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PKMζ Maintains Remote Contextual Fear Memory by Inhibiting GluA2-Dependent AMPA Receptor Endocytosis in the Prelimbic Cortex

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Abstract—Fear memories allow animals to recognize and adequately respond to dangerous situations. The prelimbic cortex (PrL) is a crucial node in the circuitry that encodes contextual fear memory, and its activity is central for fear memory expression over time. However, while PrL has been implicated in contextual fear memory storage, the molecular mechanisms underlying its maintenance remain unclear. Protein kinase M zeta (PKMζ) is a persistently active enzyme which has been shown to maintain many forms of memories by inhibiting the endocytosis of GluA2-containing AMPA receptors. Therefore, we hypothesized that PKMζ action upon GluA2-containing AMPARs could be a mechanism for contextual fear memory maintenance in the PrL. To test this hypothesis, we trained rats in a contextual fear conditioning (CFC) paradigm and administered intra-PrL infusions of the PKMζ inhibitor ZIP, the GluA2-dependent endocytosis inhibitor $GluA2_{3Y}$ or the inactive peptide $GluA2_{3Y(s)}$, either two or twenty days after conditioning, and assessed long-term memory retention twenty-four hours later. We found that acute inhibition of GluA2-dependent AMPAR endocytosis in the PrL does not affect recent or remote contextual fear memory maintenance. Also, PKMζ inhibition in the PrL does not impair the maintenance of recent contextual fear memory. However, we found that inhibition of prelimbic PKMζ at a remote time point disrupts contextual fear memory maintenance, and that blocking GluA2-dependent removal of AMPARs prevents this impairment. Our results confirm the central role of PrL in fear memory and identify PKMζ-induced inhibition of GluA2-containing AMPAR endocytosis as a key mechanism governing remote contextual fear memory maintenance.

This article is part of a Special Issue entitled: In memory of Ivan Izquierdo South American pioneer of the Neuroscience of Memory Temporal dynamics and molecular mechanisms.

Key words: memory, contextual fear conditioning, persistence, PKMzeta, GluA2, prelimbic cortex.

INTRODUCTION

Fear memories are indispensable for survival, inasmuch as they permit animals to recognize dangerous situations and display adequate defensive responses (Maren, 2001; Izquierdo et al., 2016). Learning that arises from fearful experiences generates some of the strongest and most enduring memories of an organism, which may persist for a lifetime (Izquierdo et al., 2016). In contextual fear conditioning (CFC), a form of Pavlovian conditioning, animals learn to associate an initially neutral environment with an aversive stimulus, and subsequently exhibit defensive responses to the context that predicts threat (Maren, 2001; Maren et al., 2013; Izquierdo et al., 2016). Memories are believed to be encoded through enduring physical and chemical alterations in distributed neuronal circuits throughout the brain (Takeuchi et al., 2014; Tonegawa et al., 2015; Josselyn and Tonegawa, 2020). For CFC memory, these circuits include the hippocampus, the amygdala and the medial prefrontal cortex (mPFC) (Maren et al., 2013; Izquierdo et al., 2016; Rao-Ruiz et al., 2021).

Within the mPFC, the prelimbic cortex (PrL) is a crucial hub in the fear circuitry (Giustino and Maren, 2015; Izquierdo et al., 2016). The PrL is extensively connected with both cortical and subcortical brain regions involved in fear memory processing (Vertes, 2004; Gabbott et al., 2005). It receives direct excitatory inputs from the ventral hippocampus (Jay et al., 1989; Hoover and Vertes, 2007), which transmit contextual information to this region (Twining et al., 2020). Moreover, the PrL is bidirectionally connected with the amygdala, with

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cortical axons innervating the basolateral amygdala (BLA), and inputs from the BLA terminating predominantly in PrL layers 2 and 5 (Bacon et al., 1996; McDonald et al., 1996; Vertes, 2004).

The PrL is critical for the acquisition, consolidation and expression of conditioned fear (Corcoran and Quirk, 2007; Gilmartin and Helmstetter, 2010; Do-Monte et al., 2015; Kitamura et al., 2017; DeNardo et al., 2019). CFC requires protein synthesis (Rizzo et al., 2017) and N-methyl-D-aspartate (NMDA) receptor activity (Gilmartin and Helmstetter, 2010) in the PrL, which are required for recent (few days) memory retrieval. Furthermore, activity in the PrL during fear conditioning is essential to permit the organization of the ensembles that will support memory retrieval at remote (weeks) time points (Kitamura et al., 2017; DeNardo et al., 2019). Fear conditioning recruits neurons in the PrL that already hold the memory trace one day after learning, but are unable to support memory retrieval at this point. As these cells undergo plastic modifications over time, they become crucial for remote memory expression (Kitamura et al., 2017), along with neurons recruited into the ensemble later in time (DeNardo et al., 2019; Quiñones-Laracuente et al., 2021). So, even though the role of PrL neurons in supporting fear memory across time has been firmly established, the molecular mechanisms underlying the maintenance of the memory trace in the PrL during recent and remote time points remain largely elusive.

One of the mechanisms suggested to be responsible for maintaining memories in the brain is the autonomously active enzyme protein kinase M zeta (PKMζ). PKMζ is an atypical protein kinase C isoform, which lacks the pseudosubstrate autoinhibitory domain (Sacktor et al., 1993; Hernandez et al., 2003). This unique characteristic renders the enzyme persistently active after its formation (Hernandez et al., 2003; Sacktor, 2011). PKMζ has been shown to be both necessary and sufficient for the maintenance of long-term potentiation (LTP; Ling et al., 2002), a putative cellular model of learning and memory (Lynch, 2004; Malenka and Bear, 2004; Takeuchi et al., 2014). Also, PKMζ has been shown to maintain many forms of memories (Pastalkova et al., 2006; Serrano et al., 2008; Shema et al., 2009; Migues et al., 2010), including contextual fear memory in the amygdala for up to a week (Kwapis et al., 2009; 2012). One of the mechanisms involved on the role of PKMζ in the maintenance of synaptic potentiation and of memories is its regulation of the trafficking of GluA2-containing α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs; Yao et al., 2008; Migues et al., 2010). PKMζ inhibits the endocytosis of GluA2-containing AMPARs (Migues et al., 2010) and limits their lateral diffusion (Yu et al., 2017), which results in an augmented concentration of these receptors in the postsynaptic membrane.

Therefore, in this study, we used a pharmacological approach to investigate whether PKMζ underlies the maintenance of contextual fear memory at recent (3 days) and remote (21 days) long-term memory intervals in the PrL, and whether it does so by inhibiting the endocytosis of GluA2-containing AMPARs.

EXPERIMENTAL PROCEDURES

Animals

Male *Wistar* rats (CrlCembe:WI, 3-month-old, 300–330 g) were obtained from the Centro de Modelos Biológicos Experimentais (CeMBE) of the Pontifical Catholic University of Rio Grande do Sul (PUCRS). Rats were housed four to a cage, maintained under a 12:12-hour light/dark cycle (lights on at 7 a.m.) and allowed access food and water *ad libitum*. All procedures were approved by the Animal Committee on Ethics for the Care and Use of Laboratory Animals of PUCRS, in compliance with National Institutes of Health guidelines for the care and use of laboratory animals.

Surgery

Animals were anesthetized with intraperitoneal (i.p.) injections of ketamine (75 mg/kg) and xylazine (10 mg/kg) and submitted to stereotaxic surgery in order to implant bilateral stainless steel 22-gauge guide cannulae aimed 1.0 mm above the prelimbic cortex (PrL; anterior + 3.2 mm, lateral ± 0.8 mm, ventral −3.0 mm, relative to bregma, according to Paxinos & Watson, 2007). The guide cannulae were fixed to the skull with dental acrylic cement. All animals were allowed seven days for recovery from surgery before behavioral procedures. Animals were handled once daily for 3 consecutive days before behavioral experiments.

Contextual fear conditioning

Contextual fear conditioning was performed in a conditioning chamber placed inside a larger, sound-attenuating box (Panlab®, Barcelona-Spain). The conditioning chamber consisted of three aluminum walls (35 × 35 × 35 cm) and a transparent acrylic front door. The chamber's floor was made up of parallel 3 mm-caliber stainless steel bars spaced 1 cm apart, connected to an energy source for footshock delivery. Before each phase of the experiment, animals were transported in their home cages from the vivarium to the experimental room, and allowed 20 min for acclimation. The conditioning chamber was cleaned with 70% (v/v) ethanol prior to each experiment.

On day 1 (training day; Tr), rats were placed inside the conditioning chamber and, after 120 s, received five scrambled footshocks (2-s duration, 0.7 mA, 30-s interval). Thirty seconds after the last footshock, animals were returned to their home cages. After 3 days (recent memory) or 21 days (remote memory), animals were placed again in the conditioning chamber for a 180-s retention test, without electric shock. The time the animals spent freezing (as defined by complete immobility except for respiratory movements) was measured by an experienced researcher unaware of the animal's experimental condition, during the first 120 s of Tr session and during the 180 s of the retention test and converted into a percentage of the total time. Freezing time and total session time were measured using

stopwatches and a minimum of 1 s of immobilization was considered as freezing.

Drugs and infusions

ZIP (Myr-SIYRRGARRWRKL-OH, AnaSpec®), a PKMζ inhibitor peptide; Tat-GluA2$_{3Y}$ (YGRKKRRQRRRYKEGYNVYG-OH, AnaSpec®), a GluA2-containing AMPAR endocytosis inhibitor peptide; and Tat-GluA2$_{3Y(s)}$ (scrambled, YGRKKRRQRRRVYKYGGYNE-OH, AnaSpec®), the inactive control peptide; were dissolved to the appropriate concentrations in sterile saline (NaCl, 0.9%), which also served as the Vehicle. The doses used in all experiments were: ZIP, 10 nmol per side; Tat-GluA2$_{3Y}$, 100 pmol per side; Tat-GluA2 $_{3Y(s)}$, 100 pmol per side (Pastalkova et al., 2006; Migues et al., 2010; Dong et al., 2015). All infusions were bilateral and a total volume of 1.0 μl per side was infused into the PrL. The infusion procedure was performed using a Hamilton microsyringe tightly connected via polyethylene tubing to a 30-gauge infusion needle, which was introduced into the guide cannulae and protruded 1.0 mm beyond its ends. Infusion was carried out at a 1.0-μl/min rate and the needle was left in place for additional 1.0 min to ensure proper drug diffusion and prevent backflow. At the end of the infusion, needle was carefully withdrawn, and the procedure was repeated on the other side.

Cannulae placements

Correct cannulae placements were verified 2 days after the end of the last behavioral procedure. Animals were infused with 1.0 μl of 4% methylene blue, as described above, anesthetized and killed by decapitation 15 min later. Cannula placement was considered correct when the spread was 1.0 mm^3 from the intended infusion site. Fig. 1 depicts a schematic drawing of the infusion sites.

Statistical analyses

Data are expressed as mean and standard error of the mean (S.E.M). The percentage of time spent freezing was analyzed using two-way ANOVA with treatment and session as factors, followed by Bonferroni's *post hoc* test. Data from the test session were confirmed for normality using the Shapiro-Wilk test, with $p > 0.05$. All data were analyzed using GraphPad Prism® software. A p-value < 0.05 was considered statistically significant.

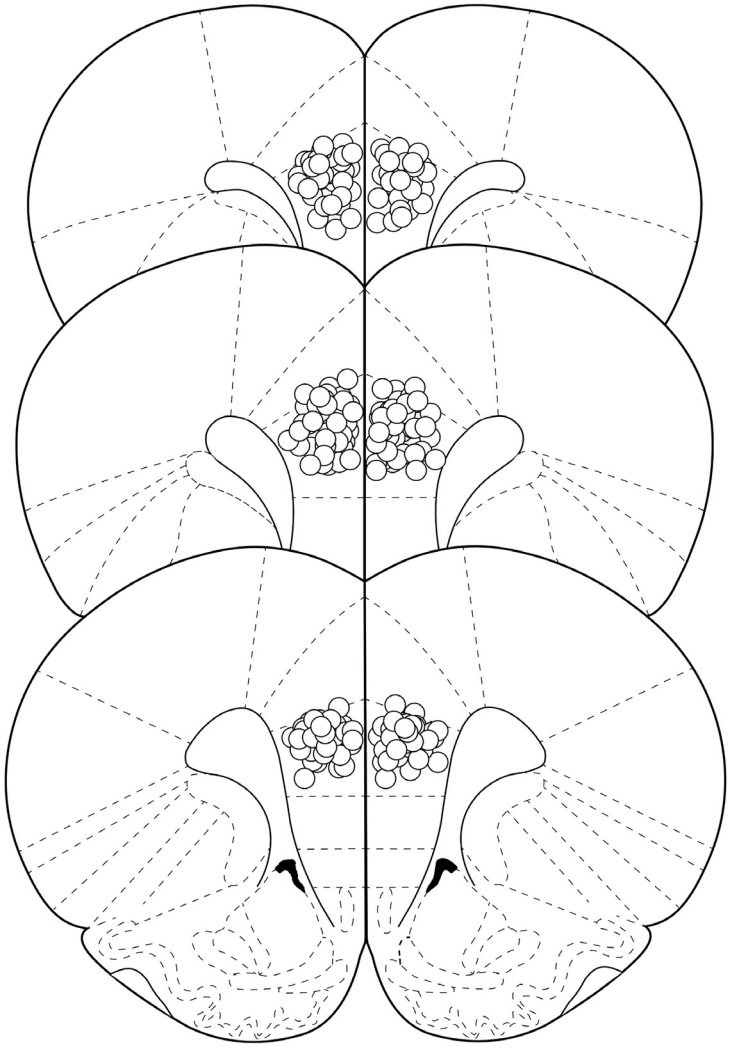

Fig. 1. Schematic representation of the infusion sites in the prelimbic cortex. Schematic drawing depicting the position of the injection needle tips (circles) in the prelimbic cortex. Adapted from Paxinos and Watson, 2007.

RESULTS

Effect of ZIP infusion into the PrL on the maintenance of recent contextual fear memory

First, we investigated whether PKMζ is required for the maintenance of recent contextual fear memory in the PrL. Animals were submitted to CFC and, two days later, different groups of animals received bilateral intra-PrL infusions of either ZIP (10 nmol) or vehicle (Veh; NaCl 0.9%). Twenty-four hours after the infusions, long-term memory was evaluated in the retention test (Fig. 2). Two-way repeated-measures ANOVA demonstrated effects of session ($F_{(1,15)} = 129.7$, $p < 0.0001$), but no effect of treatment ($F_{(1,15)} = 0.2876$, $p = 0.5996$) or session × treatment interaction ($F_{(1,15)} = 0.2670$, $p = 0.6129$). Bonferroni's *post hoc* test detected no differences in time spent freezing between groups during training session ($p > 0.9999$) or during the

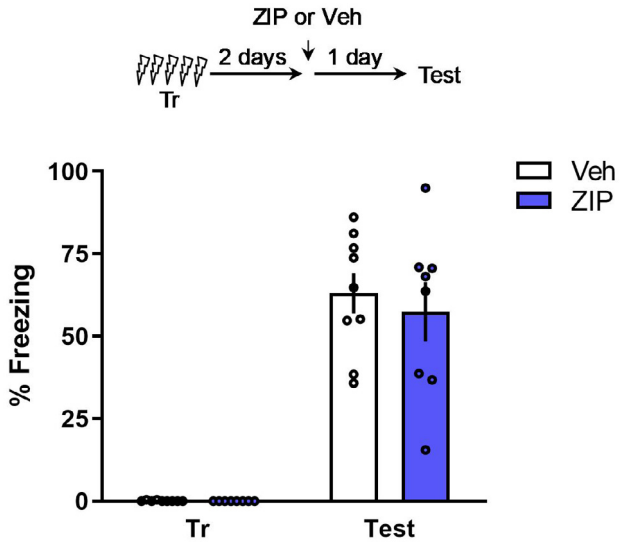

Fig. 2. Intra-PrL infusion of ZIP does not affect recent CFC memory. Animals were subjected to CFC training session (Tr) and two days later received bilateral intra-PrL infusions of vehicle (Veh; NaCl 0.9%) or of the PKMζ inhibitor, ZIP (10 nmol). Twenty-four hours later, long-term memory was evaluated in a 3 min-retention test. Animals treated with Veh or ZIP did not differ in the amount of time spent freezing (*n* Veh = 9, *n* ZIP = 8). Percentages of time spent freezing on the first 2 min of Tr and on the 3-min retention test were analyzed by two-way repeated measures ANOVA followed by Bonferroni's multiple comparison test. Data expressed as mean ± S.E.M. (Upper) Schematic representation of the behavioral protocols.

retention test (Veh *vs.* ZIP, $p = 0.9246$; *n* Veh = 9, *n* ZIP = 8). This result suggests that PKMζ is not required in the PrL for the maintenance of recent CFC memory.

Effect of GluA2$_{3Y}$ and GluA2$_{3Y(s)}$ infusions into the PrL on the maintenance of recent contextual fear memory

We investigated whether inhibition of GluA2-containing AMPA receptor endocytosis in the PrL interferes with the maintenance of recent CFC memory. To address this issue, animals were trained in the CFC paradigm and, 2 days later, received bilateral intra-PrL infusions of GluA2$_{3Y}$ (100 pmol), GluA2$_{3Y(s)}$ (100 pmol) or vehicle (Veh; NaCl 0.9%). Twenty-four hours after the infusion, animals underwent a recent CFC memory test (Fig. 3). Two-way repeated-measures ANOVA showed an effect of session ($F_{(1,20)} = 108.7$, $p < 0.0001$), but no effect of treatment ($F_{(2,20)} = 0.5229$, $p = 0.6007$) or session × treatment interaction ($F_{(2,20)} = 0.5182$, $p = 0.6034$). Bonferroni's posttest revealed no differences between groups during training session ($p > 0.9999$) and no differences during the retention test (Veh *vs.* GluA2$_{3Y}$, $p = 0.6583$; Veh *vs.* GluA2$_{3Y(s)}$, $p > 0.9999$; GluA2$_{3Y}$ *vs.* GluA2$_{3Y(s)}$, $p = 0.6694$; *n* Veh = 7, *n* GluA2$_{3Y}$ = 8, *n* GluA2$_{3Y(s)}$ = 8), when animals from all groups froze during similar percentages of time. This result suggests that inhibition of GluA2-containing AMPAR endocytosis does not affect the maintenance of recent CFC memory.

Effect of ZIP infusion into the PrL on the maintenance of remote contextual fear memory

In order to verify whether PKMζ is necessary in the PrL for the maintenance of remote CFC memory, different groups of animals received bilateral intra-PrL infusions of ZIP (10 nmol) or vehicle (Veh; NaCl 0.9%) twenty days after CFC training session and were tested 24 h later (Fig. 4). Two-way repeated-measures ANOVA showed effects of session ($F_{(1,19)} = 92.99$, $p < 0.0001$), treatment ($F_{(1,19)} = 21.83$, $p = 0.0002$) and interaction between factors ($F_{(1,19)} = 22.09$, $p = 0.0002$). Bonferroni's posttest revealed that while there were no differences between groups during training session ($p > 0.9999$), animals that were treated with ZIP displayed significantly less freezing behavior during the retention test than the Veh-treated ones (Veh *vs.* ZIP, $p < 0.0001$; *n* Veh = 11, *n* ZIP = 10). This result suggests that ZIP impaired the maintenance of remote CFC memory and, therefore, PKMζ activity in the PrL is required for the persistence of remote CFC memory.

Effect of GluA2$_{3Y}$ and GluA2$_{3Y(s)}$ infusions into the PrL on the maintenance of remote contextual fear memory

In order to investigate whether the inhibition of GluA2-dependent AMPAR endocytosis alters the maintenance of remote CFC memory, different groups of animals received bilateral intra-PrL infusions of GluA2$_{3Y}$ (100 pmol), GluA2$_{3Y(s)}$ (100 pmol) or vehicle (Veh; NaCl 0.9%) twenty days after CFC training session and were tested 24 h later (Fig. 5). Two-way repeated-measures ANOVA showed an effect of session ($F_{(1,29)} = 95.24$, $p < 0.0001$), but no effect of treatment ($F_{(2,29)} = 0.2178$, $p = 0.8056$) or session × treatment interaction ($F_{(2,29)} = 0.2198$, $p = 0.8040$). Bonferroni's posttest revealed neither difference between groups during training session ($p > 0.9999$) nor during the retention test (Veh *vs.* GluA2$_{3Y}$, Veh *vs.* GluA2$_{3Y(s)}$, GluA2$_{3Y}$ *vs.* GluA2$_{3Y(s)}$, $p > 0.9999$; *n* Veh = 11, *n* GluA2$_{3Y}$ = 11, *n* GluA2$_{3Y(s)}$ = 10), when all groups spent similar amounts of time in freezing behavior. Therefore, blocking the endocytosis of GluA2-containing AMPAR *per se* does not affect remote CFC memory persistence.

Effect of GluA2$_{3Y}$ and GluA2$_{3Y(s)}$ infusions into the PrL on the impairment induced by ZIP on the maintenance of remote contextual fear memory

Since PKMζ has been shown to maintain memories in the amygdala and in the hippocampus by inhibiting GluA2-containing AMPAR endocytosis (Migues et al., 2010), we sought to investigate whether PKMζ acts via the same mechanism to maintain remote CFC memory in the prelimbic cortex. For this purpose, animals received bilateral intra-PrL infusions of ZIP (10 nmol), ZIP (10 nmol) plus GluA2$_{3Y}$ (100 pmol), ZIP (10 nmol) plus GluA2$_{3Y(s)}$

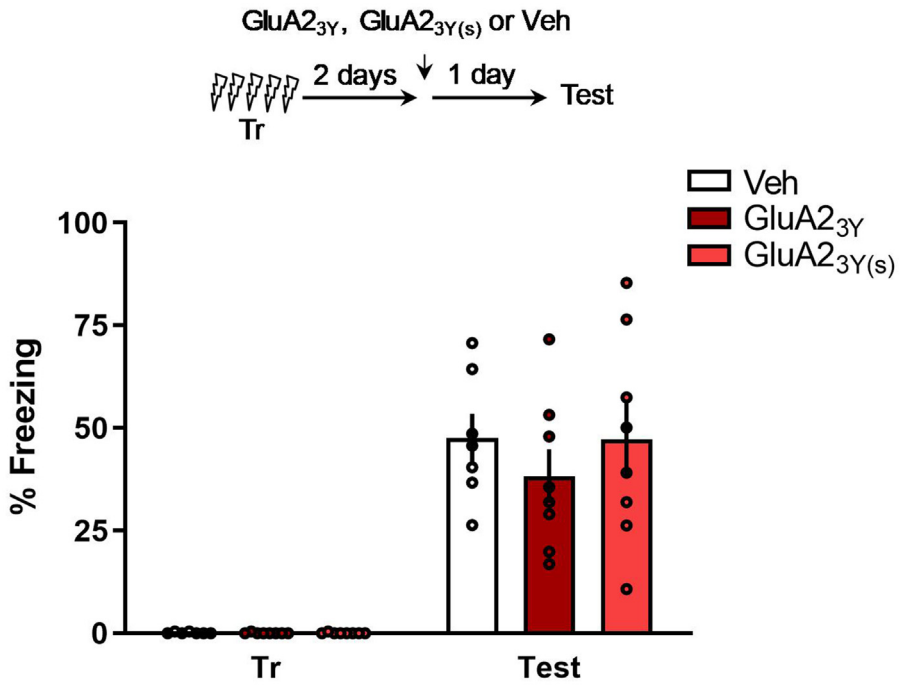

Fig. 3. Intra-PrL infusions of GluA2$_{3Y}$ or GluA2$_{3Y(s)}$ do not affect recent CFC memory. Animals were subjected to CFC training session (Tr) and two days later received bilateral intra-PrL infusions of vehicle (Veh; NaCl 0.9%), the GluA2-containing AMPAR endocytosis inhibitor peptide, GluA2$_{3Y}$ (100 pmol), or the scrambled control peptide, GluA2$_{3Y(s)}$ (100 pmol). Twenty-four hours later, long-term memory was evaluated in a 3 min-retention test. Animals treated with Veh, GluA2$_{3Y}$ or GluA2$_{3Y(s)}$ did not differ in the amount of time spent freezing (n Veh = 7, n GluA2$_{3Y}$ = 8; n GluA2$_{3Y(s)}$ = 8). Percentages of time spent freezing on the first 2 min of Tr and on the 3-min retention test were analyzed by two-way repeated measures ANOVA followed by Bonferroni's multiple comparison test. Data expressed as mean ± S.E.M. (Upper) Schematic representation of the behavioral protocols.

(100 pmol) or vehicle (Veh; NaCl 0.9%) twenty days after CFC training session and were tested 24 h later (Fig. 6). Two-way repeated-measures ANOVA showed effects of session ($F_{(1,33)}$ = 145.6, p < 0.0001), treatment ($F_{(3,33)}$ = 10.28, p < 0.0001) and session × treatment interaction ($F_{(3,33)}$ = 10.37, p < 0.0001). Bonferroni's posttest detected no differences among groups during training (p > 0.9999), but revealed that animals that received infusions of ZIP (n = 10) or ZIP + GluA2$_{3Y(s)}$ (n = 10) spent significantly less time freezing when compared with those who received Veh (ZIP *vs.* Veh, ZIP + GluA2$_{3Y(s)}$ *vs.* Veh, p < 0.0001; n Veh = 10) or ZIP + GluA2$_{3Y}$ (ZIP *vs.* ZIP + GluA2$_{3Y}$, ZIP + GluA2$_{3Y(s)}$ *vs.* ZIP + GluA2$_{3Y}$, p < 0.0001; n ZIP + GluA2$_{3Y}$ = 7). Also, the group that received ZIP + GluA2$_{3Y}$ did not differ from the group that received Veh (ZIP + GluA2$_{3Y}$ *vs.* Veh, p > 0.9999), neither did the group that received ZIP + GluA2$_{3Y(s)}$ differ from ZIP (ZIP + GluA2$_{3Y(s)}$ *vs.* ZIP, p > 0.9999). This set of results suggests that inhibition of GluA2-dependent AMPARs internalization with GluA2$_{3Y}$ prevents ZIP's amnesic effect, while the inactive GluA2$_{3Y(s)}$ fails to impede the memory deficit. Therefore, the results indicate that PKMζ maintains remote CFC memory in the prelimbic cortex by preventing the removal of GluA2-containing AMPARs from the synapses.

DISCUSSION

In the present study we investigated the role of PKMζ and GluA2-containing AMPARs in the PrL on the maintenance of recent and remote contextual fear memories. We found that inhibition of PKMζ in the PrL does not affect the maintenance of contextual fear memory at a recent time point. We also show that acute inhibition of GluA2-containing AMPAR endocytosis in the PrL *per se* does not alter the maintenance of either recent or remote contextual fear memories. However, we demonstrated that PKMζ activity in the PrL supports the persistence of remote contextual fear memory. Moreover, we showed that PKMζ maintains remote contextual fear memory in the PrL by inhibiting the internalization of GluA2-containing AMPARs.

The theory of systems consolidation posits that as memories get older their dependence shifts from the hippocampus to neocortical areas (Squire, 1992; Frankland et al., 2004; Frankland and Bontempi, 2005; Kitamura et al., 2017). In this regard, it has been demonstrated that contextual fear conditioning forms engram neurons in the prelimbic region early during conditioning, but these cells are in a silent state and cannot drive natural memory retrieval at this stage. Over time, with inputs from the hippocampus, these cells are unsilenced and become central in promoting remote contextual fear memory retrieval (Kitamura et al., 2017). Another study found that, in cued fear conditioning, although neurons in the PrL recruited at the time of conditioning make smaller contributions to remote memory retrieval than cells recruited later in time, early PrL activity is required for the reorganization of the ensemble (DeNardo et al., 2019). Also, recent studies found enduring changes in the PrL following CFC (Pan et al., 2020; Chaaya et al., 2021). It has been described that CFC induces oligodendrogenesis and myelin formation in the PrL, which are essential for the preservation of remote, but not recent, contextual fear memory (Pan et al., 2020). CFC has also been shown to produce immunohistochemical alterations in the PrL that are detectable two weeks after conditioning, such as an increase in the number of phospho-mitogen activated protein kinase-expressing neurons and brain-derived neurotrophic factor-expressing cells (Chaaya et al., 2021). In our study, we found that PKMζ underlies the maintenance of remote, but not recent, contextual fear memory in the PrL by inhibiting the internalization of GluA2-containing AMPARs. Our findings corroborate the notion

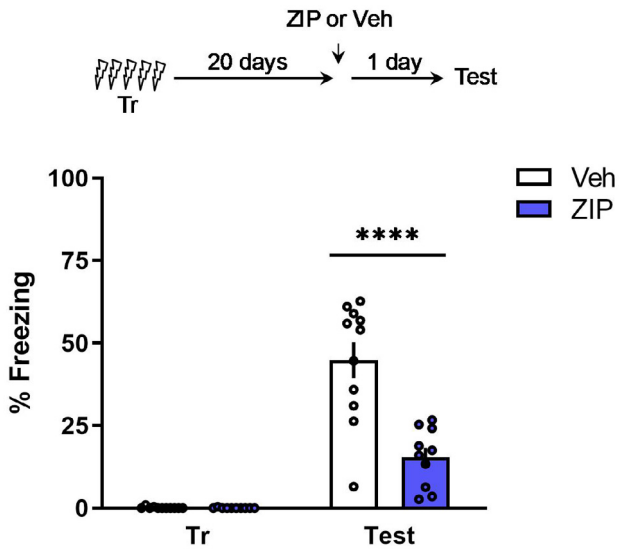

Fig. 4. Intra-PrL infusion of ZIP impairs remote CFC memory. Animals were subjected to CFC training session (Tr) and twenty days later received bilateral intra-PrL infusions of vehicle (Veh; NaCl 0.9%) or of the PKMζ inhibitor, ZIP (10 nmol). Twenty-four hours later, long-term memory was evaluated in a 3 min-retention test. Animals treated with ZIP spent significantly less time in freezing than animals treated with Veh (n Veh = 11, n ZIP = 10). Percentages of time spent freezing on the first 2 min of Tr and on the 3-min retention test were analyzed by two-way repeated measures ANOVA followed by Bonferroni's multiple comparison test. Data expressed as mean ± S.E.M. **** $p < 0.0001$ Veh *vs.* ZIP. (Upper) Schematic representation of the behavioral protocols.

that PrL serves as a fundamental site for memory storage, especially during remote time points, and provide a molecular mechanism for the maintenance of the prelimbic memory trace.

Moreover, our results are in line with previous studies that have pointed to a role for prelimbic PKMζ in fear memory processing. CFC increases the expression of PKMζ and GluA2 in the PrL of young and adult rats, but not in aged rats with cognitive impairment, however, PKMζ overexpression is able to block the memory deficits in the latter (Chen et al., 2016). Furthermore, overexpression of PKMζ in the PrL, but not in the infralimbic cortex, enhances memory formation for tone fear conditioning and increases membrane levels of the GluA2 subunit (Xue et al., 2015).

Persistence of contextual fear memory following reactivation has also shown to be dependent on prelimbic PKMζ (da Silva et al., 2020). This study found no impairment in remote memory expression when ZIP was infused in the PrL without memory reactivation. However, their infusion took place one day after conditioning, a time point when prelimbic engram neurons might not yet require PKMζ, since they are still not able to support memory retrieval (Kitamura et al., 2017). Moreover, reports have shown that memory maintenance mechanisms are insensitive to ZIP during or immediately after memory encoding, and ZIP's ability to induce amnesia might be reengaged roughly two days after acquisition or retrieval (Shema et al., 2009; Levitan et al., 2016).

It is interesting to notice that although the dorsal hippocampus is critically involved in the formation and retrieval of contextual fear memory (Anagnostaras et al., 2001; Izquierdo et al., 2016; Kitamura et al., 2017), the maintenance of CFC memory is unaffected by PKMζ inhibition in this area, either by ZIP infusions (Serrano et al., 2008; Kwapis et al., 2009) or shRNA-induced knockdown (Wang et al., 2016). In stark contrast, ZIP disrupts 1-day and 7-day CFC memory in the BLA (Kwapis et al., 2012) and 21-day CFC memory in the PrL, as demonstrated here. This suggests that storage of CFC memory at enduring sites in the BLA and PrL circuitries is maintained by PKMζ, whilst maintenance at the transitory hippocampal sites might be achieved through a different mechanism.

Amnesia in experiments that involve inhibition of PKMζ during the memory maintenance phase has traditionally been interpreted as a result of memory erasure (Sacktor, 2011; Sacktor and Fenton, 2018). However, the distinction between retrieval and storage failure explanations for retrograde amnesia has been a long-standing debate in neuroscience (Gold and King, 1974; Hardt et al., 2009). It has been recently proposed that synaptic plasticity may not be required for the actual storage of learned information, but rather to permit access to it, i.e., to enable memory retrieval (Ryan et al., 2015; Roy et al., 2017). Taking this into account, amnesia that results from experiments that interfere with synaptic plasticity mechanisms, including ours, could possibly reflect that the memory is inaccessible albeit still inscribed in the brain. Nevertheless, since memory utility for organisms depends intrinsically on its ability to be retrieved, PKMζ and other synaptic plasticity mechanisms remain key for the physiology of memory.

It is important to mention that we chose to compare ZIP with vehicle and not with its scrambled version (Scr-ZIP) to avoid potential confounding factors in our results, since reports indicate that Scr-ZIP retains the ability to inhibit PKMζ, even though with considerably lower affinity (Lee et al., 2013). ZIP also seems to be less specific than originally thought (Lee et al., 2013; Volk et al., 2013; Song et al., 2020). Besides PKMζ, ZIP also inhibits PKCι/λ, a related atypical enzyme from the PKC family (Tsokas et al., 2016; Wang et al., 2016). However, while PKMζ acts on the maintenance of long-term potentiation (Sacktor et al., 1993; Ling et al., 2002; Yao et al., 2008) and the persistence of long-term memories (Pastalkova et al., 2006; Shema et al., 2007; Serrano et al., 2008; Kwapis et al., 2012), PKCι/λ is involved in the induction of long-term potentiation and early memory consolidation (Wang et al., 2016), and it only supports memory maintenance in PKMζ-knockout animals (Tsokas et al., 2016). Besides that, PKCι/λ exerts its actions via interactions with the GluA1 subunit (Ren et al., 2013), while PKMζ interacts specifically with the GluA2 subunit (Yao et al., 2008; Migues et al., 2010; Xue et al., 2015).

Recently, additional mechanisms were described by which ZIP interferes with the maintenance of memories. ZIP was found to activate glycogen synthase kinase 3 beta (GSK-3β) *in vitro*, and GSK-3β blockade attenuated both ZIP-induced depotentiation of lateral amygdala synapses potentiated by fear and ZIP-induced

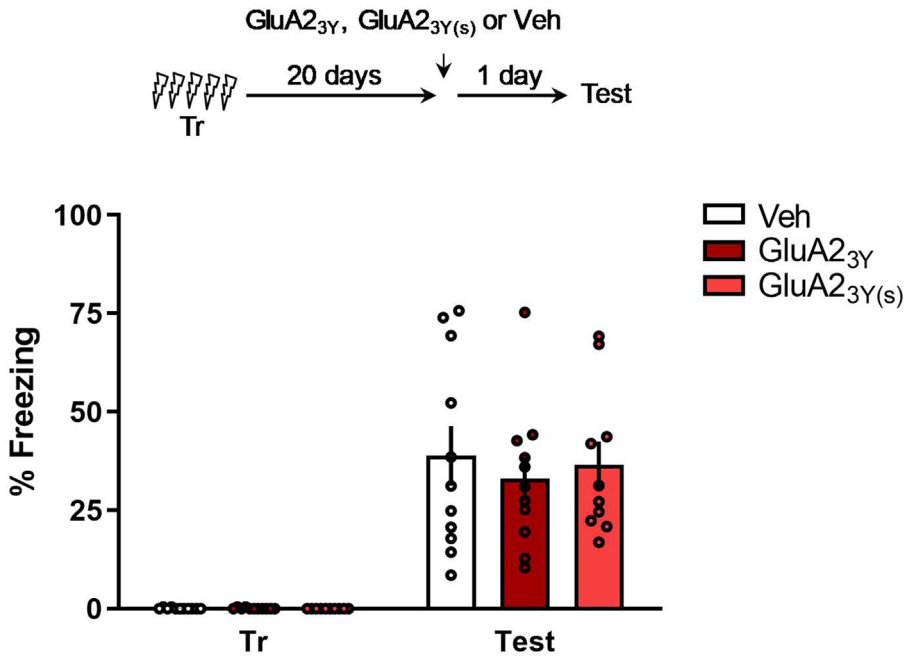

Fig. 5. Intra-PrL infusions of GluA2$_{3Y}$ or GluA2$_{3Y(s)}$ do not affect remote CFC memory. Animals were subjected to CFC training session (Tr) and twenty days later received bilateral intra-PrL infusions of vehicle (Veh; NaCl 0.9%), the GluA2-containing AMPAR endocytosis inhibitor peptide, GluA2$_{3Y}$ (100 pmol), or the scrambled control peptide, GluA2$_{3Y(s)}$ (100 pmol). Twenty-four hours later, long-term memory was evaluated in a 3 min-retention test. Animals treated with Veh, GluA2$_{3Y}$, GluA2$_{3Y(s)}$ did not differ in the amount of time spent freezing (n Veh = 11, n GluA2$_{3Y}$ = 11; n GluA2$_{3Y(s)}$ = 10). Percentages of time spent freezing on the first 2 min of Tr and on the 3-min retention test were analyzed by two-way repeated measures ANOVA followed by Bonferroni's multiple comparison test. Data expressed as mean ± S.E.M. (Upper) Schematic representation of the behavioral protocols.

disruption of learned fear (Song et al., 2020). Another work found that ZIP might act as an arginine donor, and that its effects on synaptic plasticity and memory would depend on a nitric-oxide-dependent downregulation of AMPARs that contain the GluA1 subunit (Bingor et al., 2020). Although we cannot completely rule out the influence of such mechanisms on the results we observed, because PKMζ potentiates synaptic transmission specifically by blocking GluA2-containing AMPAR endocytosis, the fact that administration of the selective GluA2-containing AMPAR endocytosis inhibitor peptide, GluA2$_{3Y}$, completely reversed the memory deficits induced by ZIP, strongly supports our hypothesis that PKMζ inhibition in the PrL degrades the remote memory trace by promoting GluA2-AMPAR internalization.

AMPA receptors are composed of four subunits, GluA1-GluA4, which assemble into a tetrameric structure. Different subunit combinations endow the receptor with distinct functional properties (Shepherd and Huganir, 2007). While AMPARs lacking GluA2 subunit allow the passage of calcium ions, GluA2-containing AMPARs are calcium-impermeable (Diering and Huganir, 2018). GluA2-lacking AMPA receptors (e.g. GluA1 homomers) are involved in acute synaptic plasticity, being rapidly delivered to synapses following LTP induction and experience-dependent synaptic plasticity (Clem and Barth, 2006; Plant et al., 2006; Choquet, 2010). These receptors, however, are less stable at the synapse, and are only transiently expressed, being subsequently replaced by GluA2-containing AMPARs (Shi et al., 2001; Malinow and Malenka, 2002; Man, 2011; Morita et al., 2014). GluA2 interacts with synaptic proteins to stabilize AMPARs at the synapse (Song et al., 1998; Shi et al., 2001; Yao et al., 2008; Migues et al., 2010) and also with extracellular molecules to maintain dendritic spines (Saglietti et al., 2007). Importantly, the amount of GluA2 in the synaptic membrane correlates both with the duration and the strength of long-term memories (Yao et al., 2008; Migues et al., 2010; Tsokas et al., 2016; Gao et al., 2018). Hence, given that permeability to calcium influx renders GluA2-lacking AMPARs permissive to plastic events, these receptors are recruited early during learning, when the memory trace is still labile. On the other hand, during consolidation, GluA2-containing AMPARs replace those that lack the GluA2 subunit in order to stabilize the memory trace and support its maintenance.

Several studies have demonstrated that manipulation of GluA2-dependent AMPAR endocytosis during different epochs alters memory processes (Rao-Ruiz et al., 2011; Hong et al., 2013; Dong et al., 2015; Lopez et al., 2015; Migues et al., 2016; Awasthi et al., 2019; Ferrara et al., 2019). Fear memory retrieval has been shown to depend on GluA2-containing AMPAR trafficking to synapses in the amygdala (Lopez et al., 2015). Also, fear memory retrieval induces GluA2-containing AMPAR endocytosis in both the hippocampus and the amygdala, which are critical for memory destabilization and subsequent reconsolidation, when they are reinserted into the membrane (Rao-Ruiz et al., 2011; Hong et al., 2013; Ferrara et al., 2019). Blockade of GluA2-dependent AMPAR endocytosis inhibits the decay of long-term potentiation (Dong et al., 2015; Awasthi et al., 2019) and promotes the conversion of a short-term inhibitory avoidance memory into a long-term one, when the blockade is performed immediately after training (Dong et al., 2015). On the other hand, forgetting requires the removal of GluA2-containing AMPARs from synapses (Awasthi et al., 2019), and chronic blockade of GluA2-dependent AMPAR endocytosis prolongs the retention of associative memories (Migues et al., 2016). Our results corroborate and expand these findings by showing that acute inhibition of GluA2-dependent AMPAR endocytosis in the PrL during the maintenance phase does not affect recent or remote contextual fear memory persistence, and that prelimbic PKMζ is in charge of maintaining these receptors at the membrane to preserve the remote contextual fear memory trace.

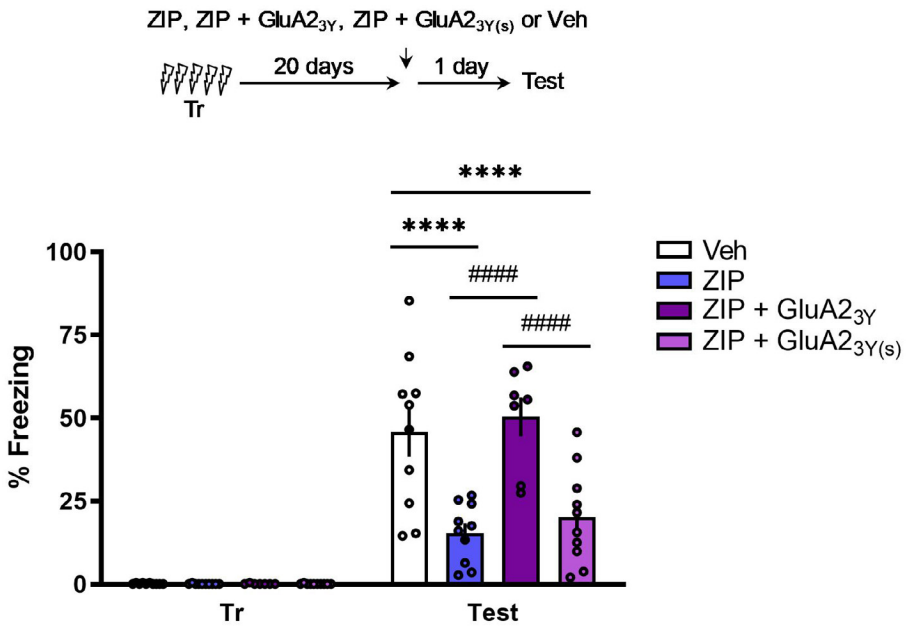

Fig. 6. Intra-PrL infusion of GluA2$_{3Y}$ prevents the impairment induced by ZIP on remote CFC memory. Animals were subjected to CFC training session (Tr) and twenty days later received bilateral intra-PrL infusions of vehicle (Veh; NaCl 0.9%), the PKMζ inhibitor ZIP (10 nmol), ZIP (10 nmol) + GluA2$_{3Y}$ (100 pmol) or ZIP (10 nmol) + GluA2$_{3Y(s)}$ (100 pmol). Twenty-four hours later, long-term memory was evaluated in a 3 min-retention test. Animals treated with ZIP or with ZIP + GluA2$_{3Y(s)}$ spent significantly less time in freezing than animals treated with Veh or with ZIP + GluA2$_{3Y}$ (*n* Veh = 10, *n* ZIP = 10, *n* ZIP + GluA2$_{3Y}$ = 7, *n* ZIP + GluA2$_{3Y(s)}$ = 10). Percentages of time spent freezing on the first 2 min of Tr and on the 3-min retention test were analyzed by two-way repeated measures ANOVA followed by Bonferroni's multiple comparison test. Data expressed as mean ± S.E.M. **** $p < 0.0001$ *vs.* Veh; #### $p < 0.0001$ *vs.* ZIP + GluA2$_{3Y}$. (Upper) Schematic representation of the behavioral protocols.

In summary, our study confirms the PrL as a cardinal site for remote fear memory storage and identifies PKMζ-driven inhibition of GluA2-containing AMPAR endocytosis as a molecular mechanism underlying the persistence of the remote contextual fear memory trace in this region. These findings may help to understand the differential contribution of the prelimbic cortex to fear memories over time and also to elucidate the molecular underpinnings of memory persistence.

DECLARATION OF INTEREST

None.

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