

Overexpression of Protein Kinase M ζ in the Hippocampus Enhances Long-Term Potentiation and Long-Term Contextual But Not Cued Fear Memory in Rats

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The persistently active protein kinase M ζ (PKM ζ) has been found to be involved in the formation and maintenance of long-term memory. Most of the studies investigating PKM ζ , however, have used either putatively unselective inhibitors or conventional knock-out animal models in which compensatory mechanisms may occur. Here, we overexpressed an active form of PKM ζ in rat hippocampus, a structure highly involved in memory formation, and embedded in several neural networks. We investigated PKM ζ 's influence on synaptic plasticity using electrophysiological recordings of basal transmission, paired pulse facilitation, and LTP and combined this with behavioral cognitive experiments addressing formation and retention of both contextual memory during aversive conditioning and spatial memory during spontaneous exploration. We demonstrate that hippocampal slices overexpressing PKM ζ show enhanced basal transmission, suggesting a potential role of PKM ζ in postsynaptic AMPAR trafficking. Moreover, the PKM ζ -overexpressing slices augmented LTP and this effect was not abolished by protein-synthesis blockers, indicating that PKM ζ induces enhanced LTP formation in a protein-synthesis-independent manner. In addition, we found selectively enhanced long-term memory for contextual but not cued fear memory, underlining the theory of the hippocampus' involvement in the contextual aspect of aversive reinforced tasks. Memory for spatial orientation during spontaneous exploration remained unaltered, suggesting that PKM ζ may not affect the neural circuits underlying spontaneous tasks that are different from aversive tasks. In this study, using an overexpression strategy as opposed to an inhibitor-based approach, we demonstrate an important modulatory role of PKM ζ in synaptic plasticity and selective memory processing.

Key words: contextual fear; hippocampus; LTP; memory; overexpression; PKM ζ

Significance Statement

Most of the literature investigating protein kinase M ζ (PKM ζ) used inhibitors with selectivity that has been called into question or conventional knock-out animal models in which compensatory mechanisms may occur. To avoid these issues, some studies have been done using viral overexpression of PKM ζ in different brain structures to show cognitive enhancement. However, electrophysiological experiments were exclusively done in knock-out models or inhibitory studies to show depletion of LTP. There was no study showing the effect of PKM ζ overexpression in the hippocampus on behavior and LTP experiments. To our knowledge, this is the first study to combine these aspects with the result of enhanced memory for contextual fear memory and to show enhanced LTP in hippocampal slices overexpressing PKM ζ .

Introduction

Protein kinase M ζ (PKM ζ) is an atypical PKC isoform and attracted attention when Todd Sacktor's group described an increase of postsynaptic EPSCs using whole-cell recordings from CA1 pyramidal cells perfused with PKM ζ (Ling et al., 2002).

PKM ζ is a brain-specific kinase highly expressed in neocortex and hippocampus and independently transcribed from the *PRKCZ* gene by its own internal promoter (Naik et al., 2000; Hernandez et al., 2003; Oster et al., 2004). Interestingly, it lacks the PKC ζ autoinhibitory regulatory domain and thus is thought

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to be persistently active once activated by phosphoinositide-dependent protein kinase 1 (Kelly et al., 2007). Expression of PKM ζ is increased postsynaptically after electrophysiological stimulation of hippocampal slices (Kelly et al., 2007) or stimulation of cortical primary neurons with mGluR1 agonist (Eom et al., 2014). Many studies have been performed both *in vivo* and *in vitro* to link PKM ζ with the maintenance phase of LTP (Ling et al., 2006; Kelly et al., 2007) and its ability to modulate the storage of episodic memories using aversive reinforced experiments (Pastalkova et al., 2006; Madroñal et al., 2010; Dong et al., 2015). However, most of this evidence was obtained using the inhibitors chelerythrine or myristoylated ζ inhibitory pseudosubstrate (ZIP), the selectivity of which has been questioned regarding additional kinases important for LTP, such as CaMKII and other isoforms of PKC (Ling et al., 2002; Lee et al., 2013; Ren et al., 2013). Moreover, a recent study found ZIP to be excitotoxic in hippocampal primary neurons (Sadeh et al., 2015). In fact, ZIP reversed established LTP in mice lacking PKM ζ , further underscoring selectivity concerns (Volk et al., 2013). In addition, LTP could be established in these KO mice, raising questions about the integral role of PKM ζ in the maintenance of LTP, suggesting that it rather plays a modulatory role to augment LTP.

The use of unselective inhibitors complicates assignment of a specific protein function to observed results. For example, two studies investigated the performance of spontaneous exploration in the object location task (OLT) after stereotactic injection of ZIP into the dorsal hippocampus of rats. Both studies showed complete loss of memory to object location, suggesting that the neural networks underlying OLT are influenced by PKM ζ (Hardt et al., 2010; Miguez et al., 2010). However, due to the unselective nature of ZIP, it is unclear whether PKM ζ inhibition was responsible for these deficits.

In general, even if selective inhibition of PKM ζ leads to memory deficits, it is unclear whether overexpression in the same structure necessarily shows memory enhancement. To explore the consequences of PKM ζ overexpression, studies exploring increased levels of PKM ζ in the insular cortex or mPFC of rats have been performed. These studies provided evidence that overexpression of PKM ζ enhances memory in aversive behavioral tasks such as conditioned taste aversion or conditioned cued response (Shema et al., 2011; Xue et al., 2015). To address the mechanism of this modulatory effect, experiments using shRNA knock-down linked PKM ζ to synaptic plasticity and memory and suggested a role in AMPA receptor trafficking (Hara et al., 2012; Ron et al., 2012; Dong et al., 2015).

To avoid off-target inhibition and to explore the consequences of overexpression, we conducted experiments using adeno-associated virus (AAV) to bilaterally overexpress PKM ζ -WT or a kinase-dead (KD) mutant in the hippocampus of rats. Our goal was to combine electrophysiological approaches to characterize the influence of PKM ζ on synaptic plasticity with behavioral experiments for both contextual and spatial memory. To address these two types of memory, we used reinforced cued and contextual fear conditioning (CFC) and spontaneous exploration of relocated objects in the OLT, both of which are known to be hippocampus dependent. Through this combination of approaches, we demonstrate enhanced basal transmission and LTP and memory for CFC in rats overexpressing PKM ζ -WT. The enhancement, as opposed to occlusion, of LTP suggests a modulatory, but not integral, role of PKM ζ in synaptic plasticity and memory retention.

Materials and Methods

Animals

All animal procedures were performed according to the institutional and European Union guideline (Directive 2010/63/EU) and were approved by the Ethical Committee of the responsible regional council (Tübingen, Germany).

Adult male Wistar rats of 250–300 g were obtained from Janvier Labs. The animals were housed 4 per cage with *ad libitum* access to food and water, living under a 12/12 h light/dark cycle (lights on at 6:00 A.M.). All experiments were conducted during the light phase of the cycle.

AAV

Molecular cloning of rAAV constructs. The CMV promoter from the pAAV-MCS plasmid (Agilent Technologies, catalog #240071) was replaced by a human synapsin promoter to have neuronal specific transgene expression. Therefore a synthetic piece of DNA harboring the human synapsin promoter followed by a KpnI and HindIII restriction site and the human growth hormone poly adenylation signal was cloned via the NotI restriction sites into pAAV-MCS and termed pAAV_hSyn. The two expression cassettes consisting of a Kozak sequence followed by tdTomato, a 2A peptide from *Thosea signa* virus, either rat PKM ζ wild-type or rat PKM ζ K98W KD (groups receiving this construct will be referred to as “PKM ζ -WT” or “PKM ζ -KD,” respectively, in the following) and finally a WPRE were cloned into the pAAV-hSyn via the KpnI and HindIII restriction sites. These plasmids, which were used for production of rAAV5, were termed pAAV_hSyn-tdTomato-2A-ratPKM ζ and pAAV_hSyn-tdTomato-2A-ratPKM ζ K98W, respectively.

Packaging/helper plasmid used for rAAV production. A commercially available packaging plasmid consisting of AAV2 rep, AAV5 cap, and Ad5 helper functions (plasmidFactory, catalog #pDP5rs) was used for packaging the rAAV constructs containing AAV2-ITRs into an AAV5 capsid.

Production and purification of rAAV5 vectors. The rAAV5 vectors were produced as described previously (Aschauer et al., 2013).

Stereotactic surgery

Rats were deeply anesthetized with a ketamine/xylazine mixture of 70/6 mg/kg intraperitoneally and fixed in a stereotactic frame (David Kopf Instruments) in flat skull position. During surgery, the animals were administered 0.5% isoflurane in air (3 L/min) provided by an inhalation mask. After 60 min of surgery, the rats received an additional treatment of ketamine/xylazine mixture of 35/3 mg/kg intramuscularly. Before the skin was opened, lidocaine was administered subcutaneously. Five small holes were drilled bilaterally into the skull and 200 nl of AAV (6.7×10^{12} viral genome/ml PBS) or PBS was injected at the following coordinates (relative to bregma in millimeters): (1) AP -2.0 , ML ± 1.6 , DV -3.6 ; (2) AP -4.2 , ML ± 2.6 , DV -3.6 ; (3) AP -4.8 , ML ± 4.8 , DV -7.0 ; (4) AP -5.3 , ML ± 4.6 , DV -5.2 ; (5) AP -5.3 , ML ± 4.6 , DV -7.0 ; and (6) AP -5.8 , ML ± 4.6 , DV -5.2 with a flow rate of 2.3 nl/s. Glass pipettes were mounted into the Nanoliter 2010 injector that was injection controlled by SYS-Micro4-controller (World Precision Instruments). The glass pipette was held in position for additional 120 s after the injection to allow diffusion. To avoid possible tissue damage by overloading one hemisphere with six consecutive injections, we alternated between the hemispheres after each infusion until all 12 injections were done. The rats experienced daily monitoring after surgery until the behavioral tests began.

Behavioral tests

CFC and cued fear conditioning. Three weeks after AAV injection, the animals were handled and habituated to the experimenter 2 d before the experiment started. The rats were then placed into a sound-attenuated chamber with a grid floor (Med Associates) for 9 min in total with house lights on and white background noise (100 Hz, 65 db) provided by the built-in fan. After 5 min of habituation, they received 3 consecutive tone stimuli for 15 s (1000 Hz, 80 db, 50 ms rising time), followed by a mild foot shock of 0.3 mA for 2 s after a delay of 2 s. The intervals between the first and second tone and between second and third tone were 124 and 160 s, respectively. The boxes were cleaned with 70% ethanol. One week later, the animals were tested for contextual fear retention memory in the

boxes with same interior and odor but without the presentation of the tone stimuli. The animals were placed in the boxes for 5 min and freezing behavior was recorded and analyzed automatically during this period. One day later, the animals were tested for cued fear retention memory in the boxes with different interior and odor (1% acetic acid) but with the presentation of the tone. The rats were placed in the boxes for 8 min. After 2 min, the presentation of the tone were initiated for 6 min consecutively and freezing behavior was recorded and analyzed automatically during this period. Data are shown as mean \pm SEM of the defensive response (% of total time). For multiple comparisons, statistical significance was assessed by one- or two-way ANOVA with Bonferroni's *post hoc* test. Values of $p < 0.05$ were considered to reflect statistically significant differences.

Object location task. Four weeks after AAV injection, the animals were handled by the experimenter for 2 d before the experiment started. On days 3 and 4, the rats were placed into an empty circular arena of 85 cm diameter for 15 min to habituate with the arena. Outside of the arena, different landmarks were placed to allow allocentric orientation. Between each appearance of an animal in the arena, the whole arena was wiped clean with 70% ethanol to destroy all olfactory traces and to prevent the animals from orientating olfactorily. The OLT experiment took place on day 5. Rats were placed into the empty arena for 10 min. Afterward, two identical objects (white pyramids) were presented in the arena for 5 min (T1). After an intertrial interval (ITI) of 30 or 45 min, the animals again explored the arena for 5 min with 1 of the 2 objects placed at another location (T2). On day 8, the rats again underwent the habituation, T1, and T2 procedures but with an ITI of 8 min to ensure the discrimination as a positive control. Exploration of both objects was measured at T1 and T2 and the location index for T2 was calculated using the following:

$$\text{Location Index} = \frac{T2_{\text{sec reloc}} \times 100}{T2_{\text{sec reloc}} + T2_{\text{sec unmov}}}$$

Where *reloc* is the relocated object and *unmov* the unmoved one. Data are shown as mean \pm SEM of the location index. For multiple comparisons, statistical significance was assessed by one- or two-way ANOVA with the Bonferroni's *post hoc* test. Values of $p < 0.05$ were considered to reflect statistically significant differences.

Electrophysiology

Preparation of brain slices. The animals were anesthetized with isoflurane and, after decapitation, the brains were quickly removed and immersed in ice-cold (4°C) sucrose-containing cutting ACSF containing the following (in mM): 185 sucrose, 2.45 KCl, 8.8 MgSO₄, 1.2 KH₂PO₄, 25.6 NaHCO₃, and 10 D-glucose, pH 7.4, saturated with 95% O₂ and 5% CO₂. Transversal hippocampal slices of 400 μ m were cut using a Vibratome. The slices were left to rest at room temperature (22–24°C) for at least 90 min before recording in a holding chamber containing the same ACSF but with 2.25 mM CaCl₂ and sucrose replaced by 124 mM NaCl.

Electrophysiological measurements. The slices were transferred to integrated brain slice chambers (Kroger et al., 2011b) and continuously superfused (2.5 ml/min, room temperature) with the same ACSF used for the recovering phase except for 1.2 mM MgSO₄ (regular ACSF). Field EPSPs (fEPSPs) were recorded in the stratum radiatum of area CA1 in response to Schaffer collateral pathway stimulation by a monopolar glass electrode. The stimulation and field recording electrodes (2–6 M Ω) were filled with regular ACSF. The slopes of the fEPSPs were used as parameter of interest and were determined by linear regression over the maximum initial slope points. At the beginning of each experiment, an input–output curve was obtained using a procedure similar to that described previously (Taqatqeh et al., 2009). Briefly, with a stimulus duration of 200 μ s, the intensity was adjusted to evoke a maximal fEPSP response. Once this intensity was achieved, it was held constant while the duration of the stimulation was changed from 20 to 200 μ s in increasing 20 μ s steps every 30 s. Because basal transmission was enhanced in the PKM ζ -overexpressing slices, all subsequent experiments [paired-pulse facilitation (PPF) and LTP] were performed using a stimulus intensity that evoked 30% of the maximal fEPSP response that was determined for each

individual slice, thereby normalizing the slices from the different groups to each other. PPF was studied by the application of pairs of stimuli (50 ms interstimulus interval) every 30 s. The PPF ratio was calculated by dividing the slope of the second synaptic response by the slope of the first response. LTP was induced by repeated HFS consisting of 100 pulses at the frequency of 100 Hz applied 4 times in 5 min intervals (Lu et al., 1999; Kroger et al., 2011b) unless otherwise stated. Changes in the fEPSP slope were calculated in relation to the baseline fEPSP responses during the last 10 min before drug administration (100%). Long-term synaptic changes were evaluated by comparing the last 10 min of recording 1, 2, and 3 h after HFS. A modular electrophysiology system, supplied by npi electronic, conducted the low-noise recordings of extracellular signals. Signals were 1000 \times amplified and subsequently filtered with a low-pass (5 kHz) and a high-pass (3 Hz) filter. For data acquisition and analysis, the software NOTOCORD-hem was used. Data are shown as mean \pm SEM of the PPF ratio or the percentage to the baseline fEPSP slope. In each experiment, *N* represents the number of animals whereas *n* represents the number of slices. The significance of the differences between the mean values obtained in two different conditions was evaluated by the paired Student's *t* test. For multiple comparisons, statistical significance was assessed by either one- or two-way ANOVA with the Tukey's *post hoc* test. Values of $p < 0.05$ were considered to reflect statistically significant differences.

Drug application to slices. (2R,3S,4S)-2-(4-Methoxybenzyl)-3,4-pyrrolidinediol-3-acetate (anisomycin), 3-[2-(3,5-Dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]glutarimide (cycloheximide, CHX), and 6-Chloro-3,4-dihydro-3-(2-norbornen-5-yl)-2H-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide (cyclothiazide, CTZ) were obtained from Sigma-Aldrich. All drugs were initially dissolved in a small amount of dimethyl sulfoxide (DMSO) and diluted further by regular ACSF to a final DMSO concentration of 0.05%.

Histology

Rats were anesthetized with ketamine/xylazine (140/12 mg/kg) and subjected to perfusion–fixation via cardiac puncture with PBS followed by 4% paraformaldehyde in PBS solution. Afterward, the brains were continuously fixed in the fixative for 48 h and frozen sectioned in 25 μ m coronal slices. Each slice was dried in a 12-well plate with high performance German cover glass (In Vitro Scientific) and finally covered with Fluoroshield histology mounting medium (with DAPI; Sigma-Aldrich). Images were acquired on a PerkinElmer OperaQEHs system at a 20 \times magnification, with 1404 fields/well. The images were transferred to PerkinElmer's Columbus image storage and analysis system. The fields were stitched in Columbus using a magnification correction of 0.95. Images show cell nuclei in the 450 nm and tdTomato Red in the 650 nm fluorescence channel, respectively.

Immunoblotting

Rats were anesthetized with 3% isoflurane in air and immediately decapitated using a guillotine. Brains were removed and further processed on ice on a filter paper soaked with PBS. Both hippocampi were removed, weighed, and shock frozen in nitrogen until further processing. The hippocampi were thawed in 500 μ l of cell lysis buffer (Cell Signaling Technology) with additional 0.1% SDS, and protease and phosphatase inhibitor compounds (cOmplete mini EDTA-free tablet; Roche Diagnostics; Phosphatase Inhibitor Cocktail 2 and 3 and 1 mM PMSF (Sigma-Aldrich) and immediately homogenized by sonification for 9 bursts at 10% intensity. Homogenates were left on ice for 15 min, stirred, and then centrifuged at 14,000 \times g for 10 min to remove nuclei and cell debris. The supernatant was used for blotting experiments. Total protein concentration was measured using the Pierce BCA protein assay kit and protein content was balanced by diluting high content samples with lysis buffer. Gel electrophoresis was performed using NuPAGE 4–12% Bis-Tris Gel and MOPS SDS Running buffer (Thermo Fisher Scientific), followed by blotting on Immobilon-FL membrane (Millipore) with NuPAGE Transfer buffer (Thermo Fisher Scientific). Immunostaining was performed using antibodies against catalytic domain of PKC ζ (Santa Cruz Biotechnology) and β -actin (Sigma-Aldrich), followed by IRDye secondary antibodies, and visualized with the Odyssey Imaging system and analyzed with Image Studio Software (LI-COR Biosciences).

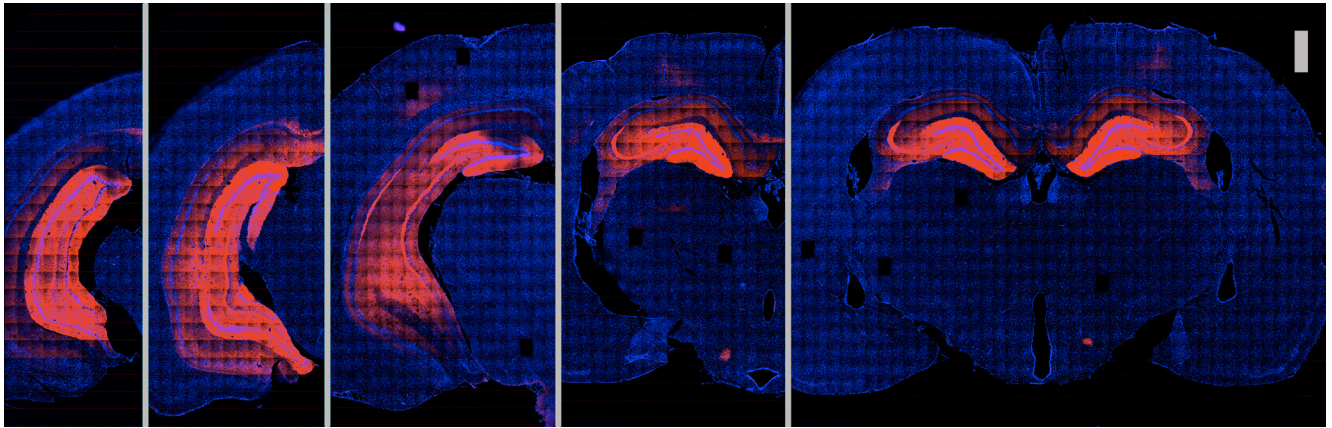


Figure 1. PKM ζ -WT-coexpressed tdTomato fluorescent protein is expressed selectively in the hippocampus of rats. Representative images of rat's brain slices from (left) posterior to (right) anterior are shown. Cell nuclei are represented by blue staining, tdTomato fluorescent protein is represented by red signal. Scale bar, 1 mm.

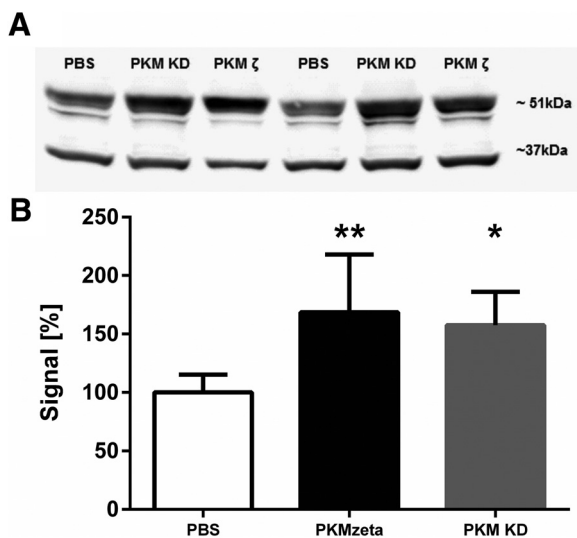


Figure 2. Quantitative analysis of protein expression in hippocampal tissue 5 weeks after AAV-mediated PKM ζ -WT and PKM KD overexpression in rats. **A**, Representative Western blot image. PKM ζ is blotted at 51 kDa and β -actin at 37 kDa was loaded as a normalizing protein. Hippocampal PKM ζ -WT and PKM ζ -KD are 1.5-fold increased compared with PBS-injected animals. **B**, Plot of six to seven hippocampi per group, normalized to endogenous PKM ζ level in PBS-treated rats. **/ $p < 0.05/0.01$ compared with PBS.

Results

Imaging and immunoblotting

To investigate the extent and selectivity of viral vector overexpression in hippocampus, we first inspected coronal slices of the rat's brain and measured the signal of tdTomato red fluorescence protein throughout the whole slice. Figure 1 shows representative coronal images suggesting a viral expression that extends throughout but is limited to the hippocampus. To quantify the level of exogenous PKM ζ -WT or PKM ζ -KD compared with endogenous PKM ζ of PBS-injected rats, we lysed hippocampi and performed immunoblots using antibodies against the catalytic domain of PKC ζ and against β -actin. Figure 2A shows immunoblots of the hippocampi of the three groups in a representative manner. A plot of all experiments is shown in Figure 2B, demonstrating an increase of PKM ζ levels to ~150% of endogenous level in the PBS group (one-way ANOVA $F_{(2,17)} = 7.937$, $n = 6-7$, $p = 0.0037$, followed by Bonferroni's *post hoc* test).

Basal transmission and short-term plasticity properties of PKM ζ -overexpressing hippocampal slices

We compared input–output relationships of extracellular recorded fEPSPs in hippocampal slices from the different groups of animals. Although both control rats (PBS and PKM ζ -KD) showed comparable input–output curves upon Schaffer-collateral stimulation, fEPSP evoked in slices obtained from PKM ζ -WT showed a significant increase in basal fEPSP responses, with stimulation durations of 50–200 μ s (Fig. 3; two-way ANOVA, $F_{(2,250)} = 88.79$, $n = 8-12$, $p < 0.0001$, followed by Tukey's *post hoc* test). Importantly, the stimulation strength giving 30% of the maximal response did not differ significantly among the different groups (one-way ANOVA $F_{(2,41)} = 2.56$, $n = 12-16$, $p = 0.0900$) and this stimulation was exclusively used for the following PPF and LTP experiments unless otherwise stated.

To elucidate whether a presynaptic mechanism could account for the enhanced basal transmission of the PKM ζ -WT slices, PPF was studied by the application of pairs of stimulations with 50 ms interstimulus intervals. Under our standard conditions of stimulation (30% of maximal response), we found no significant differences in the PPF ratio obtained in the different groups (Fig. 4; PBS, 1.21 ± 0.04 ; PKM ζ -KD, 1.18 ± 0.04 ; PKM ζ -WT, 1.22 ± 0.04 , one-way ANOVA $F_{(2,12)} = 0.38$, $n = 5$, $p = 0.6892$).

Slices from PKM ζ -WT-overexpressing rats show a protein-synthesis-independent LTP enhancement

To study long-term plasticity, we attempted to induce LTP in the Schaffer collateral to CA1 pathway in hippocampal slices from our different groups of rats. After 1 h of baseline recordings to verify stability, 4 trains of high-frequency stimulation (100 Hz, 1 s) were applied to potentiate synaptic transmission. Although LTP could be induced reliably in all the groups tested, its duration was different among groups. Therefore, in slices from PBS rats, this potentiation lasted for ~2 h and fEPSP responses returned to baseline levels 3 h after HFS (LTP 1 h post-HFS, $126.4 \pm 4.8\%$; LTP 3 h post-HFS, $107.9 \pm 5.0\%$; $n = 6$). Similarly, hippocampal slices from KD rats showed consistent potentiation 1 h after HFS, which dropped to baseline levels 3 h after HFS (LTP 1 h post-HFS, $126.4 \pm 3.9\%$; LTP 3 h post-HFS, $101.5 \pm 3.2\%$; $n = 5$). On the contrary, hippocampal slices from PKM ζ -WT rats still showed LTP 3 h after HFS (LTP 1 h post-HFS, $143.7 \pm 8.6\%$; LTP 3 h post-HFS, $121.6 \pm 5.3\%$; $n = 4$). This increase in LTP was significantly different from the PBS and PKM ζ -KD groups 2 h

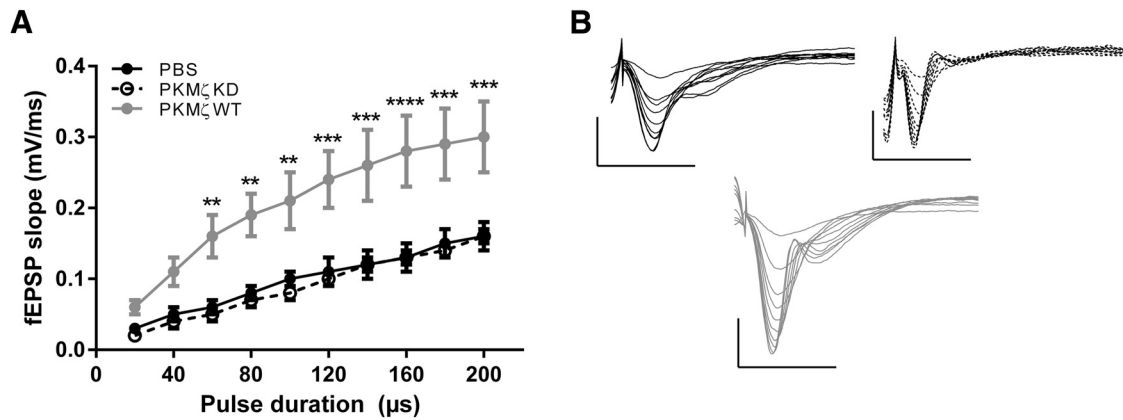


Figure 3. PKM ζ overexpression induces an increase of basal synaptic transmission compared with the PBS and KD groups. **A**, Basal transmission was measured by giving pulses of increasing duration at the stimulation intensity necessary to induce a maximal fEPSP response at 200 μ s in hippocampal slices from the different groups of rats. $^{***}/^{****}$ $p < 0.01/0.001$ compared with PBS and PKM ζ -KD. Group sizes: PBS and PKM ζ -WT, $N = 3$, $n = 8$; PKM ζ -KD, $N = 3$, $n = 12$. **B**, Example of traces elicited in each animal type for increasing pulse durations (20–200 μ s) (calibration: horizontal, 20 ms; vertical, 0.5 mV).

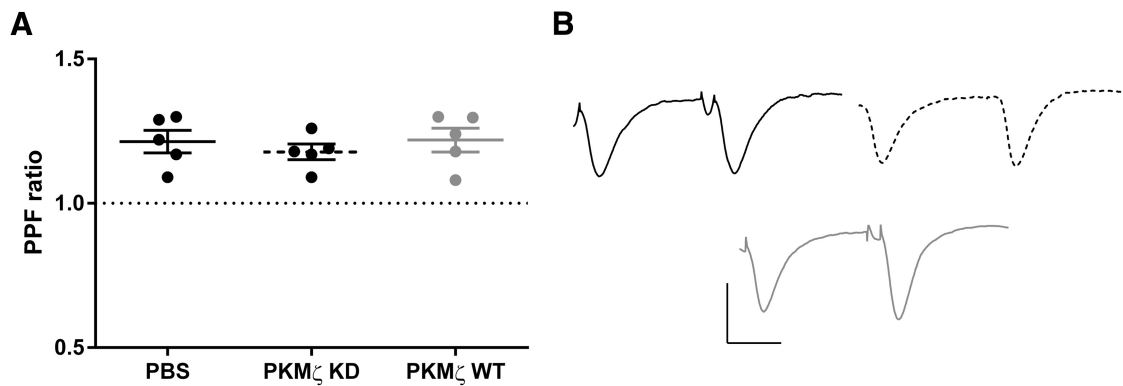


Figure 4. PKM ζ overexpression does not affect PPF. **A**, The PPF ratio values for each animal group were comparable, indicating that presynaptic function is not affected by PKM ζ overexpression. Group sizes: PBS and PKM ζ -KD, $N = 2$, $n = 5$; PKM ζ -WT, $N = 3$, $n = 5$. **B**, Example of paired pulses induced in the different animal types (calibration: horizontal, 20 ms; vertical, 0.3 mV).

after HFS (Fig. 5A; LTP 2 h post-HFS: PBS, $109.7 \pm 5.5\%$; PKM ζ -WT, $130.4 \pm 7.8\%$; PKM ζ -KD, $108.7 \pm 4.9\%$; two-way ANOVA $F_{(2,48)} = 12.10$, $n = 4-6$, $p < 0.0001$, followed by Tukey's *post hoc* test).

Late LTP is considered to hold two main criteria: potentiation lasting longer than 3 h and protein synthesis dependency (Lu et al., 1999). Having in mind that synaptic transmission was still potentiated 3 h after HFS in the PKM ζ -WT group, we wanted to confirm the second of these criteria by the acute application of the protein synthesis inhibitor anisomycin in the PKM ζ -WT slices. The slices were preincubated in anisomycin during the resting period for at least 2 h and again 30 min before and after HFS. Intriguingly, LTP was not changed upon treatment with 30 μ M anisomycin, a concentration shown previously to be sufficient to abolish protein-synthesis-dependent LTP (Frey et al., 1988; Fonseca et al., 2006; Kroker et al., 2011a). New controls in the presence of the same amount of DMSO (0.05%) used with anisomycin were performed simultaneously, showing no differences with respect to the earlier experiments; for this reason, this DMSO data were pooled together with the previous data (Fig. 5B; LTP 3 h post-HFS: DMSO 0.05%, $119.8 \pm 3.7\%$; 30 μ M anisomycin, $123.5 \pm 7.3\%$; Student's *t* test; $n = 5-7$; $p = 0.3186$). To confirm the results with anisomycin, we performed similar experiments, this time in the presence of a different protein synthesis inhibitor, CHX (Aoto et al., 2008; Martin et al., 2009). Again, 100 μ M CHX (2 h preincubation during resting period and bath

application during the whole experiment) did not affect LTP induction or maintenance in the PKM ζ -WT slices (Fig. 5B; LTP 3 h post-HFS: 100 μ M CHX, $131.9 \pm 8.7\%$; Student's *t* test; $n = 4$; $p > 0.3853$). To confirm that protein synthesis blockage can be demonstrated, we induced protein-synthesis-dependent late LTP in slices from our PBS-treated animals. For this purpose, we applied a stronger protocol of HFS consisting of four trains at a frequency of 200 Hz (instead of 100 Hz). This protocol reliably induced late LTP, which was potentiated 3 h after HFS to levels comparable to those achieved previously in the PKM ζ -WT slices upon 100 Hz HFS. Moreover, this 200 Hz-induced LTP in the PBS group was completely abolished in the ubiquitous presence of 100 μ M CHX 2 h after tetanization, suggesting its dependence on protein synthesis (Fig. 5C; LTP 3 h post-HFS: DMSO 0.05%, $119.2 \pm 7.6\%$; 100 μ M CHX, $101.9 \pm 5.2\%$; Student's *t* test; $n = 5$; $p = 0.0486$). Altogether, these experiments suggest that the mechanisms behind the late form of LTP in the PKM ζ -WT slices appear to be protein-synthesis independent even though protein-synthesis-dependent late LTP can be reliably induced in our setup.

Blockade of AMPA receptor desensitization mimics and occludes the PKM ζ -WT-induced LTP enhancement

As a next step, we wanted to know whether intrinsic changes in the surface AMPAR properties can account for the enhancement of potentiation seen in the PKM ζ -WT group. To address this

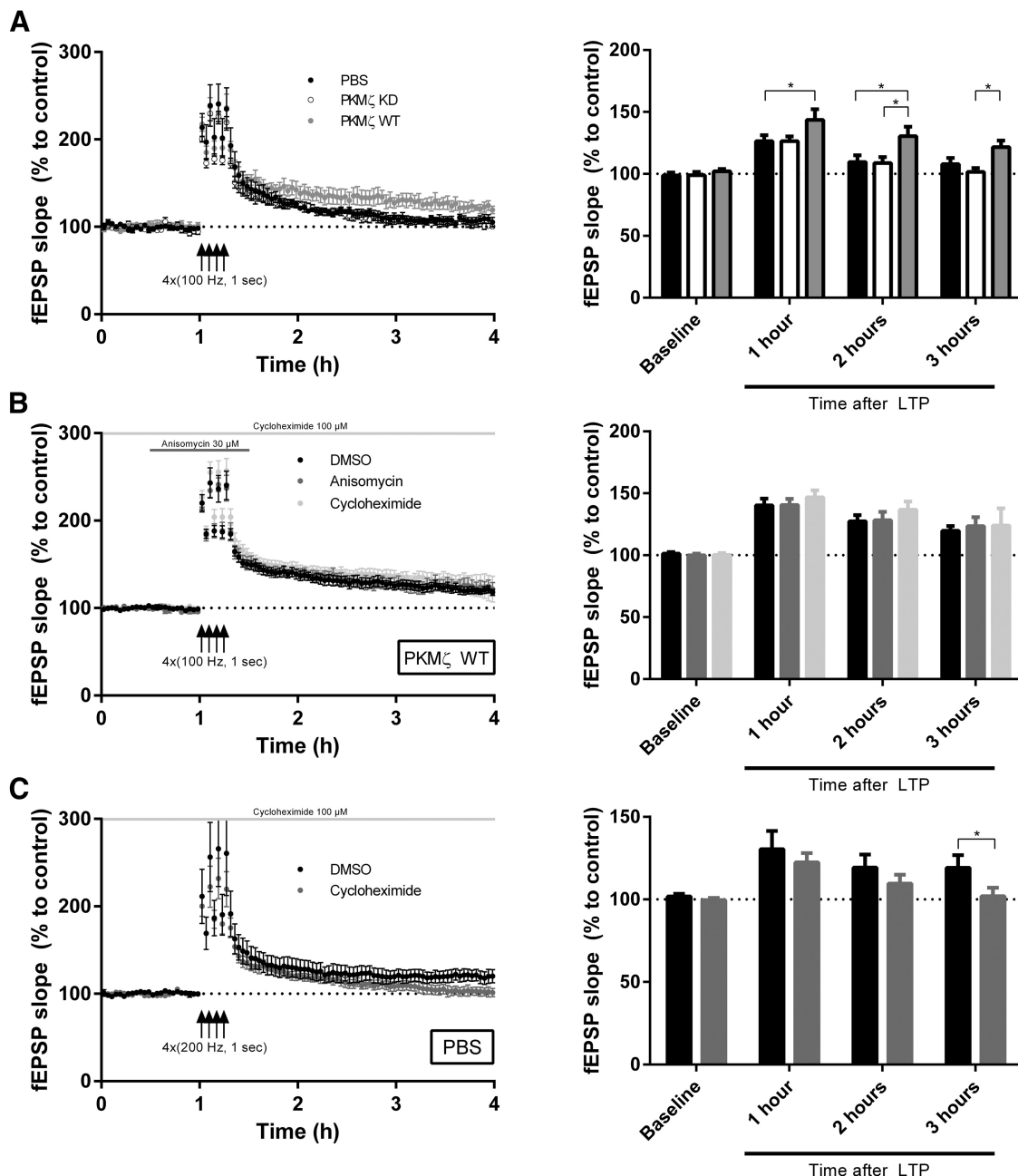


Figure 5. PKM ζ overexpression induces a protein-synthesis-independent form of late LTP. **A**, LTP was induced by application of 4 100 Hz (1 s) trains every 5 min in the different groups of slices. PKM ζ -WT slices express an LTP enhancement 3 h post-HFS, whereas both the PBS and KD groups show no potentiation at this point. Group sizes: PBS, $N = 2$, $n = 6$; PKM ζ -WT, $N = 2$, $n = 4$; PKM ζ -KD, $N = 2$, $n = 5$. **B**, Both protein synthesis inhibitors anisomycin (30 μ M, applied 30 min before and after HFS) and CHX (100 μ M, applied during the whole experiment) were unable to block the PKM ζ -induced late LTP. Group sizes: DMSO, $N = 5$, $n = 7$; anisomycin, $N = 3$, $n = 5$; CHX, $N = 2$, $n = 6$. **C**, In the PBS group, 4 200 Hz (1 s) trains every 5 min induced LTP, which was still enhanced 3 h after HFS and was completely abolished by the application of CHX (100 μ M, applied during the whole experiment). * $p < 0.05$. Group sizes: DMSO and CHX, $N = 3$, $n = 5$.

question we used CTZ, which functions as a desensitization blocker (Traynelis et al., 2010). Strikingly, bath application of CTZ at a concentration shown previously to block receptor desensitization (Constals et al., 2015) significantly enhanced LTP 3 h after HFS in slices from PBS-treated rats (Fig. 6A; LTP 3 h post-HFS: DMSO 0.05%, $100.5 \pm 6.7\%$; 20 μ M CTZ, $125.6 \pm 6.9\%$; Student's t test, $n = 5$ – 9 ; $p = 0.0374$), an effect that was also confirmed in the slices from PKM ζ -KD rats (Fig. 6B; LTP 3 h post-HFS: DMSO 0.05%, $101.5 \pm 2.6\%$; 20 μ M CTZ, $135.6 \pm 10.1\%$; Student's t test; $n = 5$ – 6 ; $p = 0.0063$). Interestingly, LTP magnitude in PKM ζ -WT slices was not further increased upon treatment with CTZ (Fig. 6C; LTP 3 h post-HFS: DMSO 0.05%,

$119.8 \pm 3.7\%$; 20 μ M CTZ, $131.9 \pm 8.7\%$; Student's t test, $n = 4$ – 7 ; $p = 0.1692$), suggesting that blocking AMPAR desensitization mimics and occludes the PKM ζ -WT-induced LTP enhancement. As before, new controls in the presence of the same amount of DMSO used with CTZ (0.05%) were performed simultaneously, showing no differences with respect to the earlier experiments, so these DMSO data were pooled together with the previous data. Importantly, CTZ did not affect basal transmission in any experimental group. Moreover, we tested PPF before and after application of 20 μ M CTZ, which did not induce any significant change in the PPF ratio. The fEPSP duration was likewise unaffected by CTZ treatment (data not shown).

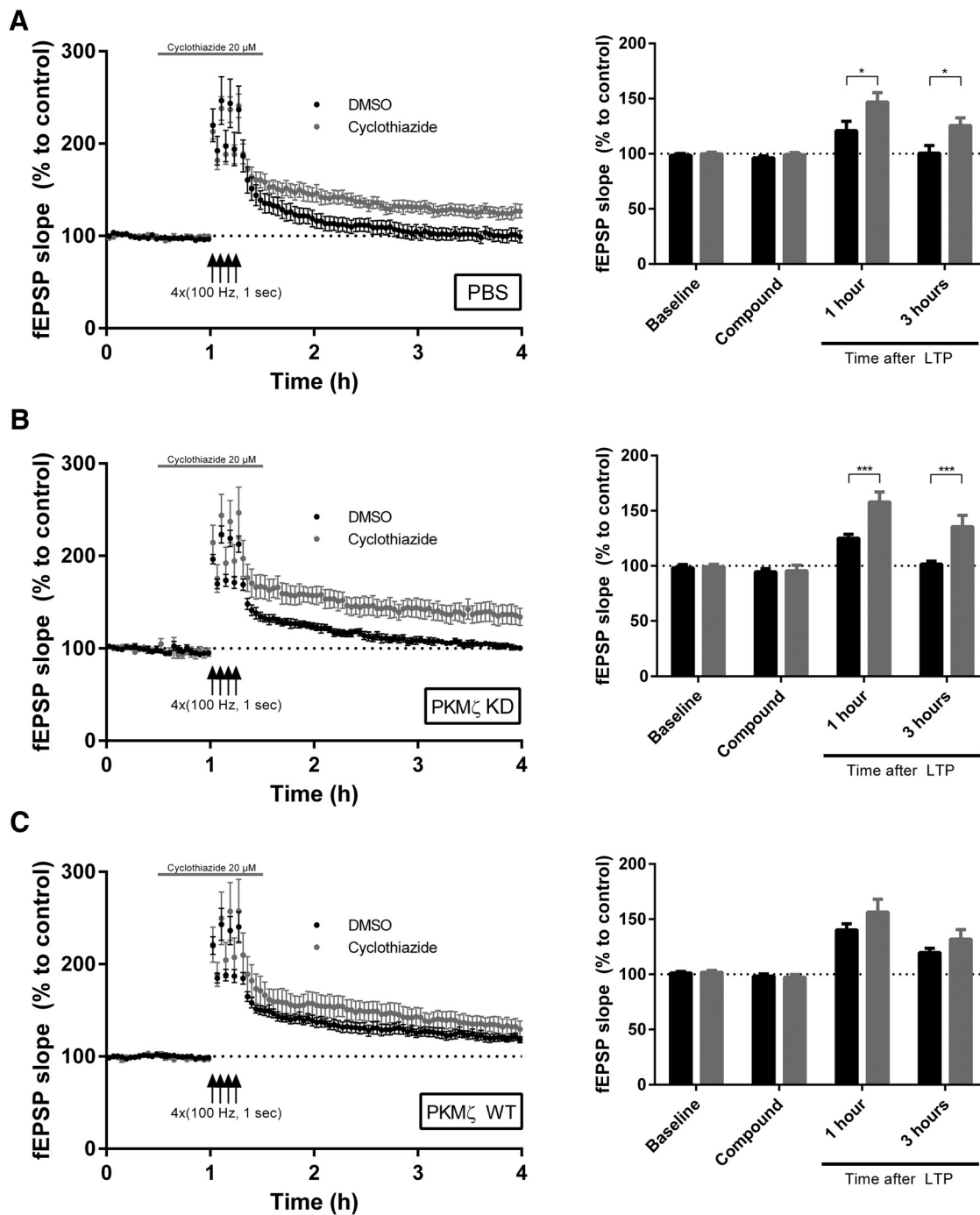


Figure 6. Blockade of AMPAR desensitization mimics and occludes PKM ζ -induced LTP enhancement. LTP was induced by application of 4 100 Hz (1 s) trains every 5 min in hippocampal slices from the PBS (**A**), PKM ζ -KD (**B**), and PKM ζ -WT (**C**) animals in the presence and in the absence of the AMPAR desensitization blocker CTZ (20 μ M, applied 30 min before and after HFS). In the PBS and PKM ζ -KD groups, CTZ induced and enhanced LTP 3 h after HFS. On the contrary, LTP in the PKM ζ -WT group was unaffected by CTZ. **/**** $p < 0.05/0.001$. Group sizes: **A**, DMSO, $N = 4, n = 9$; CTZ, $N = 2, n = 5$; **B**, DMSO, $N = 3, n = 6$; CTZ, $N = 2, n = 5$; **C**, DMSO, $N = 5, n = 7$; CTZ, $N = 3, n = 4$.

Behavioral experiments

CFC

To address associative memory formation and retention, we measured the expression of freezing response in the CFC task. Figure 7A shows increasing freezing responses over time to consecutive presentations of the conditioned stimulus (CS) paired with a foot shock, serving as an unconditioned stimulus (US) in all groups during the acquisition phase (two-way ANOVA $F_{(2,46)} = 85.01, n = 6-10, p < 0.0001$). No significant differences

in the level of freezing after each foot shock between the three groups were observed ($F_{(2,23)} = 0.359, n = 6-10, p = 0.702$). Testing contextual memory in the absence of auditory stimuli 1 week later, a significantly increased freezing behavior was found only in PKM ζ -WT-overexpressing rats (Fig. 7B; one-way ANOVA $F_{(2,23)} = 5.212, n = 6-10, p < 0.014$, followed by Bonferroni's *post hoc* test). Interestingly, no differences were detected between the groups tested for freezing response to tone recognition in a new context (Fig. 7C; one-way ANOVA $F_{(2,23)} = 0.866$,

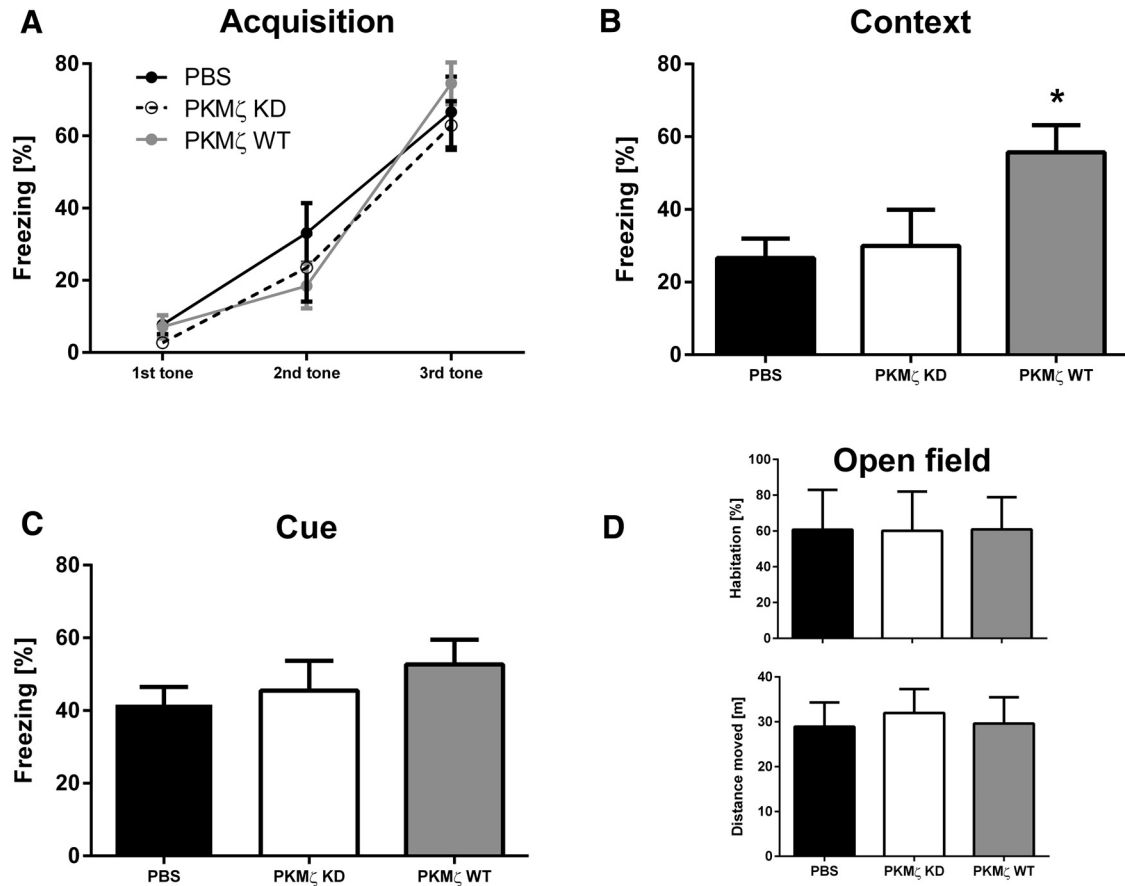


Figure 7. Effects of different treatments on performance of CFC in rats. Shown is the mean percentage of freezing in response to presentation of consecutive tone stimuli paired with a foot shock during acquisition (**A**), in response to known context 7 d after acquisition without tone or foot shock (**B**), and in response to tone 8 d after acquisition in new context without foot shock (**C**). Group sizes: **A–C**, PBS and PKM ζ -WT, $N = 10$; PKM ζ -KD, $N = 6$. **D**, Open-field analysis showing no difference between groups in time spent in center (top) and distance moved (bottom). * $p < 0.05$ compared with PBS. Group sizes: $N = 10$.

$n = 6–10$, $p = 0.434$). Next, we conducted open-field experiments to exclude potential behavioral implications of PKM ζ -WT and PKM ζ -KD overexpression, such as effects on anxiety-related behavior (center habitation) or locomotor activity that could influence the measured outcomes of the CFC paradigm. These experiments demonstrated no alterations between the groups in either center habitation or locomotor activity (Fig. 7D; one-way ANOVA $F_{(2,27)} = 0.003$, $n = 10$, $p = 0.9966$ and $F_{(2,27)} = 0.83$, $n = 10$, $p = 0.4469$, respectively).

OLT

To investigate spontaneous exploration combined with retention of spatial memory, we additionally performed an OLT behavioral task. To demonstrate the influence of both spatial cues and ITI in naive rats, different ITIs (8, 30, and 45 min) in the presence of spatial cues and an 8 min ITI in the absence of spatial cues were tested initially. Figure 8A shows that the naive animals were able to discriminate the relocated object with an ITI of 8 min compared with the unbiased T1. Next, we measured the OLT at the same ITI of 8 min but without the spatial cues, demonstrating no significant differences in exploration time compared with the unbiased T1 measurement and thus underscoring the role of the hippocampus in this task (one-way ANOVA $F_{(2,45)} = 9.876$, $n = 12–24$, $p = 0.0003$, followed by Bonferroni's *post hoc* test). Furthermore, we could show that the naive rats at longer ITIs of 30 min or 45 min were no longer able to discriminate the relocated object (Fig. 8B), showing a statistically significant difference to 8 min ITI but not to unbiased T1 (shown as a

line), suggesting a time window to investigate memory enhancement at those ITIs (one-way ANOVA $F_{(3,55)} = 7.183$, $n = 11–24$, $p = 0.0004$, followed by Bonferroni's *post hoc* test). To assess whether overexpression of PKM ζ -WT or KD improved performance in the spatial memory, animals were tested at ITIs of both 30 and 45 min, and naive animals showed no difference between T1 and T2 exploration. Furthermore, to confirm that overexpression of PKM ζ has no confounding effect in this task, the animals were also tested at an ITI of 8 min, when naive animals showed differences between T1 and T2 exploration. Figure 8C illustrates a retention of object location memory when tested 8 min after T1 (two-way ANOVA $F_{(2,79)} = 60.89$, $n = 9–10$, $p < 0.0001$) in all three groups, serving as a positive control and indicating that the treated animals were able to perform OLT comparable to naive rats. However, no statistically significant differences were detected in the PKM ζ -overexpressing animals when tested at ITIs of 30 or 45 min (two-way ANOVA $F_{(2,79)} = 0.011$, $n = 9–10$, $p = 0.98$), suggesting that PKM ζ does not mediate memory enhancement in this spatial memory task.

Discussion

In this study, we tested the effect of AAV-mediated bilateral hippocampal PKM ζ overexpression on fear memory, spontaneous behavior, and electrophysiological recordings in rats. The selective hippocampal overexpression of PKM ζ was confirmed to be ~ 1.5 -fold over endogenous PKM ζ . Slice fEPSP recordings revealed that both basal transmission and LTP are

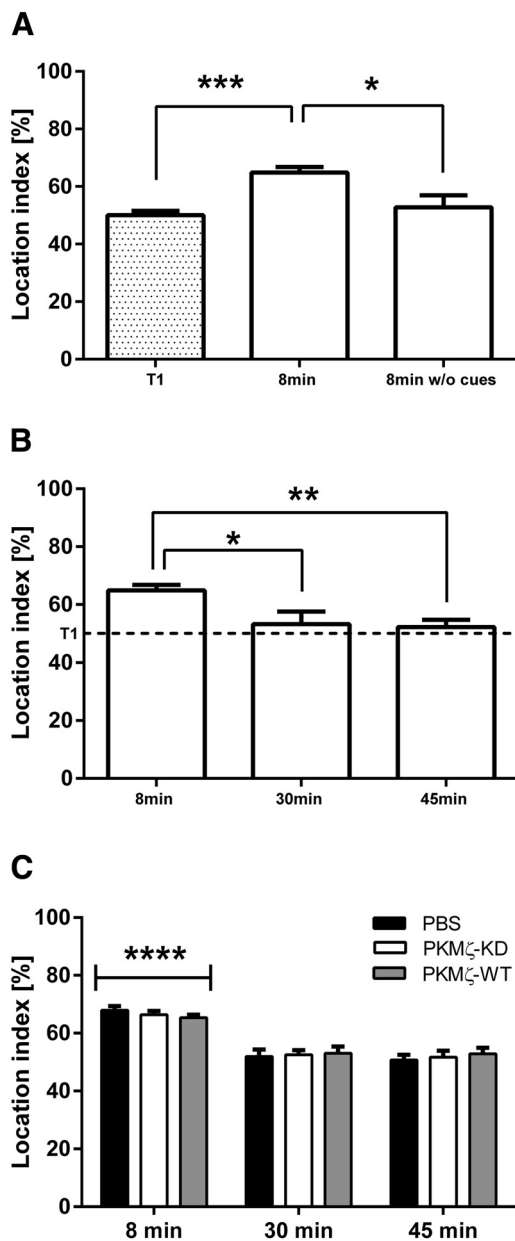


Figure 8. Effects of different ITI and treatments on object location memory in rats. **A**, Naive rats in OLT performing T2 of OLT 8 min after T1 with or without (w/o) spatial cues. Group sizes: T1, $N = 24$; 8 min, $N = 12$; 8 min w/o cues, $N = 12$. **B**, Naive rats in OLT performing T2 OLT in different ITI after T1 and with spatial cues. $^{*/**}p < 0.05/0.01$. Group sizes: 8 min, $N = 12$; 30 min, $N = 11$; 45 min, $N = 12$. **C**, PBS, PKM ζ -KD, and PKM ζ -WT treated rats in OLT performing T2 in different ITI after T1 with spatial cues 3 weeks after hippocampal AAV-mediated PKM ζ overexpression. $^{****}p < 0.0001$ compared with ITI of 30 and 45 min. Group sizes: 8 min, $N = 10$; 30 min, $N = 9$ –10; 45 min, $N = 10$.

augmented in the PKM ζ -WT slices, suggesting that PKM ζ functions as an important modulator of synaptic plasticity. Moreover, in the CFC paradigm, PKM ζ -WT rats showed enhanced contextual, but not cued, memory. However, no spatial memory enhancement was observed in the OLT. To exclude potential confounding effects for these cognitive tasks, both locomotor activity and anxiety-related behavior were assessed in an open-field test and were not influenced by PKM ζ overexpression.

Classically, LTP is subdivided into early and late LTP. Late LTP is believed to be protein-synthesis dependent and to last >3

h (Lu et al., 1999; Raymond, 2007). Historically, PKM ζ has been associated with late LTP maintenance (Ling et al., 2002; Serrano et al., 2005; Yao et al., 2008; Mei et al., 2011; Monti et al., 2012), yet the exact mechanisms behind this phenomenon remain unclear. Here, we combined a compilation of electrophysiological data in hippocampal PKM ζ -WT-overexpressing rats to further clarify the contribution of PKM ζ to LTP maintenance.

Basal synaptic transmission was significantly increased in our PKM ζ -WT slices. In our setup, basal fEPSPs are composed mainly by AMPAR currents (Fernández-Fernández et al., 2015), so this PKM ζ -WT effect is putatively due to an increase in the number of functional synaptic AMPARs or to a modification of their intrinsic properties, as described previously (Ling et al., 2006; Miguez et al., 2010; Hara et al., 2012; Dong et al., 2015). Therefore, the increased basal transmission detected from PKM ζ -WT overexpression may be due to modulated AMPAR trafficking, increasing the number of receptors ready to respond to the evoked presynaptic release of glutamate under basal conditions and resulting in an augmented synaptic potentiation.

Because LTP is saturable (Malenka et al., 1986), mechanisms that induce synaptic potentiation via LTP-associated signaling cascades such as CaMKII occlude subsequent LTP after tetanization (Pettit et al., 1994; Lledo et al., 1995). Here, we demonstrate that LTP is augmented as opposed to occluded in the PKM ζ -WT slices, suggesting that our results likely reflect a modulatory role of PKM ζ on synaptic plasticity rather than an integral role in LTP maintenance. However, it needs to be considered that our normalization protocol may have masked any potential occlusion effects.

It is important to emphasize that most of the data supporting PKM ζ as the maintenance molecule are based on experiments using ZIP. However, the selectivity of ZIP has been questioned (Kwapis and Helmstetter, 2014). In fact, it was demonstrated recently that ZIP is able to reduce LTP to an equivalent extent when comparing WT mice with transgenic mice lacking both PKC ζ and PKM ζ (Volk et al., 2013), underscoring the lack of ZIP's specificity. Interestingly, those investigators could induce late LTP in these transgenic animals, suggesting that PKM ζ may not be required for hippocampal LTP maintenance. Together with our enhancement but not occlusion of LTP, we interpret our electrophysiological data to reflect the modulatory role of PKM ζ on synaptic plasticity rather than an integral role in LTP maintenance.

In addition, we found that the augmented LTP in the PKM ζ -WT-overexpressing slices after tetanization is protein-synthesis independent. This is in agreement with a previous report describing a protein-synthesis-independent interaction between PKM ζ and BDNF associated with LTP (Mei et al., 2011). It should be noted that PKM ζ -WT microinjection took place 4 weeks before LTP measurements. Therefore, the extended presence of exogenous PKM ζ may have altered Pin1 phosphorylation (Westmark et al., 2010), making additional protein synthesis induced by the LTP augmentation less pronounced and thereby occluding an effect of the protein synthesis blocker.

Glutamate stimulation increases AMPAR mobility by inducing its desensitization (Constals et al., 2015). We show that blocking AMPAR desensitization by CTZ mimics (in the control groups) and occludes (in the PKM ζ -WT group) the PKM ζ -induced increase in LTP. Therefore, PKM ζ function may favor retaining the receptor in the PSD, thereby preserving the postsynaptic response to glutamate and modulating synaptic plasticity. Supporting this, blocking AMPAR endocytosis rescued LTP in PKM ζ knock-down rats (Dong et al., 2015). In contrast to a pre-

vious study in which 100 μM CTZ treatment altered basal transmission (Mainen et al., 1998), we observed neither an increase in basal transmission upon CTZ treatment in any of the groups nor an increase of the mean channel open time, as reflected by a significant change of the fEPSP shape in the presence of CTZ. CTZ has been demonstrated to block GABA_A receptors, which are widely expressed throughout the hippocampus with an IC₅₀ of 60 μM (Deng and Chen, 2003), \sim 3-fold higher than the EC₅₀ on AMPARs (Bertolino et al., 1993). To avoid this issue, we tested CTZ at 20 μM . Another explanation is that, in our experiments, integrated fEPSPs were studied in the absence of GABAergic transmission blockers, implying that the inhibitory currents (the onset of which coincides with the end of the fEPSP) may mask the putative enhancement of the time course of recovery from desensitization of AMPARs (Rozov et al., 2001).

The hippocampus plays a significant role in spatial orientation as well as in encoding and retrieving spatial memory (Morris et al., 1982; Nadel, 1991; Moser and Moser, 1998; Rolls, 1999). Lesion studies showed that the hippocampus also acts as the processing component of CFC (Kim and Fanselow, 1992; Phillips and LeDoux, 1992; Maren et al., 1998) and plays a role in spatial recognition memory during OLT (Barker and Warburton, 2011). In this study, we present evidence for memory enhancement of contextual, but not cued, fear in the PKM ζ -WT group. In contrast to previous results (Shema et al., 2011), memory formation in the PKM ζ -KD group was unaffected and therefore did not act as a dominant-negative mutant. Here, we overexpressed PKM ζ -KD 1.5-fold, whereas Shema et al. (2011) overexpressed 6-fold above endogenous levels. Moreover, we tested for memory and synaptic plasticity 4 weeks after surgery instead of 6 d, perhaps allowing compensatory mechanisms to occlude negative effects of PKM ζ -KD, for example, by enhancing PKM ζ synthesis. Interestingly, we detected no enhanced object location memory in OLT. This could be due to an absence of emotional significance in this task because it does not provide aversive or reinforced motivation or may reflect that PKM ζ function is not linked with short-term memory.

The hippocampus projects into several cortical and noncortical brain regions, including the amygdala (Canteras and Swanson, 1992; Maren and Fanselow, 1995) and the mPFC (Jay et al., 1995; Parent et al., 2010). Both structures are involved in providing emotional significance to an aversive situation (LeDoux, 2000; Banks et al., 2007; Meloni et al., 2008), as measured in CFC. In contrast, we are unaware of any studies that connect the amygdala with the OLT. Furthermore, Barker and Warburton (2011) found that the mPFC is not involved in OLT performance.

The role of these structures in fear acquisition and expression after conditioning is complex and distinct. Both context-US and auditory CS-US pairing are thought to be processed in the basal nuclei and CeA to generate the freezing response (Maren and Quirk, 2004; Herry et al., 2008; Biedenkapp and Rudy, 2009). Lesion of the BLA disrupted both acquisition and defensive response (Phillips and LeDoux, 1992; Maren et al., 1996; Cousens and Otto, 1998). Here, we demonstrate enhanced response to contextual conditioning, supporting the role of the hippocampus in memory of contextual fear. Interestingly, no effect was detected in the cued conditioning paradigm, which has been shown to be hippocampus independent (Phillips and LeDoux, 1992). However, it should be acknowledged that the high freezing levels in the PBS-treated animals may represent a ceiling effect, occluding PKM ζ -WT enhancement.

In addition to the amygdala, the mPFC plays an important role in the acquisition and expression of conditioned fear for both

context and auditory stimuli (Morgan and LeDoux, 1995; Quirk and Beer, 2006; Gilmartin et al., 2014). Interestingly, two of the main projections from the prelimbic cortex of the ventral mPFC innervate the BLA and CeA (Vertes, 2004), perhaps suggesting an important role of a neural circuit involving hippocampus, mPFC, and amygdala in the processing of aversive stimuli. Therefore, it is possible that supporting any of these structures leads to enhanced response in aversive behavioral tasks. This is supported by a study reporting enhanced freezing to cued conditioning after PKM ζ overexpression in the prelimbic cortex (Xue et al., 2015). Although no data for contextual response were shown, the mPFC may also be involved in contextual fear memory.

In conclusion, to our knowledge, this study is the first to combine electrophysiological measurements with behavioral experiments in animals overexpressing PKM ζ in the hippocampus and therefore constitutes an experimental approach different from previous inhibitor-based strategies. As opposed to previous studies linking PKM ζ with LTP maintenance, our results confirm and expand upon the modulatory role of PKM ζ in memory processes and synaptic plasticity and provide a novel link to AMPAR desensitization, bringing new insights for the selective role of PKM ζ in different neural circuits.

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