

Overexpression of Protein Kinase M ζ in the Prelimbic Cortex Enhances the Formation of Long-Term Fear Memory

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Neuroplasticity in the prefrontal cortex (PFC) after fear conditioning has been suggested to regulate the formation and expression of fear memory. Protein kinase M ζ (PKM ζ), an isoform of protein kinase C with persistent activity, is involved in the formation and maintenance of memory. However, less is known about the role of PKM ζ in the PFC in the formation of fear memory. We investigated whether the overexpression of PKM ζ enhances the formation of auditory fear memory in rats. We found that microinfusion of lentiviral vector-expressing PKM ζ into the prelimbic cortex (PrL) selectively enhanced the expression of PKM ζ without influencing the expression of other isoforms of PKC. The overexpression of PKM ζ in the PrL enhanced the formation of long-term fear memory without affecting short-term fear memory, whereas the overexpression of PKM ζ in the infralimbic cortex had no effect on either short-term or long-term fear memory. The overexpression of PKM ζ in the PrL had no effect on anxiety-like behavior or locomotor activity. We also found that PKM ζ overexpression potentiated the fear conditioning-induced increase in the membrane levels of glutamate subunit 2 of AMPA receptors in the PrL. These results demonstrate that the overexpression of PKM ζ in the PrL but not infralimbic cortex selectively enhanced the formation of long-term fear memory, and PKM ζ in the PrL may be involved in the formation of fear memory.

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INTRODUCTION

Fear normally causes individuals to generate an adaptive response to danger, whereas extreme fear and anxiety can become harmful and maladaptive and lead to fear and anxiety disorders, such as posttraumatic stress disorder (LeDoux, 2000; Maren *et al*, 2013). Thus, a deeper understanding of the neurobiological mechanisms of fear memory has significant clinical implications and may aid in the development of more effective therapies for amnesia, cognitive decline, or fear and anxiety disorders (Duvarci and Pare, 2014; Maren and Quirk, 2004). In animal studies, the mechanisms that underlie fear can be investigated using Pavlovian fear conditioning, in which an originally neutral conditioned stimulus (CS; eg, a tone) is paired with an aversive unconditioned stimulus [US; eg, a shock; (LeDoux 2000; Maren and Quirk 2004)].

The medial prefrontal cortex (mPFC) is a neocortical structure that can be cytoarchitectonically divided into different subregions, including the prelimbic cortex (PrL) and infralimbic cortex (IL; Heidbreder and Groenewegen, 2003). Although most studies have focused on the role of the amygdala and hippocampus in fear memory (Bannerman *et al*, 2004; Duvarci and Pare, 2014; Fanselow and Gale 2003; Goosens 2011; Maren 2003; Riccio and Joynes 2007), a growing body of evidence demonstrates that the mPFC has an important role in modulating the formation, expression, and extinction of fear (Gilmartin *et al*, 2014; Quirk and Beer, 2006a; Sotres-Bayon and Quirk, 2010). For example, reducing mPFC activity, blocking the signaling pathways of the dopamine D₁ receptor and N-methyl-D-aspartate (NMDA) receptor, and ubiquitin-proteasome system-mediated protein degradation in the mPFC disrupted the formation of trace or delayed fear conditioning (Gilmartin and Helmstetter, 2010; Reis *et al*, 2013; Runyan and Dash, 2004). Different subregions of the mPFC appear to have distinct functions in fear memory, and the IL appears to control extinction learning, in which a new conditioned stimulus-no unconditioned stimulus association is formed (Milad and Quirk 2002; Milad *et al*, 2007; Peters *et al*, 2010; Quirk *et al*, 2006b). The PrL was found to modulate the expression of learned fear (Corcoran and Quirk, 2007; Guhn *et al*, 2014; Lee and Choi, 2012; Sotres-Bayon *et al*, 2012; Vidal-Gonzalez *et al*, 2006). Recently, the PrL has also been

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suggested to participate in the storage of fear memory. NMDA receptor blockade or perineuronal net disruption in the PrL impaired the storage of learned fear in rats (Gilmartin and Helmstetter, 2010; Hylin *et al*, 2013).

Protein kinase M ζ (PKM ζ) is a brain-specific protein kinase C (PKC) isoform with persistent activity (Sacktor 2008). In the past few years, PKM ζ has been found to be critical for the maintenance of long-term potentiation (LTP) and long-term memories (Pastalkova *et al*, 2006; Sacktor 2008). Microinfusion of the PKC ζ inhibitory peptide ZIP abolished the maintenance of various types of long-term memories, including hippocampus-dependent spatial learning (Pastalkova *et al*, 2006), amygdala-dependent auditory fear conditioning (Serrano *et al*, 2008), insular cortex-dependent conditioned taste aversion (Shema *et al*, 2007), and nucleus accumbens—and amygdala-dependent drug-associated memories (He *et al*, 2011; Li *et al*, 2011). Evuarherhe *et al*, (2014) found that PKM ζ in the medial PFC may be involved in the formation of recognition memory. The underlying mechanisms of PKM ζ in memory regulation may involve the glutamate subunit 2 trafficking of α -amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid receptors (GluA2; (Migues *et al*, 2010; Yao *et al*, 2008). However, in these studies, the chemical compound that was used to modulate PKM ζ activity was ZIP, the specificity of which for PKM ζ has been challenged (Kwapis and Helmstetter, 2014). ZIP has been shown to suppress the activity of PKC ζ (Ren *et al*, 2013a), and the application of ZIP in PKC ζ knockout mice abolished the maintenance of LTP and long-term memory (Lee *et al*, 2013; Volk *et al*, 2013). Using viral transfection of the insular cortex to selectively modulate the expression of PKM ζ , Shema *et al*, (2011) found that PKM ζ overexpression enhanced the memory trace of conditioned taste aversion, whereas dominant-negative PKM ζ overexpression disrupted memory, even long after the memory was formed. Inspired by these studies, using a lentiviral vector-expressing PKM ζ gene, we investigated whether the overexpression of PKM ζ in the mPFC enhances the formation of auditory fear memory.

MATERIALS AND METHODS

Subjects

All of the experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and Biomedical Ethics Committee of Peking University for animal use and protection. Male Sprague-Dawley rats (3-month old, 200–220 g) were obtained from the Laboratory Animal Center, Peking University Health Science Center. These rats were housed five per cage with *ad libitum* access to food and water under a 12 h/12 h light/dark cycle. The behavioral experiments were conducted during the dark phase of the cycle.

Design, Construction, and Validation of Lentiviral Vectors for PKM ζ Overexpression

The construction and use of the lentiviral vectors were based on our previous studies with minor modifications (Zhu *et al*, 2012). pSinRep5-pPKM ζ -Ires-ZsGreen vector plasmids (kindly provided by Todd Sacktor, SUNY Downstate

Medical Center, Brooklyn, NY, USA) were constructed for the production of lentiviruses that express PKM ζ . PKM ζ was amplified by PCR from the vectors and subcloned into the GV208 vector using *Bam*HI and *Age*I restriction sites. All of the vectors contained the enhanced green fluorescence protein (eGFP) coding sequence, and PKM ζ was separated from eGFP by IRES. All of the vectors were then transfected into human embryonic kidney 293 cells. Approximately 48 h post-transfection, cells were collected, purified by centrifugation, and stored at -80°C (Genechem, Shanghai, China).

Surgery

The rats were anesthetized with sodium pentobarbital (50 mg/kg, ip) and then placed in a stereotaxic apparatus. Identical stainless-steel guide cannulae (22 gauge) were bilaterally implanted into the IL (anterior/posterior, +2.9 mm; medial/lateral, \pm 2.3 mm; dorsal/ventral, -4.8 mm) and PrL (anterior/posterior, +2.9 mm; medial/lateral, \pm 2.3 mm; dorsal/ventral, -3.0 mm; (He *et al*, 2011; Paxinos and Watson, 2005). The cannulae were placed at a 16° angle toward the midline to avoid penetration of the lateral ventricle. After surgery, the rats were allowed 7 days to recover, during which time they were handled until the start of training.

Intracerebral Injections of Lentiviruses and Drugs

The experimental parameters that were used for the virus injections were based on previous work from our and other laboratories (Shema *et al*, 2011; Zhu *et al*, 2012). The LV_{PKM ζ -GFP} and LV_{GFP} lentiviruses (1×10^9 viral genomes, dissolved in phosphate-buffered saline (PBS)) were injected into the IL or PrL with 10 μ l Hamilton syringes that were connected via polyethylene-50 tubing to 30-gauge injectors. The Hamilton syringes were connected to an infusion pump (World Precision Instruments, Sarasota, FL, USA). The viruses were delivered bilaterally over 10 min at an infusion rate of 0.1 μ l/min (total volume, 1 μ l per side), and the injectors were left in place for an additional 2 min to allow diffusion before removing them. At the end of the experiments, the rats were anesthetized with sodium pentobarbital (100 mg/kg, ip) and transcardially perfused. Cannula placements were assessed using Nissl staining with a section thickness of 40 μ m under light microscopy. Rats with misplaced cannulae were excluded from the statistical analysis.

Apparatus and Procedures

Apparatus. Fear conditioning was conducted using a video-based fear system (Beijing Macro Ambition S&T Development, Beijing, China) as in our previous study (Yang *et al*, 2013). The system consists of four Plexiglas chambers (30 cm height \times 30 cm width \times 30 cm length) with floors made of metal stainless-steel rods connected to a shock generator. Each chamber was enclosed within a ventilated acoustic isolation box. Across the experiments, two different contexts (A and B) were used. Context A consisted of a chamber with a grid floor and was illuminated by a white light. Context A (conditioning context) consisted of the original conditioning chamber with a 20% ethanol odor. In context B, several modifications were introduced based on

the original training chambers to create a novel testing context. In context B, the stainless-steel rod floor and rear walls were covered with black Plexiglas, and a 2% acetic acid odor was introduced.

Training. The fear-conditioning procedure was conducted based on methods used in previous studies from our and other laboratories with minor modifications (Monfils *et al*, 2009; Si *et al*, 2012; Yang *et al*, 2013). The animals were placed into training chamber A and allowed to explore it for 10 min. Fear conditioning was then conducted with three 20 s, 2 kHz, 80 dB tones (CS), each coterminating with a 1 s, 0.75 mA footshock (US). The interval between each CS was an average of 120 s. After conditioning, the rats were allowed to explore the conditioning chamber for an additional 1 min before being returned to its home cage.

Test. During the long-term memory tests, the rats were first placed in the test chamber (context B) for 2 min to determine pre-CS freezing behavior. The test sessions consisted of three presentations of the CS with a CS-CS interval of 120 s. The rats were returned to their home cage 120 s after the last CS. The data represent the average of the two trials.

Behavioral score. Behavioral scores were recorded and analyzed using computer software (Beijing MacroAmbition S&T Development, Beijing, China) by an experimenter who was blind to the treatment. Conditioned fear was assessed by measuring the percentage of time spent freezing during the 20-s period of each CS presentation. Freezing behavior was measured using an animal behavior video analysis system (Beijing MacroAmbition S&T Development, Beijing, China).

Locomotor activity. Locomotor activity was assessed based on our previous studies (Shi *et al*, 2012; Zhu *et al*, 2012). Briefly, the open-field apparatus consisted of a 75 \times 75 \times 40 cm square arena that was divided into 25 equal squares (15 \times 15 cm) on the floor. Each rat was placed in the center of the apparatus, and the number of crossings into adjacent squares was counted for 5 min to assess locomotor activity.

Elevated plus maze. The elevated plus maze test was based on our previous studies (Suo *et al*, 2013). Briefly, each rat was first placed in the central zone of the elevated plus maze. The rat was allowed to freely explore the maze for 5 min, and the entire test was conducted under dim illumination. The number of entries into and time (in seconds) spent on the open arms were recorded by two independent observers who were blind to the animal groups and sat quietly 2.5 m from the maze.

Tissue Sample Preparation

Tissue samples were prepared based on our previous studies (Ren *et al*, 2013b; Xue *et al*, 2014). After the behavioral experiments, the rats were decapitated, and the brains were rapidly extracted and frozen in -40°C *N*-hexane. The brains were then stored in a -80°C freezer. Bilateral tissue punches (12 gauge) of the PrL and IL were then taken. Afterward, each tissue punch was exposed to 100 μl of radioimmuno-precipitation assay (RIPA) lysis buffer (20 mM Tris (pH 7.5),

150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM EDTA, 1% Na_3VO_4 , 0.5 g/ml leupeptin, and 1 mM phenylmethanesulfonyl fluoride; Beyotime Biotechnology) for 30 min. The tissue samples were then homogenized (10–15 s three times at 5-s intervals) with an electrical disperser (Wiggenhauser). The tissue homogenates were then subjected to 10 000 $\times g$ centrifugation at 4°C for 20 min. The supernatant was used for subsequent western blot. The membrane fractions were prepared using a membrane protein extraction kit (Applygen, Beijing, China). The protein concentrations of all of the samples were determined using the bicinchoninic acid assay (Beyotime Biotechnology). The samples were further diluted in RIPA lysis buffer to equalize the protein concentrations.

Western Blot Assays

The western blot assays were based on our previous studies (Xue *et al*, 2012; Xue *et al*, 2014). Loading buffer (5 \times ; 16% glycerol, 20% mercaptoethanol, 2% sodium dodecyl sulfate (SDS), and 0.05% bromophenol blue) was added to each sample (4 : 1, sample:loading buffer) before boiling for 3 min. The samples were cooled and subjected to SDS-polyacrylamide gel electrophoresis (10% acrylamide/0.27% *N,N'*-methylenebisacrylamide resolving gel) for ~ 40 min at 80 V in stacking gel and ~ 1 h at 120 V in resolving gel. Proteins were electrophoretically transferred to polyvinylidene fluoride transfer membranes (Millipore, Bedford, MA, USA) at 0.25 A for 2.5 h. Membranes were washed with TBST (tris-buffered saline plus 0.05% Tween-20, pH 7.4) and then dipped in blocking buffer (5% bovine serum albumin (BSA) in TBST) overnight at 4°C . The next day, the membranes were incubated for 1 h at room temperature on an orbital shaker with anti-PKC ζ antibody (1 : 1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA; catalog no. sc-216), anti-PKC α antibody (1 : 1000; Santa Cruz Biotechnology; catalog no. sc-208), anti-PKC λ antibody (1 : 1000; Santa Cruz Biotechnology; catalog no. sc-11399), anti-GluA1 antibody (1 : 1000; Abcam, Cambridge, UK; catalog no. ab109450), anti-GluA2 antibody (1 : 1000; Abcam; catalog no. ab52932), anti-GluA3 antibody (1 : 1000; Abcam, catalog no. ab40845), anti-Na-K ATPase antibody (1 : 1000; Abcam, catalog no. ab7671), or β -actin (1 : 1000; Santa Cruz Biotechnology; catalog no. sc-47778) in TBST plus 5% BSA and 0.05% sodium azide. After three 5-min washes (three times) in TBST buffer, the blots were incubated for 45 min at room temperature on a shaker with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG for PKC α , PKC λ , PKM ζ , GluA1, GluA2, and GluA3; goat anti-mouse IgG for β -actin and Na-K ATPase Santa Cruz Biotechnology) diluted 1 : 5000 in blocking buffer. The blots were then washed three times for 5 min each in TBST and incubated with a layer of Super Signal Enhanced chemiluminescence substrate (Detection Reagents 1 and 2, 1 : 1 ratio, Pierce Biotechnology, Rockford, IL, USA) for 1 min at room temperature. Excess mixture was dripped off before the blots were wrapped with a clean piece of plastic wrap (no bubbles between blot and wrap), and the blots were then screened using the ChemiDoc MP System (BioRad, Hercules, CA, USA) for 5–60 s. The band intensities for PKM ζ were quantified by two observers who were blind to the experimental groups using Quantity One 4.4.0 software

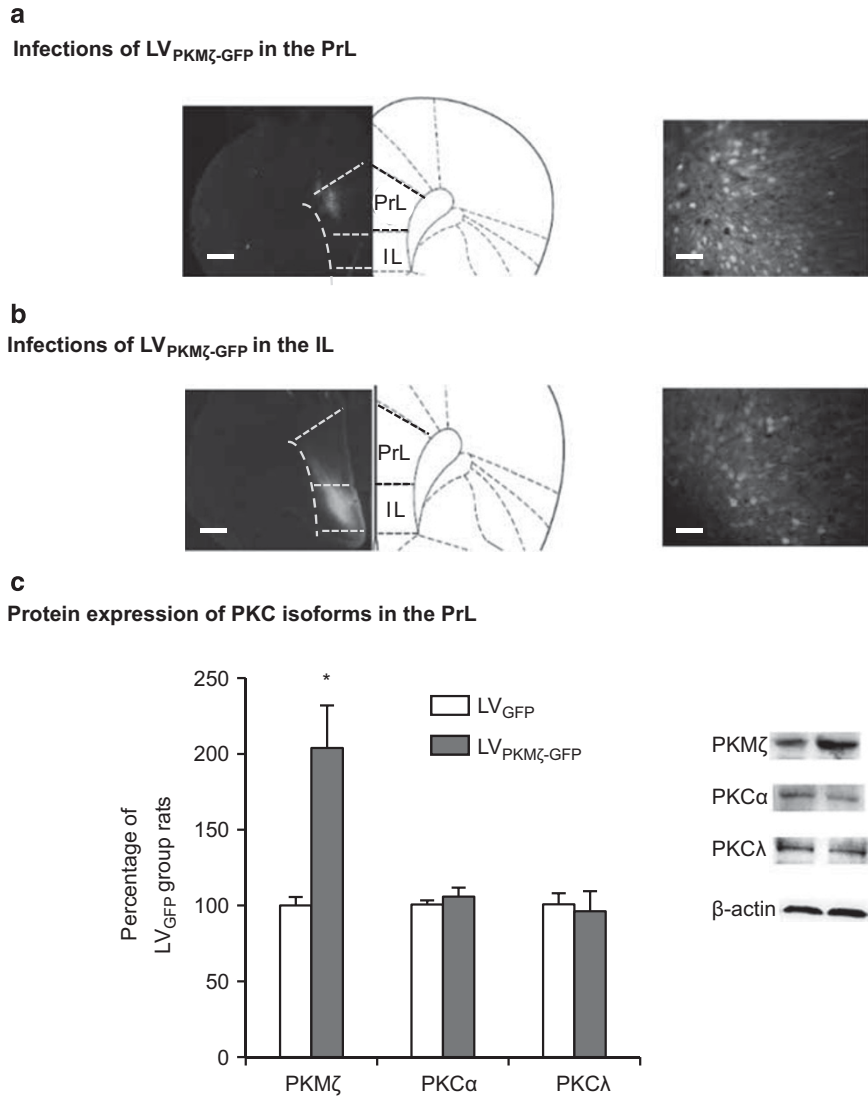


Figure 1 Lentiviral PKM ζ -GFP infection in the PrL enhances the expression of PKM ζ but not other isoforms of PKC in the PrL. (a, b) Representative pictures of the injection sites and coronal sections of the rat brain with LV_{PKM ζ -GFP} infusion into the PrL and IL. Scale bars, 500 μ m (low-power images) and 50 μ m (high-power images). (c) Western blot with an PKM ζ antibody that depicts a significant increase in PKM ζ expression in the PrL after infusion of a lentiviral vector that contained the PKM ζ gene (LV_{PKM ζ -GFP}) compared with infusion of GFP alone (LV_{GFP}). No significant differences in any PKC isoforms were found in the PrL. * $p < 0.05$. The data are expressed as mean \pm SEM. $n = 6-7$ per group.

(BioRad, Hercules, CA, USA). The band intensities from each test sample were compared with the band intensities from the standard curves. The amount of the protein of interest in each sample was interpolated from the standard curve. The standard curve runs in all of the western blots in our study showed that the band intensities for each of our test samples were within the linear range of detection.

Immunohistochemistry

Immunofluorescence was based on our previous study (Jiang *et al*, 2013). After the behavioral experiments, the rats were anesthetized and perfused with 0.01 mol/l PBS and 4% paraformaldehyde, pH 7.4. The brains were then extracted and removed in 4% paraformaldehyde for 24 h. Subsequently, the brains were placed in 30% sucrose for $\sim 24-48$ h, frozen, coronally sectioned at 10 μ m using a sliding microtome, and stored in PBS. Brain slices were then mounted on Superfrost/

plus slides, and eGFP expression was screened using a fluorescent microscope at the injection sites. Representative images were captured at the same time.

Statistical Analysis

Group sizes were balanced to ensure equal variance between the tested groups, and this was verified using Levene's weighted F-test and SPSS software for all comparisons. Each molecular experiment was replicated 2-4 times (an average of three times). All of the data showed a normal distribution and are expressed as mean \pm SEM. The data were analyzed using analysis of variance (ANOVA) with appropriate between—and within—subjects factors for each experiment (see Results). Significant main effects and interactions ($p < 0.05$, two-tailed) in the factorial ANOVAs were followed by one-way ANOVAs and the least significant difference *post hoc* test.

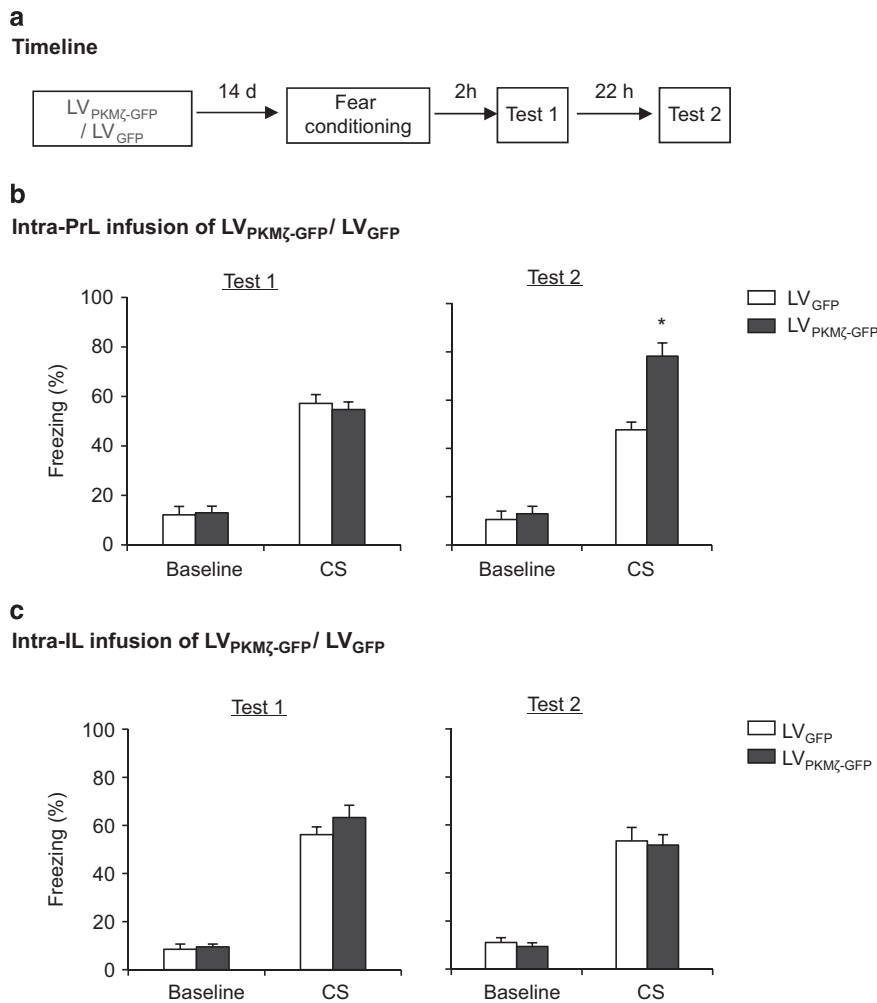


Figure 2 PKM ζ overexpression in the PrL but not IL enhances the consolidation of fear memory. (a) Timeline of the experiment. One week after LV_{PKM ζ -GFP} or LV_{GFP} infusions, all of the rats underwent fear conditioning and were tested for the fear response 2 and 24 h later. (b) The group that received intra-PrL infusion of LV_{PKM ζ -GFP} exhibited a significant fear response 24 h but not 2 h post-conditioning compared with the LV_{PKM ζ -GFP} group. (c) The two groups that received infusions in the IL showed similar fear responses in all of the fear tests. * $p < 0.05$. The behavioral data are expressed as mean \pm SEM. $n = 8-10$ per group.

RESULTS

Experiment 1: Intra-PrL Injection of LV_{PKM ζ -GFP} Increased PKM ζ Protein Levels But Not PKC α or PKC λ Levels

We first constructed and microinjected LV_{PKM ζ -GFP} into the PrL and examined whether LV_{PKM ζ -GFP} specifically increased the protein expression of PKM ζ but not other types of PKC. Two groups of rats ($n = 6-7$ per group) received microinjections of LV_{PKM ζ -GFP} or LV_{GFP}. Fourteen days later, all of the rats were euthanized to detect PKC α , PKC λ , and PKM ζ levels in the PrL.

Figure 1 a and 1b show representative pictures of coronal sections of the rat brains with LV_{PKM ζ -GFP} infusion into the PrL or IL. The analysis of the western blot data using one-way ANOVA revealed that PKM ζ expression in the PrL was significantly increased 14 days after lentivirus infusion in the group infused with LV_{PKM ζ -GFP} compared with the group infused with LV_{GFP} in the PrL ($F_{1,12} = 17.372$, $p = 0.002$; Figure 1c) but not IL ($p > 0.05$). No significant differences were found in the levels of PKC α or PKC λ in the PrL (all $p > 0.05$; Figure 1c).

Experiment 2: Effect of PKM ζ Overexpression in the IL and PrL on Short-Term Memory and Long-Term Memory After Fear Conditioning

We then assessed whether PKM ζ overexpression in the PrL and IL enhances the formation of fear memory. Four groups of rats ($n = 8-10$ per group) received LV_{PKM ζ -GFP} or LV_{GFP} infusions into the PrL or IL. Two weeks later, the rats underwent fear conditioning and were tested 2 h (short-term memory) and 24 h (long-term memory) later (Figure 2a).

The freezing scores 2 and 24 h after fear conditioning were analyzed using one-way ANOVA (LV_{PKM ζ -GFP} and LV_{GFP}). This analysis revealed a significant effect of lentivirus type in the PrL 24 h after fear conditioning ($F_{1,17} = 20.382$, $p = 0.000$; Figure 2b, right column) but not 2 h after fear conditioning ($p > 0.05$; Figure 2b, left column). In the IL, no significant effect of lentivirus type was found 2 or 24 h after fear conditioning (all $p > 0.05$; Figure 2c). These data indicate that the overexpression of PKM ζ in the PrL had no effect on short-term fear memory, but promoted the formation of long-term fear memory.

Experiment 3: Effect of PKM ζ Overexpression in the PrL on Locomotor Activity and Anxiety-Like Behavior

In this experiment, we examined whether prelimbic PKM ζ overexpression affects locomotor activity and anxiety-like behavior. Two groups of rats ($n = 8-10$ per group) received LV_{PKM ζ -GFP} or LV_{GFP} infusions in the PrL. Two weeks later, all of the rats underwent the open field and elevated plus maze tests (Figure 3a).

The open field and elevated plus maze data were analyzed using one-way ANOVA (LV_{PKM ζ -GFP} and LV_{GFP}). This analysis revealed no significant effect of lentivirus type on the number of crossings (Figure 3b) or time spent in the central area (Figure 3c) in the open field or time spent on the open arms (Figure 3d) in the elevated plus maze (all $p > 0.05$). These data indicate that PKM ζ overexpression in the PrL had no effect on locomotion or anxiety-like behavior.

Experiment 4: Effect of PKM ζ Overexpression on the Membrane Expression of AMPA Receptors in the PrL During the Phases of Short-Term and Long-Term Memory

AMPA receptor trafficking has been shown to have a role in the involvement of PKM ζ in LTP and long-term memory (Migues *et al*, 2010; Yao *et al*, 2008). We explored the relationship between the membrane expression of AMPA receptors and the enhancing effect of PKM ζ overexpression on fear memory. Eight groups of rats ($n = 5$ per group) received LV_{PKM ζ -GFP} or LV_{GFP} infusions into the PrL. Two weeks later, the rats were fear-conditioned or exposed to the conditioning chamber without shock. The rats were then decapitated 2 or 24 h later to assess the membrane expression of GluA1, GluA2, and GluA3 in the PrL (Figure 4a).

The western blot data were analyzed using two-way ANOVA, with conditioning (conditioning and no conditioning) and lentivirus type (LV_{PKM ζ -GFP} and LV_{GFP}) as factors. The analysis of protein levels 2 h after fear conditioning revealed significant effects of conditioning for GluA1 ($F_{1,16} = 26.390$, $p = 0.000$) and lentivirus type for GluA2 ($F_{1,16} = 16.948$, $p = 0.001$; Figure 4b). The *post hoc* analysis revealed that the LV_{PKM ζ -GFP}+fear conditioning and LV_{GFP}+fear conditioning groups exhibited an increase in the membrane levels of GluA1 compared with the other two groups (all $p > 0.05$). The LV_{PKM ζ -GFP} + fear conditioning and LV_{PKM ζ -GFP} + no conditioning groups exhibited an increase in the membrane levels of GluA2 compared with the other two groups. No significant effect of conditioning or lentivirus type on the membrane levels of GluA3 in PrL was found ($p > 0.05$). The analysis of protein levels 24 h after fear-conditioning revealed significant effects of conditioning ($F_{1,16} = 13.967$, $p = 0.002$) and lentivirus type ($F_{1,17} = 10.555$, $p = 0.005$; Figure 4c) for GluA2, and only a significant effect of conditioning on GluA1 ($F_{1,16} = 17.770$, $p = 0.001$) and GluA3 ($F_{1,16} = 5.613$, $p = 0.03$). The *post hoc* analysis revealed that the LV_{PKM ζ -GFP} + fear conditioning and LV_{GFP} + fear conditioning groups exhibited an increase in the membrane levels of GluA1 and GluA2 compared with the other two groups (all $p > 0.05$). The LV_{PKM ζ -GFP} group exhibited an increase in the membrane levels of GluA2 compared with the other three groups. These data indicate that fear conditioning increased the membrane expression of

GluA1 during the short-term memory phase and increased the membrane expression of GluA1, GluA2, and GluA3 during the long-term memory phase. PKM ζ overexpression potentiated the fear conditioning-induced increase in the membrane expression of GluA2 during the long-term phase.

DISCUSSION

In the present study, we evaluated the effect of PKM ζ overexpression in the mPFC on the formation of fear memory. We found that microinjection of lentiviral PKM ζ -GFP selectively enhanced the expression of PKM ζ without affecting other forms of PKC. PKM ζ overexpression in the PrL selectively enhanced the formation of long-term memory but not short-term memory, whereas PKM ζ overexpression in the IL had no effect on either long-term memory or short-term memory. The augmenting effect of PKM ζ overexpression on memory formation did not appear to be attributable to alterations in locomotor activity or the emotional state of the rats because we found that PKM ζ overexpression in the PrL had no effect on locomotor activity in the open field or anxiety-like behavior in the elevated plus maze. PKM ζ overexpression increased the membrane levels of GluA2 and potentiated the increase in the membrane levels of GluA2 induced by fear conditioning. This indicates that PKM ζ overexpression in the PrL enhanced the formation of long-term fear memory, and this effect may be mediated by elevations of the membrane levels of GluA2 in the PrL.

The PrL and IL are two subregions of the mPFC that have been suggested to have different roles in the formation and expression of fear memory. The activity of PrL neurons is correlated with freezing responses to a CS (Burgos-Robles *et al*, 2009), and IL neurons fire in response to the CS only when the freezing response is successfully extinguished (Milad and Quirk, 2002). Inactivation of the PrL blocked the acquisition and expression of learned fear (Gilmartin and Helmstetter 2010; Sierra-Mercado *et al*, 2011), whereas inactivation of the IL impaired the acquisition and recall of extinction (Sierra-Mercado *et al*, 2011). In addition, cannabinoid, glutamatergic, and dopaminergic signaling regulates the formation and storage of fear memory (Gilmartin and Helmstetter, 2010; Laviolette and Grace, 2006; Runyan and Dash, 2004), whereas dopaminergic, glutamatergic, noradrenergic, and brain-derived neurotrophic factor signaling in the IL modulates the consolidation of extinction of fear memory (Burgos-Robles *et al*, 2007; Fontanez-Nuin *et al*, 2011; Mueller *et al*, 2010; Mueller *et al*, 2008; Peters *et al*, 2010). Extending these studies, we found that PKM ζ overexpression in the PrL but not IL enhanced the formation of long-term fear memory. The mPFC has been suggested to participate in the regulation of emotional states (Blanco *et al*, 2009; Covington *et al*, 2010; Meloni *et al*, 2008), which may contribute to the expression of fear (Beracochea *et al*, 2004; Ferreira and Nobre 2014). We found that PKM ζ overexpression in the PrL had no effect on short-term fear memory or anxiety-like behavior, suggesting a selective role for PKM ζ in the PrL in the regulation of long-term fear memory.

PKM ζ , a persistently active PKC isoform, has been shown to be involved in the maintenance of LTP (Ling *et al*, 2002) and long-term memories, including spatial learning (Pastalkova *et al*, 2006), auditory fear conditioning

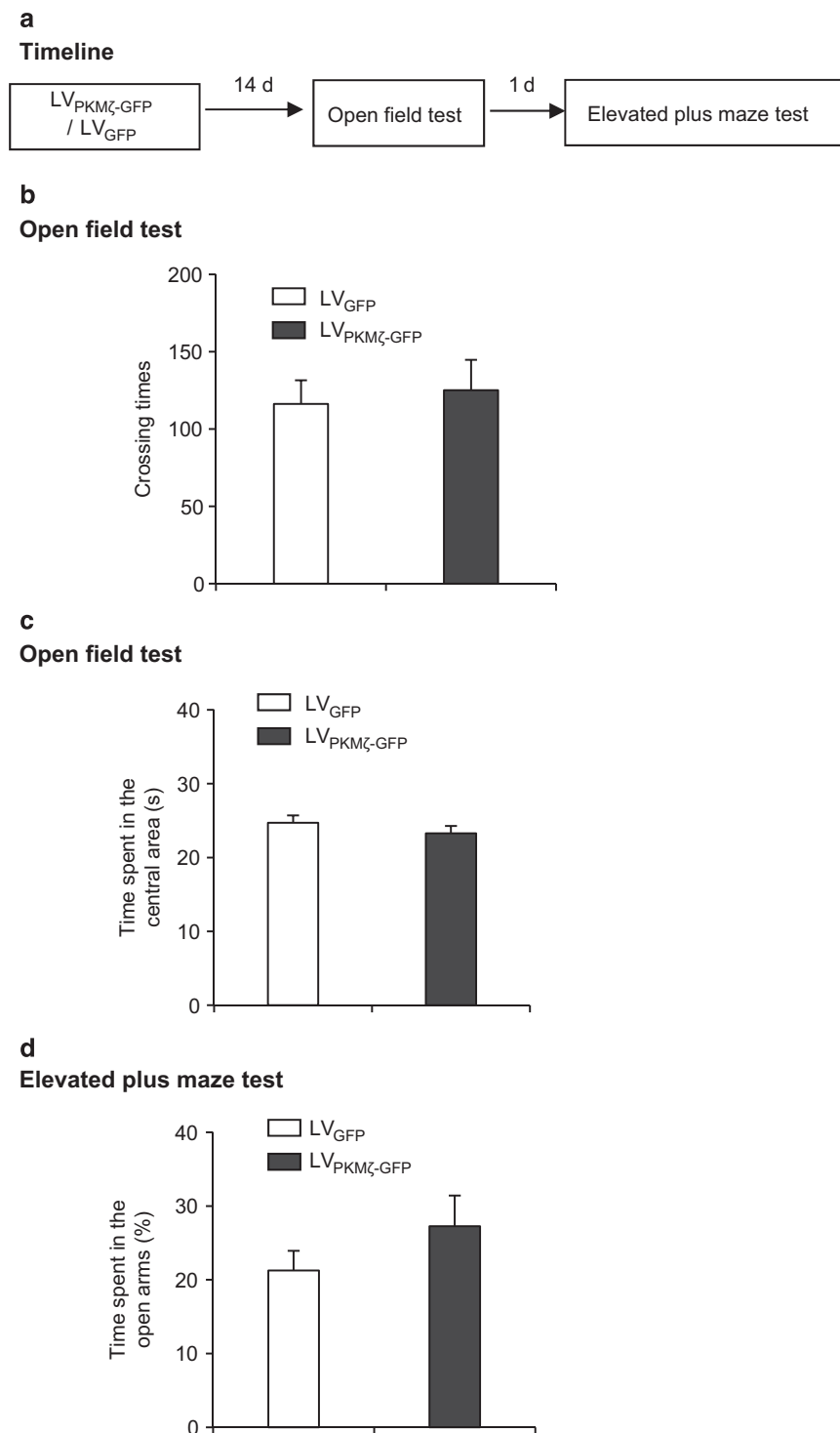


Figure 3 PKM ζ overexpression in the PrL has no effect on locomotor activity or anxiety-like behavior. (a) Timeline of the experiment. One week after LV_{PKM ζ -GFP} or LV_{GFP} infusions, all of the rats underwent the open field and elevated plus maze tests. (b-d) The two groups exhibited a similar number of crossings (b) and time spent in the central area (c) in the open field and a similar percentage of open arm time (d) in the elevated plus maze. The data are expressed as mean \pm SEM. $n = 8-10$ per group.

(Serrano *et al*, 2008), and conditioned taste aversion (Shema *et al*, 2007). We recently found that injections of the PKC ζ inhibitor ZIP into the nucleus accumbens core disrupted the maintenance of cocaine and morphine reward memory (Li *et al*, 2011). Injections of the PKC ζ inhibitor ZIP into the infralimbic mPFC abolished the extinction memories of

morphine-induced conditioned place preference and conditioned place aversion (He *et al*, 2011). However, two recent studies reported that PKM ζ is not necessary for memory maintenance. Using a global knockout approach that targeted exon 11 of PKM ζ , Volk *et al*, (2013) found that LTP induction, the maintenance of fear conditioning, and

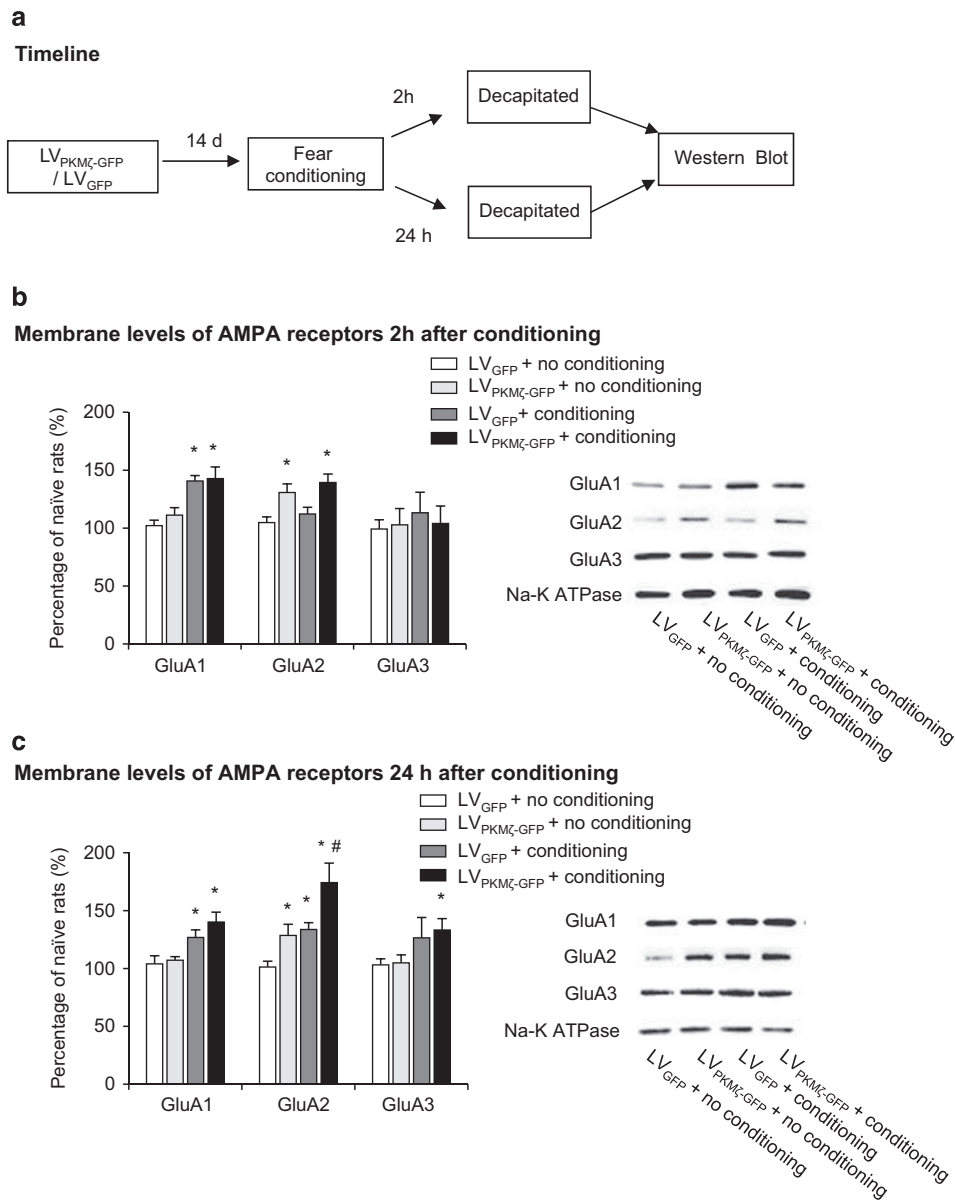


Figure 4 PKM ζ overexpression in the PrL potentiates the fear conditioning-induced increase in membrane GluA2 expression. (a) Timeline of the experiment. One week after LV_{PKM ζ -GFP} or LV_{GFP} infusions, all of the rats underwent fear conditioning or exposure to the conditioning chamber. The rats were decapitated for subsequent protein assessment 2 or 24 h later. (b) Fear conditioning significantly increased the membrane expression of GluA1 but not GluA2 or GluA3 during the phase of short-term memory. Intra-PrL infusion of LV_{PKM ζ -GFP} significantly increased the membrane expression of GluA2 in the groups with or without fear conditioning. (c) During the phase of long-term memory, fear conditioning significantly increased the membrane expression of GluA1, GluA2, and GluA3, and intra-PrL infusion of LV_{PKM ζ -GFP} significantly potentiated the increase in the membrane expression of GluA2. * $p < 0.05$, compared with LV_{GFP}+no conditioning group; # $p < 0.05$, compared with LV_{PKM ζ -GFP}+no conditioning group. The data are expressed as mean \pm SEM. $n = 5$ per group.

spatial memory in the Morris water maze were normal in knockout mice. Lee *et al*, (2013) similarly observed normal learning and memory, including cued fear conditioning, novel object recognition memory, and cocaine-induced conditioned place preference memory in knockout mice. However, both of these studies used PKM ζ knockout animals with disrupted PKM ζ expression before LTP induction or learning the behavioral task (Lee *et al*, 2013; Volk *et al*, 2013). The knockout of PKM ζ may have triggered compensatory responses that allowed for the normal maintenance of LTP and memory. Although Volk *et al*, (2013) also used

conditional knockout mice, they did not report any behavioral experiments for these mice. Furthermore, a preliminary study from Sacktor's laboratory showed that the constitutive knockout of PKC/PKM ζ resulted in a compensatory increase in phospho-Thr403 PKC ι/λ under basal conditions (Tsokas *et al*, 2012), and PKC ι/λ has been shown to be involved in the maintenance of LTP (Ren *et al*, 2013a). Others also found that ZIP may have an inhibitory effect on PKC ι/λ (Ren *et al*, 2013a), and using PKM ζ -shRNA to reduce the expression of PKM ζ without influencing PKC ι/λ abolished the fear memory (Dong *et al*, 2014). In our

studies, we used lentiviral vectors to overexpress PKM ζ , which selectively enhanced the expression of PKM ζ without affecting other isoforms of PKC. Notably, in the study by Shema *et al*, (2011), PKM ζ was overexpressed after memory formation and enhanced the maintenance of conditioned taste aversion. In the present study, PKM ζ was overexpressed before memory formation and enhanced the formation of long-term memory without influencing short-term memory. Furthermore, we found that PKM ζ overexpression potentiated the fear learning-induced increase in the membrane levels of GluA2. Thus, our findings extend Shema's study and suggest that PKM ζ overexpression may enhance synaptic plasticity and promote the formation and maintenance of long-term memory. We also found that PKM ζ overexpression in the IL had no effect on either short-term or long-term memory. However, regarding the role of the IL in fear memory (He *et al*, 2011; Quirk *et al*, 2006b), further studies are needed to determine the specific effect of PKM ζ overexpression on fear extinction and whether there is an optimal balance between PKM ζ in the PrL and IL to control the expression of fear responses after extinction.

Yao *et al*, (2008) reported that PKM ζ modulates late LTP in hippocampal slices, with the involvement of *N*-ethylmaleimide-sensitive factor (NSF)/GluA2 trafficking. *In vivo* studies showed that the magnitude of inhibition of fear memories induced by PKM ζ inhibition is correlated with the decrease in postsynaptic GluA2. Injection of Tat-GluA2_{3Y}, a peptide that inhibits GluA2 endocytosis, prevented the loss of long-term memory induced by both ZIP and lentiviral PKM ζ shRNA injections (Dong *et al*, 2014; Li *et al*, 2011; Miguez *et al*, 2010). Hara *et al*, (2012) also found that the synaptic distribution of GluA2 and PKM ζ in the monkey dentate gyrus is correlated with long-term memory performance. Extending these findings, we found that PKM ζ overexpression increased the membrane expression of GluA2 at baseline and potentiated the fear conditioning-induced increase in GluA2 during the long-term memory phase. In addition, the present study provides *in vivo* evidence that PKM ζ overexpression is sufficient to elicit the distribution of plasticity-related proteins and promote long-term memory-associated alterations of plasticity-related proteins. Our results are consistent with previous *in vitro* studies that PKM ζ overexpression is sufficient to produce the distribution of GluA2 and postsynaptic density-95 and alterations of the morphology and function of dendrite spines (Ron *et al*, 2012; Sebastian *et al*, 2013). However, the conclusion of the causal role of PKM ζ in the regulation of GluA2 trafficking during memory consolidation should be made with caution. Memory consolidation has a restricted time-window after memory acquisition (Johansen *et al*, 2011; McGaugh 2000), while LV-PKM ζ -GFP injections produce a long-lasting PKM ζ overexpression. Thus PKM ζ overexpression may potentiate the surface expression of GluA2 during the memory consolidation or after memory is consolidated. Fear conditioning induced an additive increase in membrane GluA2, which indicates some other scaffold proteins may regulate AMPAR trafficking during formation of long-term memory. Indeed, it has been demonstrated that NSF, glutamate receptor interacting protein (GRIP) and protein interacting with C-kinase-1 (PICK1), which have critical roles in the trafficking of AMPAR to synapse, are involved in the long-term memory (Carroll *et al*, 2001;

Cull-Candy *et al*, 2006; Isaac *et al*, 2007). Therefore, the causal role of these scaffold proteins in prelimbic cortex in the formation of long-term memory, and their interactions with PKM ζ to regulate GluA2 trafficking in cytoplasmic, synaptic and extrasynaptic compartments (Newpher and Ehlers 2008; Opazo and Choquet 2011) should be investigated in the future studies.

CONCLUDING REMARKS

In summary, we found that PKM ζ overexpression in the PrL but not IL enhanced the formation of fear memory, and PKM ζ in the PrL may contribute to the formation of long-term fear memory. The effect of PKM ζ overexpression on memory formation was not attributable to alterations in locomotor activity or the emotional state of rats. The potentiating effect of PKM ζ overexpression on long-term memory may be mediated by the promotion of membrane GluA2 expression. The present study extends our understanding of the role of PKM ζ in learning and memory and related neurological disorders.

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