1 h later, whereas the other group was killed 24 h later. Rats' brains were flash frozen and collected for Western blot analysis.

Experiment 8. On day 4, rats underwent a retrieval test (as in Experiment 1). Immediately after retrieval, rats received intracranial BLA infusions with either PKMζ-antisense (2 nmol/μl; 0.5 μl per side) or scrambled control and then returned to their home cage. One day later (day 5, post-reactivation long-term memory [PRLTM] test), rats were again placed in Context A and received one unpaired tone as on day 4. Freezing behavior for both the retrieval and PRLTM test days was recorded.

Experiment 9. Rats underwent the same procedure as in Experiment 8, except that infusions occurred 24 h after retrieval. As in Experiment 8, the post-infusion test occurred 24 h after infusions.

Experiment 10. In this experiment, rats underwent nearly the same procedure as in Experiment 8. However, here rats also received an intracranial infusion of APV (5 μg/μl; 0.5 μl per side) or vehicle 30 min before the retrieval test, in addition to the infusion of PKMζ antisense (2 nmol/μl; 0.5 μl per side) or scrambled control after retrieval.

During each experiment, rats were recorded during training using FreezeFrame software (Actimetrics) and during tests using GeoVision GV-600 System.

Histology

For Western blotting, rats' brains were quickly collected and flash frozen. Rats were placed in an induction chamber containing isoflurane. Once each rat was unconscious, but still breathing, it was decapitated using a guillotine and its brain quickly retrieved. The brain was immediately submerged in a beaker containing 2-methylbutane, which was seated in a container of dry ice. Once brains were frozen, they were wrapped in aluminum foil and submerged in the dry ice before final storage at −80°C.

Where rats' brains were not required for Western blotting, rats were killed in a CO_2 induction chamber. Brains were collected and stored in 20% sucrose-formalin solution. After 48 h, brains were sliced using a cryostat to check for proper cannula placement.

Subcellular fractionation

Synaptosomal fractions were obtained from BLA tissue using a previously established procedure (Bai and Witzmann, 2007). Frozen brains were mounted on a cryostat and BLA tissue was collected using a tissue puncher (Fine Science Tools). The tissue was homogenized using a Pellet Pestle (Thermo Fisher Scientific, #12141361) in 200 μl of homogenization buffer containing 20 mM HEPES, 1 mM EDTA, 2 mM EGTA, 320 mM sucrose, containing protease inhibitor (Roche Diagnostic, 05892791001) and phosphatase inhibitor (Roche Diagnostic, 04906837001) tablets. Homogenized tissue was centrifuged at 1000 × *g* for 10 min. The supernatant was collected and centrifuged at 17,000 × *g* for 15 min. The pellet was resuspended in 50 μl of homogenization buffer and layered on a sucrose gradient containing 100 μl of 0.8 M sucrose (1 mM EDTA, 2 mM EGTA) and 100 μl of 1.2 M sucrose (1 mM EDTA, 2 mM EGTA). This mixture was centrifuged at 54,000 × *g* for 90 min. The layer between the 0.8 and 1.2 M sucrose, containing the synaptosomal fraction, was collected and used for Western blotting following protein quantification with a BCA protein assay kit (Pierce).

Western blotting

After protein quantification, 15 μg of protein was loaded in 8% SDS-PAGE gels. Proteins were transferred overnight (at 4°C) onto nitrocellulose membranes. Following transfer, Ponceau solution (Sigma Millipore, P7170) was applied to membranes to reveal protein bands. Along with the molecular weight marker (Thermo Fisher Scientific, 26634), this enabled the cutting of membranes at 70 and 40 kDa to produce two membranes. One membrane contained PKMζ protein (55 kDa) and the other contained GAPDH (37 kDa). Membranes were washed in 0.1% Tween 20, TBS-Tween. Blocking of membranes was done for 1 h at room temperature in TBS-Tween containing 5% BSA. Membranes were then incubated overnight with antibodies in 5% BSA TBS-Tween: 1:1000 PKCζ (Abcam, ab59364), 1:10,000 GAPDH (Abcam, ab8245). Following overnight incubation, membranes were washed 3 times with TBS-Tween. Membranes then underwent incubation with secondary antibody (anti-rabbit IgG HRP-conjugated from GE Healthcare, NA934V; anti-mouse IgG HRP-conjugated from GE Healthcare, NA931V) for 1 h at room temperature. After secondary antibody incubation, membranes were washed four times for 10 min in TBS-Tween. Membranes were revealed using Pierce ECL 2 Western Blotting Substrate and scanned using a Storm Scanner (Molecular Dynamics). Scanned images were quantified using Image Lab (Bio-Rad). PKMζ protein values were compared with the GAPDH loading control for each sample. Values were then standardized as a percent of the control group.

Experimental design and statistical analyses

All experiments used male Sprague Dawley rats. Data were analyzed using Jamovi (version 1.0.7.0.). Statistical analyses for each experiment can be found in their respective Results sections. Briefly, PKMζ protein from Western blots was analyzed using independent-samples *t* tests (Experiments 1-7). As appropriate, freezing behavior data was analyzed using paired sample *t* tests (Experiments 1 and 2), independent-sample *t* tests (Experiments 3, 5, and 7), one-way repeated-measures ANOVAs (Experiments 8 and 9), or a two-way repeated-measures ANOVA (Experiment 10). Tukey's test was used for *post hoc* comparisons of within-subjects effects. Neither homogeneity of variance, normal distribution of data, nor sphericity assumptions was violated where relevant. For all analyses, the null hypothesis was rejected where $p < 0.05$. Figures present data as mean ± SEM.

Results

Experiment 1: Memory retrieval reduces synaptic PKMζ

While PKMζ has been much studied in regards to memory maintenance, its response to memory retrieval remains unknown. Retrieval of conditioned auditory fear can induce internalization of GluA2-containing AMPARs in the amygdala (Hong et al., 2013). Therefore, we expected that a reduction in PKMζ in the BLA would be observed following recall of an auditory fear memory. Rats were trained by pairing a tone with a footshock. The next day, one group was exposed to one unpaired tone to trigger retrieval of the fear conditioning memory and killed 1 h later (1 h Post-Retrieval group). Rats in the second group did not undergo this re-exposure, remained in their home cage, and were killed at the same time as the previous group (No Retrieval group). Rats re-exposed to the tone exhibited much more freezing to the tone than to the context (paired-samples *t* test, $t_{(5)} = -7.222$, $p < 0.001$; Fig. 1*A*), indicating that they learned and retrieved the tone-shock association.

Western blots compared the PKMζ expression in BLA samples of rats killed without retrieval or 1 h after retrieval. Notably, rats killed 1 h after the retrieval session showed less PKMζ than animals that remained in their home cage (independent-samples *t* test, $t_{(9)} = 2.755$, $p = 0.022$; Fig. 1*B*). Thus, memory retrieval seems to induce a decrease in synaptic PKMζ in the BLA.

Experiment 2: Reduction of PKMζ requires tone-shock pairing

We next sought to determine whether this reduction in PKMζ expression that occurred after re-exposure to the tone required prior learning of a tone-shock association. Following habituation, we exposed rats to a tone without any shock. The next day we re-exposed one group of rats to the tone and killed them 1 h

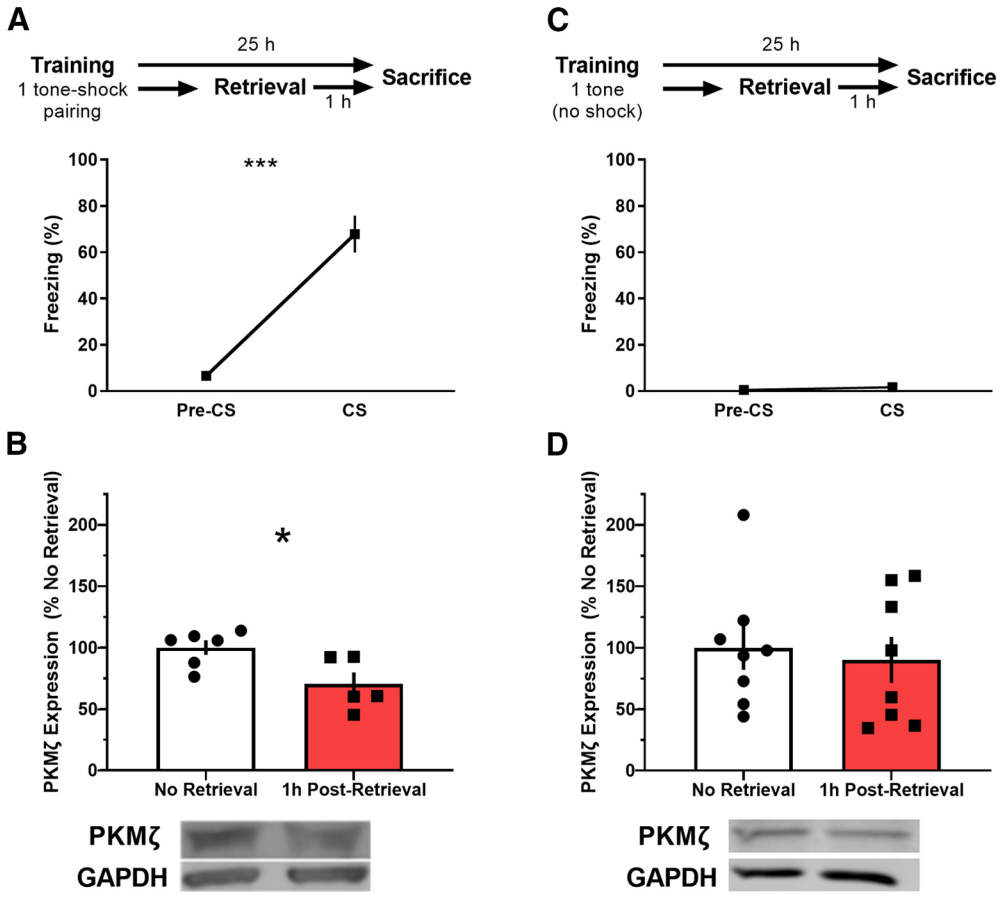

Figure 1. Synaptic PKMζ expression decreases within 1 h after retrieval of an auditory fear conditioning memory. ***A***, For those rats that were re-exposed to the tone conditioned stimulus (CS), freezing behavior was considerably elevated during the tone (CS) than to the context, before the onset of the tone (Pre-CS, paired-samples *t* test, $t_{(5)} = -7.222$, $p = 0.001$). ***B***, Rats trained in auditory fear conditioning and killed 1 h after retrieval showed significantly lower PKMζ protein in BLA synapses compared with rats that did not undergo retrieval (independent-samples *t* test, $t_{(9)} = 2.755$, $p = 0.022$). ***C***, Rats were trained with tone and no shock. For those rats that were re-exposed to the tone, freezing behavior was equally low to both the context and the tone (paired-samples *t* test, $t_{(7)} = -1.409$, $p = 0.202$). ***D***, Without prior tone-shock pairing, rats re-exposed to the tone showed similar PKMζ protein in BLA tissue compared with rats that were not re-exposed to the tone (independent-samples *t* test, $t_{(14)} = 0.378$, $p = 0.711$). Data are mean ± SEM. $^*p < 0.05$. $^{***}p < 0.001$.

later (1 h Post-Retrieval group). The other group (No Retrieval group) was not re-exposed to the tone and killed at the same time as the 1 h Post-Retrieval group. Rats that were re-exposed to the tone showed very little freezing to the context or the tone (paired-samples *t* test, $t_{(7)} = -1.409$, $p = 0.202$; Fig. 1*C*). Further, both groups showed similar PKMζ expression, suggesting that exposure to a tone is not sufficient to reduce synaptic PKMζ but rather requires prior tone-shock conditioning (independent-samples *t* test, $t_{(14)} = 0.378$, $p = 0.711$; Fig. 1*D*).

Experiment 3: Reduction in PKMζ is destabilization-dependent

Activation of NMDARs is necessary to initiate memory destabilization and internalization of GluA2-AMPARs (Ben Mamou et al., 2006; Hong et al., 2013). Therefore, we sought to determine whether the drop in PKMζ previously observed requires NMDAR activation. Rats were fear conditioned as before and, before retrieval, infused with APV, an NMDAR antagonist known to prevent labilization (Ben Mamou et al., 2006), or vehicle. Rats were then killed 1 h after retrieval to compare PKMζ protein in the BLA. Both APV- and vehicle-infused rats showed similar freezing to the tone at the retrieval test (independent-samples *t* test, $t_{(18)} = 0.746$, $p = 0.465$; Fig. 2*A*). However, we found that rats infused with APV did not show reduced levels of PKMζ seen in control animals (independent-samples *t* test, $t_{(18)} = 2.358$, $p = 0.030$; Fig. 2*B*). This suggests PKMζ protein decreases in response to NMDAR-dependent memory destabilization.

Experiment 4: APV does not increase synaptic PKMζ

To ensure that the difference observed in Experiment 3 was driven by a reduction of PKMζ in vehicle-treated rats after retrieval, we tested whether this difference occurs in the absence of retrieval. Here we fear conditioned rats and infused either APV or vehicle in the BLA the next day. However, unlike in Experiment 3, rats did not undergo a retrieval test and were simply killed 90 min after the infusion (in keeping with the timing of Experiment 3). We found that rats infused with APV or vehicle showed similar expression of PKMζ protein (independent-samples *t* test, $t_{(15)} = 0.098$, $p = 0.923$; Fig. 2*C*), suggesting that APV does not increase synaptic PKMζ.

Experiment 5: Proteasome activation is necessary for post-retrieval decrease of PKMζ

Following NMDAR activation, there is a CaMKII-dependent increase in proteasome activity (Jarome et al., 2016). Protein degradation seems necessary for destabilization since administration of the proteasome inhibitor β-lac prevents the amnesic effect of anisomycin on reconsolidation (S. H. Lee et al., 2008). Considering the outcomes of Experiments 1 and 3 showing that

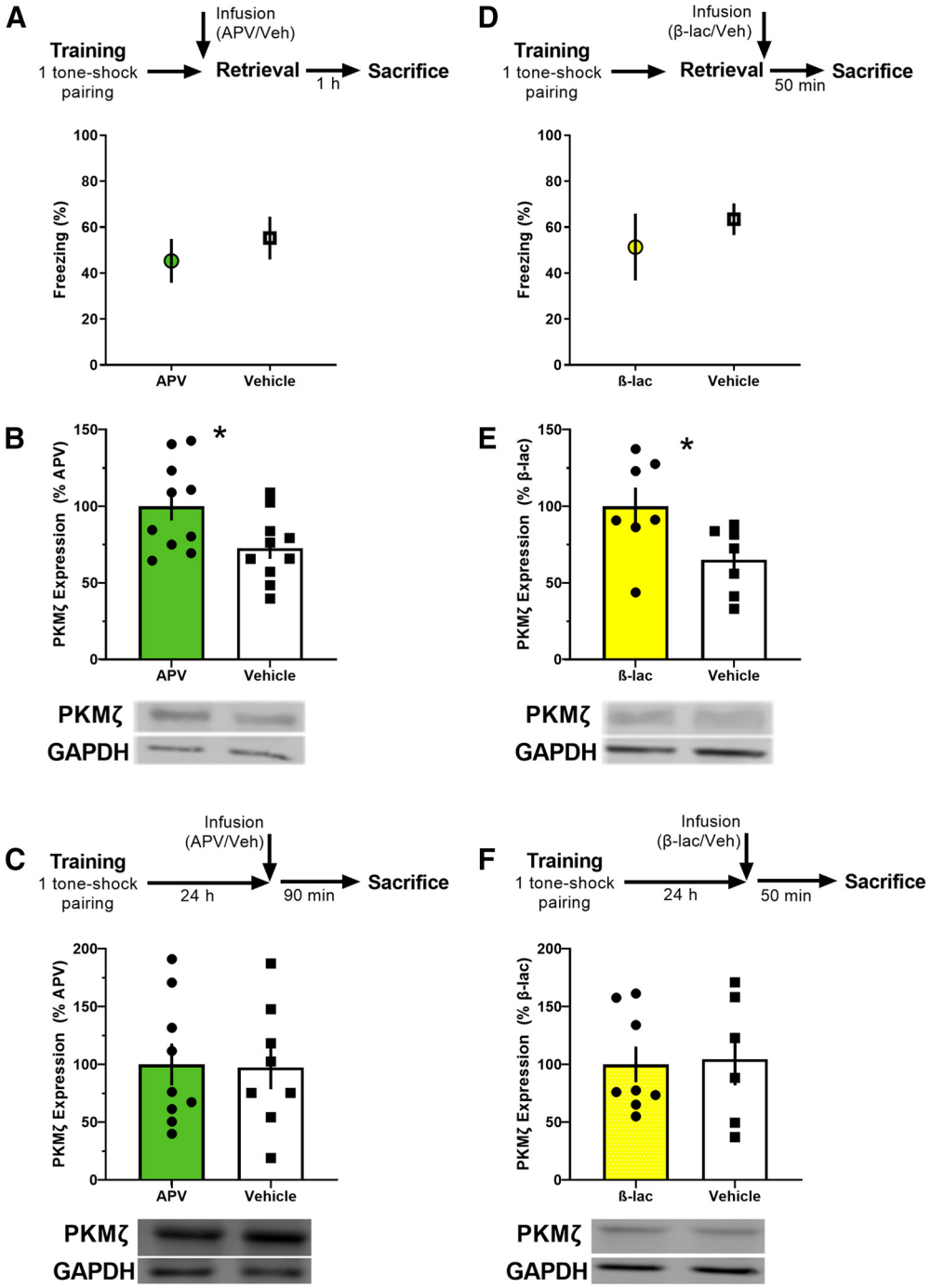

Figure 2. Post-retrieval reduction of PKMζ is destabilization-dependent. ***A***, Freezing behavior at retrieval test. Both APV- and vehicle-infused rats showed similar freezing behavior (independent-samples *t* test, $t_{(18)} = 0.746$, $p = 0.465$). ***B***, PKMζ protein levels from Western blots. Rats that received APV infusion before retrieval showed significantly higher PKMζ protein compared with rats that received vehicle (independent-samples *t* test, $t_{(18)} = 2.358$, $p = 0.030$). ***C***, PKMζ protein levels from Western blots. Rats that received APV infusion without retrieval showed similar PKMζ protein expression compared with vehicle-infused rats (independent-samples *t* test, $t_{(15)} = 0.098$, $p = 0.923$). ***D***, Freezing behavior at retrieval test. Both β-lac- and vehicle-infused rats showed similar freezing behavior (independent-samples *t* test, $t_{(12)} = 0.755$, $p = 0.465$). ***E***, PKMζ protein levels from Western blots. Rats that received β-lac infusion after retrieval showed significantly higher PKMζ protein compared with rats that received vehicle (independent-samples *t* test, $t_{(12)} = 2.377$, $p = 0.035$). ***F***, PKMζ protein levels from Western blots. Rats that received β-lac infusion without retrieval showed similar PKMζ protein expression compared with vehicle-infused rats (independent-samples *t* test, $t_{(12)} = -0.169$, $p = 0.869$). Data are mean ± SEM. *$p < 0.05$.

retrieval leads to a drop in expressed PKMζ, we tested whether this decrease depends on proteasome activity. We infused β-lac or vehicle into the BLA of rats immediately following the retrieval of conditioned auditory fear. We killed rats 1 h after retrieval and compared synaptosomal PKMζ protein using Western blotting. Both β-lac- and vehicle-infused groups showed similar freezing at the retrieval test (independent-samples *t* test, $t_{(12)} = 0.755$, $p = 0.465$; Fig. 2*D*). However, we found that rats infused with vehicle showed less PKMζ after retrieval than rats infused with β-lac (independent-samples *t* test, $t_{(12)} = 2.377$, $p = 0.035$; Fig. 2*E*). Therefore, it seems that protein degradation following retrieval leads to the reduction in PKMζ we previously observed.

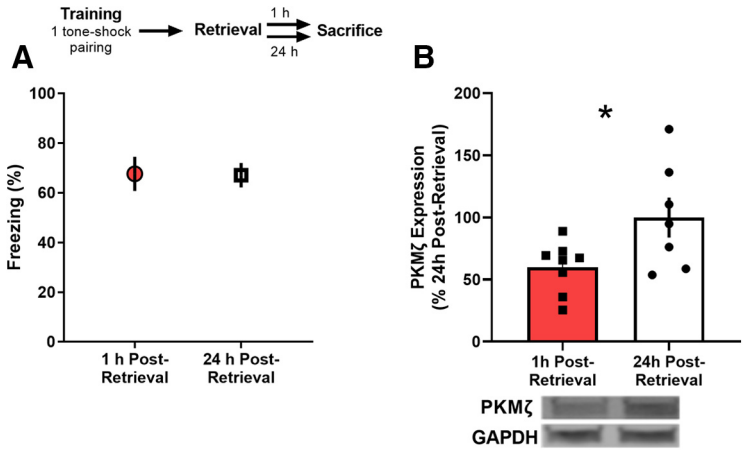

Figure 3. Increased expression of PKMζ following memory reconsolidation. ***A***, Freezing behavior from rats killed either 1 or 24 h after retrieval. Both groups showed similar freezing to the tone at the retrieval test (independent-samples *t* test, $t_{(13)} = -0.064$, $p = 0.950$). ***B***, PKMζ protein levels from Western blots. Rats killed 1 h after retrieval showed significantly lower PKMζ protein in BLA tissue compared with rats killed 24 h after retrieval (independent-samples *t* test, $t_{(13)} = 2.365$, $p = 0.034$). Data are mean ± SEM. *$p < 0.05$.

Experiment 6: β-lac does not increase synaptic PKMζ

In Experiment 4, we tested whether infusion of APV alone is sufficient to increase PKMζ in the absence of retrieval. Here we performed a similar experiment to test whether memory retrieval specifically drives the difference in β-lac- and vehicle-treated rats observed in Experiment 5 above. We fear conditioned rats and infused either β-lac or vehicle in the BLA the next day. However, unlike in Experiment 4, rats did not undergo a retrieval test and were simply killed 50 min after the infusion (in keeping with the timing of Experiment 5). We found that rats infused with β-lac or vehicle showed similar expression of PKMζ protein (independent-samples *t* test, $t_{(12)} = -0.169$, $p = 0.869$; Fig. 2*F*), suggesting that β-lac does not increase synaptic PKMζ.

Experiment 7: Reconsolidation increases synaptic PKMζ

Since destabilization reduces the availability of PKMζ at the synapse, we next tested whether reconsolidation involves a complementary increase in PKMζ protein. We re-exposed rats to one unpaired tone to retrieve the auditory fear conditioning memory and killed them either 1 h later (during destabilization) or 24 h later (following reconsolidation). Both groups showed similar freezing at the retrieval test (independent-samples *t* test, $t_{(13)} = -0.064$, $p = 0.950$; Fig. 3*A*). Western blotting showed that the quantity of PKMζ was higher in rats killed 24 h after retrieval compared with those killed 1 h after retrieval (independent-samples *t* test, $t_{(13)} = 2.365$, $p = 0.034$; Fig. 3*B*). This suggests that reconsolidation involves an increase of synaptic PKMζ to restabilize the memory.

Experiment 8: Synthesis of PKMζ is necessary for reconsolidation

Given that reconsolidation increases the availability of synaptic PKMζ, we then investigated whether synthesis of new PKMζ is necessary to stabilize the memory. Immediately following a retrieval test, rats were infused with PKMζ-antisense ODNs or a scrambled ODN sequence in the BLA. Rats were then tested 24 h later to determine whether PKMζ-antisense impaired reconsolidation and long-term memory retention. We found that rats' memory differed depending on whether they were infused with PKMζ-antisense or the scrambled control (one-way repeated-measures ANOVA, $F_{(1,18)} = 11.005$, $p = 0.004$; Fig. 4*A*). Specifically, rats receiving PKMζ-antisense showed impaired memory following infusion (Tukey's test, $p < 0.001$). However, rats receiving the scrambled control showed no difference in performance (Tukey's test, $p = 0.210$). Thus, these findings reveal that reconsolidation requires *de novo* synthesis of PKMζ.

Experiment 9: Acute inhibition of PKMζ synthesis does not impair stable memory

Next, we evaluated whether transiently blocking PKMζ synthesis can induce a memory impairment on its own, or whether this effect requires memory retrieval. To this end, rats received BLA infusions with either PKMζ-antisense or a scrambled ODN 24 h after a retrieval test. Thus, infusions occurred well after reconsolidation, which is believed to occur by 6 h after retrieval (Nader et al., 2000). Rats were tested again 24 h after infusion. We found that rats' freezing behavior did not differ as a result of which ODN infusion they received (one-way repeated-measures ANOVA, $F_{(1,14)} = 0.055$, $p = 0.818$; Fig. 4*B*). Neither group showed a memory impairment. Therefore, acute infusion of PKMζ-antisense after reconsolidation has already occurred does not disrupt long-term memory. That is, memories are vulnerable to PKMζ-antisense immediately, but not 24 h, after retrieval.

Experiment 10: Preventing destabilization protects memory from amnesic effect of PKMζ-antisense

Finally, we investigated whether it is specifically destabilization that renders memory vulnerable to PKMζ-antisense. That is, if we block NMDAR activation following retrieval, will PKMζ-antisense still impair retention of the retrieved memory? We infused rats before retrieval with either vehicle or APV to prevent memory destabilization. Immediately following retrieval, rats were then infused with either PKMζ-antisense or a scrambled control sequence and tested 24 h later. We found that which drug (APV or vehicle) rats received before retrieval determined whether the post-retrieval ODN infusion could impair memory at the final test (two-way repeated-measures ANOVA, $F_{(1,31)} = 6.65$, $p = 0.015$; Fig. 5). Rats infused with vehicle before retrieval followed by PKMζ-antisense showed impaired performance (Tukey's test, $t_{(31)} = 5.045$, $p < 0.001$) as described above. However, rats that received APV before retrieval followed by PKMζ-antisense did not show impaired memory (Tukey's test, $t_{(31)} = -0.5428$, $p = 0.999$). The scrambled control sequence did not affect memory in any group. This suggests that, absent destabilization, retrieved memories are not vulnerable to acute inhibition of PKMζ synthesis.

Discussion

Here we show dynamic expression of PKMζ during memory destabilization and reconsolidation. After retrieval, there is a decrease in the availability of PKMζ at the synapse. This reduction requires both the activation of NMDARs and protein degradation. Within 24 h after retrieval, PKMζ protein returns to the synapse. Indeed, *de novo* synthesis of PKMζ is necessary for reconsolidation; and without it, long-term memory is impaired. However, transient disruption of PKMζ synthesis outside of the

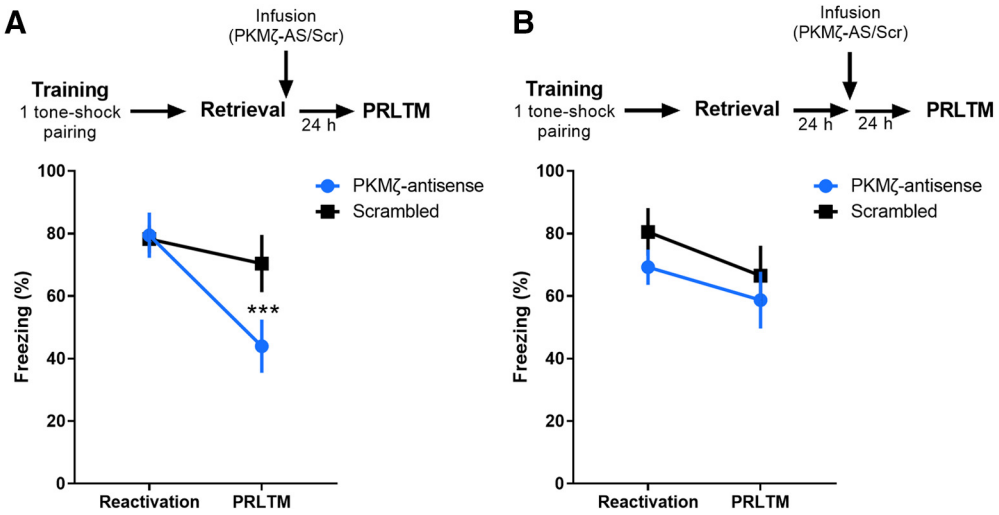

Figure 4. PKMζ-antisense disrupts memory reconsolidation. ***A***, Behavior data showed a significant effect of the infusion on performance (one-way repeated-measures ANOVA, $F_{(1,18)} = 11.005$, $p = 0.004$). Rats that received PKMζ-antisense showed a significant impairment at the PRLTM test 24 h after infusion (Tukey's test, $p < 0.001$), whereas rats that received the scrambled sequence showed no impairment (Tukey's test, $p = 0.210$). ***B***, Behavior data showed no effect of infusion on performance when the infusion occurred 24 h after retrieval (one-way repeated-measures ANOVA, $F_{(1,14)} = 0.055$, $p = 0.818$). Data are mean ± SEM. ***$p < 0.001$.

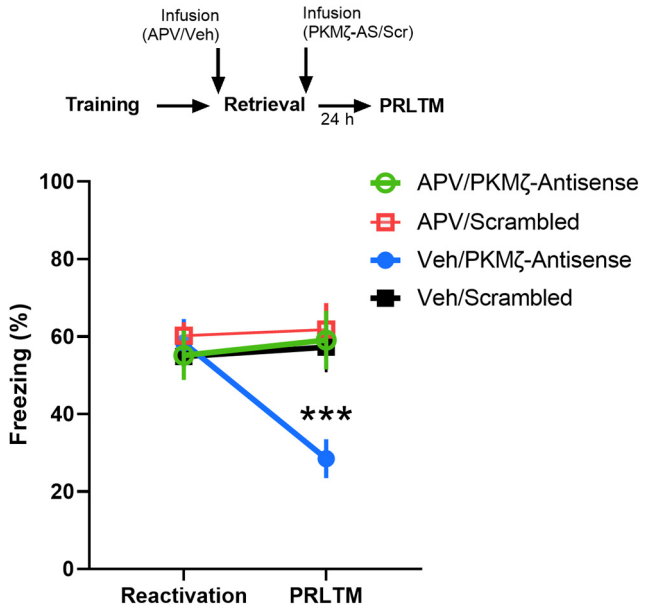

Figure 5. NMDAR activation renders memory vulnerable to impairment by PKMζ-antisense. Behavior data revealed an interaction of pre-retrieval drug and post-retrieval infusion on performance (two-way repeated-measures ANOVA, $F_{(1,31)} = 6.65$, $p = 0.015$). That is, PKMζ-antisense had an amnesic effect in vehicle-treated animals (Tukey's test, $t_{(31)} = 5.045$, $p < 0.001$) but not in APV-treated rats (Tukey's test, $t_{(31)} = -0.5428$, $p = 0.999$). Rats receiving the scrambled ODN sequence showed no impairment in performance. Data are mean ± SEM. ***$p < 0.001$.

lability window does not disrupt long-term memory. Indeed, without NMDAR activation, memories are not rendered labile after retrieval and acutely blocking translation of PKMζ does not impair memory.

While it is clear that reconsolidation requires synthesis of new proteins, which proteins in particular is mostly unknown. To date, synthesis of at least four proteins, zif268 (J. L. Lee et al., 2004; Barnes et al., 2012), Arc (Maddox and Schafe, 2011), C/EBPβ (Milekic et al., 2007), and C/EBPδ (Arguello et al., 2013), has been shown to be necessary for reconsolidation, each one important to gene expression in general. Given the importance of gene expression to reconsolidation, it should not be surprising that these proteins must be translated for it to proceed. Which other molecules, with roles beyond gene expression, are also central to reconsolidation has so far been unknown. Here we identified that reconsolidation requires PKMζ, a molecule that seems to function at the synapse to maintain GluA2-AMPARs. Thus, our work provides new insights into the reconsolidation process by demonstrating a specific synaptic protein that must be translated to restabilize a memory.

In recent years, the importance of PKMζ to memory maintenance has been debated since PKMζ-null mice show intact learning and memory and are vulnerable to memory impairment by ζ-inhibitory peptide (ZIP). ZIP has traditionally been used to disrupt PKMζ catalytic activity (Ling et al., 2002; Serrano et al., 2008; Kwapis et al., 2009; Migues et al., 2010), although it is also capable of inhibiting the activity of another atypical PKC isoform, PKCι/λ (Ren et al., 2013; Bogard and Tavalin, 2015). However, studies using more specific techniques have supported the role of PKMζ in memory. Overexpression of PKMζ enhances memory (Shema et al., 2011; Xue et al., 2015). On the other hand, expression of a dominant negative PKMζ 1 week after training impairs conditioned taste aversion memory (Shema et al., 2011). Administration of PKMζ-antisense has been shown to disrupt long-term memory *in vivo* (Hsieh et al., 2017) and late LTP *in vitro* (Tsokas et al., 2016). Further, recent work showed that PKMζ-KO mice may indeed compensate using another atypical PKC isoform, PKCι/λ, to maintain long-term memory in the absence of PKMζ (Tsokas et al., 2016). Nonetheless, to avoid the identified complications of ZIP, we limited our tools to antisense ODNs which offer sequence specificity and Western blotting. Thus, we feel confident that our results reflect the dynamics of PKMζ and not another kinase.

Interestingly, while inhibition of PKMζ kinase activity impairs memory (Shema et al., 2007; Hardt et al., 2010; Gámiz and Gallo, 2011), PKMζ-antisense did not impair memory when infused 24 h after retrieval (after reconsolidation) or in the presence of APV, an NMDAR inhibitor that prevents memory destabilization. Precisely how long the PKMζ-antisense remains active in the brain is not yet clear, although similar ODNs persist at least 90 min and are cleared by 24 h after infusion (J. L. Lee et al.,

2004). That one infusion of PKMζ-antisense does not impair memory maintenance may reflect an oversaturation of the protein at the synapse, beyond what is required to maintain the memory. It may also indicate a long half-life for PKMζ, allowing existing protein to overcome a temporary reduction in new translation. While the half-life of PKMζ is not known, it has been suggested to be at least several hours (Osten et al., 1996; Sacktor, 2011).

Our results support several important findings in the literature. First, our work builds on data demonstrating a reorganization of AMPARs during memory destabilization and reconsolidation. During destabilization, GluA2-AMPARs are internalized (Hong et al., 2013; Haubrich et al., 2020b) at the same time that we observed reductions in PKMζ expression. Further, the return of these AMPARs (Hong et al., 2013) coincides with the increase in PKMζ we observed here.

Second, our work also supports the finding that infusing ZIP 1 h after retrieval does not impair reconsolidation and long-term memory (Levitan et al., 2016). We show that PKMζ expression is significantly reduced at this time point, leaving ZIP with less protein to target. Furthermore, the need to synthesize new protein during reconsolidation suggests that any PKMζ inhibited by ZIP will soon be compensated for by new protein.

While our work does not directly test the nature of amnesia caused by reconsolidation blockade, it may provide insight to resolve this question. Previous studies indicate that disrupting reconsolidation or inhibiting PKMζ activity impairs subsequent memory performance, which remains impaired for at least several weeks (Duvarci and Nader, 2004; Shema et al., 2007). These reports suggest that such interventions lead to a permanent storage impairment of memory, rather than a temporary retrieval impairment (for review, see Haubrich et al., 2020a). Since our work impaired reconsolidation by blocking synthesis of new PKMζ in particular, we believe our interventions likely led to a storage impairment. However, we did not test memory beyond 1 d after retrieval, leaving open the possibility that performance could be restored later on, which would imply a retrieval impairment.

One limitation of this work is the uncertain fate of PKMζ after reactivation. That PKMζ protein is reduced in the synaptosome could mean that it is being trafficked elsewhere or perhaps degraded. One report showed that PKMζ can be degraded by the proteasome (Vogt-Eisele et al., 2014). Indeed, our results show that proteasome activation is necessary to decrease PKMζ since infusion of the proteasome inhibitor β-lac prevents this reduction. Given these data, it seems likely that destabilization involves proteasome-dependent degradation of PKMζ. However, we cannot rule out the possibility that the loss of PKMζ is because of some other mechanism downstream of the degradation pathway.

To clarify this question, future research should study the role of KIBRA in memory destabilization. This protein binds to PKMζ and prevents its degradation (Vogt-Eisele et al., 2014). It may be that reactivation triggers a decoupling of KIBRA and PKMζ, leading to its degradation during memory destabilization. Whether such decoupling does occur and which upstream mechanism is responsible will be crucial to understand how destabilization occurs at the synapse.

What triggers the *de novo* synthesis of PKMζ during reconsolidation also remains an open question. Following tetanization in hippocampal slices, synthesis of PKMζ requires activation of proteins, such as CaMKII, MAPK, PKA, and mTOR (Kelly et al., 2007). Each of these molecules is also required for reconsolidation (Duvarci et al., 2005; Tronson et al., 2006; Jobim et al., 2012; Jarome et al., 2016), suggesting that synthesis of PKMζ involves a similar mechanism in both consolidation and reconsolidation.

Together, this work shows that memory destabilization and reconsolidation involve the disruption and recapitulation of a crucial memory maintenance process. That is, plasticity occurs at the expense of PKMζ and restoring stability requires the production of new PKMζ. In the future, other critical proteins, both to induce lability and to restabilize synapses, will need to be identified to gain a full understanding of memory plasticity.

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