

## Intra-amygdala infusion of the protein kinase Mzeta inhibitor ZIP disrupts foreground context fear memory

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### ABSTRACT

Protein kinase Mzeta has been the subject of much recent interest, as it is the only molecule currently identified to maintain memory. Despite the wealth of studies investigating PKM $\zeta$  in memory, questions remain about which types of memory PKM $\zeta$  supports. Further, it is unclear how long the inhibitor of PKM $\zeta$ ,  $\zeta$ -pseudosubstrate inhibitory peptide (ZIP) remains in the brain after infusion. Here, we demonstrate that foreground context fear memory requires PKM $\zeta$  activity in the amygdala. We also show that ZIP is fully cleared from the brain by 24 h after infusion. These data contribute to a growing body of literature that demonstrates that PKM $\zeta$  plays a key role in maintaining amygdala-dependent memory and provides new information about the degradation timecourse of the most commonly used inhibitor of PKM $\zeta$ , ZIP.

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### 1. Introduction

Protein kinase Mzeta (PKM $\zeta$ ) is an atypical isoform of protein kinase C (PKC) that has attracted intense interest in the past few years for its putative role in memory maintenance. PKM $\zeta$  lacks the autoinhibitory domain that accompanies other PKC isoforms, allowing the molecule to remain constitutively active. The constant activity of PKM $\zeta$  is believed to actively maintain the synaptic changes that occurred during memory formation, particularly the upregulation of AMPA receptors through the NSF-GluR2 pathway (Migues et al., 2010; Sacktor, 2010; Yao et al., 2008). Inhibition of PKM $\zeta$  with zeta pseudosubstrate inhibitory peptide (ZIP) can disrupt established memories for a number of learning tasks (Hardt, Migues, Hastings, Wong, & Nader, 2009; Kwapis, Jarome, Lonergan, & Helmstetter, 2009; Pastalkova et al., 2006; Serrano et al., 2008; Shema, Sacktor, & Dudai, 2007), consistent with the idea that PKM $\zeta$  supports memory maintenance.

Interestingly, it seems that not all forms of memory require PKM $\zeta$  activity for maintenance. For example, “background” fear conditioning to the training context (acquired during a session in which a discrete conditional stimulus (CS), such as a white noise cue, is paired with an aversive unconditional stimulus (UCS), such as a shock) is not disrupted by PKM $\zeta$  inhibition in the hippocampus, although hippocampal lesions disrupt this type of background

context memory (Kwapis et al., 2009; Serrano et al., 2008). Hippocampal infusions of ZIP, however, are sufficient to disrupt object location memory (Hardt et al., 2009), and other spatial memories, like active avoidance memory and memory for the 8-arm radial arm maze (Pastalkova et al., 2006; Serrano et al., 2008). These studies demonstrate a clear need to assess the role of PKM $\zeta$  in different forms of memory, particularly those that involve contextual, background, or procedural components.

More recent findings suggest that PKM $\zeta$  inhibition may only temporarily disrupt memory expression of fear-potentiated startle, rather than permanently erasing the memory (Parsons & Davis, 2011). In this study, a test 10 d after ZIP infusion had showed no effect of the drug on memory retention despite the same infusion producing profound memory deficits when animals were tested at both 1 d and 10 d after ZIP infusion. Another recent study, however, demonstrated a deficit in active avoidance responses when the memory was tested 7 d after ZIP infusion (Gamiz & Gallo, 2011). Further, many of the previous studies testing the effects of ZIP on memory maintenance use a short injection-to-initial test interval, although it is unknown how long ZIP persists in brain tissue after infusion. It is possible that the initial test in these studies occurs while ZIP is still present in brain tissue and that memory expression or retrieval is impaired, rather than memory maintenance. In order to rule out the possibility that the peptide disrupts fear expression, and to clarify the results of previous studies using ZIP to inhibit PKM $\zeta$ , it is important to know when the peptide is fully metabolized from brain tissue.

The objectives of this study were twofold. First, we wanted to determine whether PKM $\zeta$  activity in the amygdala is required to

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maintain foreground context fear memory. In standard auditory fear conditioning, a discrete CS is paired with the UCS over the background information of the context. Although context conditioning occurs, the context–UCS association is weaker than the discrete CS–UCS association and is said to be a “background” association (Rescorla & Wagner, 1972). If no discrete CS is presented, however, the contextual information is the only cue the animal can use to predict the occurrence of the UCS. This shifts the context conditioning into a foreground association (Odling-Smee, 1975, 1978). Context-only training produces strong context fear memory that (like background context conditioning) depends on both the amygdala and hippocampus (Helmstetter, Parsons, & Gafford, 2008; Maren & Fanselow, 1997; Oliveira, Nobre, Brandão, & Landeira-Fernandez, 2004; Phillips & LeDoux, 1992). While background context associations are known to require PKM $\zeta$  in the amygdala for maintenance, it is currently unknown whether PKM $\zeta$  maintains foreground context associations in a similar manner. It is possible that the relatively stronger foreground context association is resistant to disruption by ZIP in the amygdala, relying instead on other structures (such as the hippocampus) for maintenance.

Our second goal was to identify the timecourse of ZIP degradation following its infusion into the amygdala. It is currently unknown how long the peptide remains in brain tissue following intracranial infusion, even though this information is vital to interpreting previous studies using ZIP to inhibit PKM $\zeta$  activity. In order to determine whether behavioral effects observed following ZIP injections represent disruption of the memory or could potentially result from the peptide disrupting memory expression or retrieval, it is important to know how long the peptide remains in the brain after infusion. To this end, we injected a biotinylated version of ZIP into brain tissue and used immunohistochemistry to determine when the peptide is fully cleared from the tissue after infusion.

## 2. Materials and methods

### 2.1. Subjects and surgery

Subjects were 50 adult male Long-Evans rats obtained from Harlan (Madison, WI) weighing approximately 350 g. Animals were housed individually in shoebox cages with free access to water and rat chow. The colony room was maintained under a 14:10 h light/dark cycle (lights on at 7:00 a.m.) and all behavioral tests were conducted during the light portion of this cycle. All procedures were approved by the Animal Care and Use Committee at the University of Wisconsin-Milwaukee.

Subjects were adapted to handling for three consecutive days before surgery. Immediately before surgery, each rat was anesthetized with an intraperitoneal (IP) injection of sodium pentobarbital (1.5 mg/rat) followed by a second IP injection of ketamine hydrochloride (100 mg/kg). Animals were then prepared with bilateral stainless steel 26-gauge cannulae (Plastics One, Roanoke, VA) aimed at the basolateral nucleus of the amygdala (BLA) using stereotaxic coordinates (2.8 mm posterior,  $\pm$ 5.0 mm lateral, 7.2 mm ventral) relative to Bregma (Paxinos & Watson, 1998). Cannulae were secured to the skull with stainless steel screws, superglue, and dental acrylic. Following surgery, the incision site was swabbed with a lidocaine and prilocaine solution (2.5%/2.5%) to minimize discomfort during the recovery period. Stainless steel obturators remained in the cannulae when rats were not being injected to prevent occlusion. Each rat was given a recovery period of at least 7 d before behavioral testing.

### 2.2. Apparatus

Fear conditioning training was conducted in a set of four Plexiglas and stainless steel chambers housed within sound-attenuating

boxes. The floor was composed of stainless steel rods spaced 1.5 cm apart through which footshocks were delivered. Each chamber was illuminated by an overhead 7.5-W bulb and was connected to its own shock generator–scrambler (Grason-Stadler, West Concord, MA). Ventilation fans provided constant background noise (approximately 60 dB). Chambers were cleaned with a solution of 5% ammonium hydroxide between animals.

### 2.3. Drug preparation and infusion

All subjects were exposed to the restraint and injection procedure for the 3 d preceding training or infusion. Each rat was transported to the laboratory, wrapped in a towel, and gently restrained by hand for several minutes while the infusion pump was activated to allow rats to habituate to its noise. The obturators were temporarily removed at this time and the area was gently swabbed to clean and remove debris from the cannulae. Immediately following restraint handling, rats were returned to their homecages.

Drugs were prepared fresh on the day of infusion. In Experiment 1, myristoylated ZIP (myr-SIYRRGARRWRKL-OH, Invitrogen, Carlsbad, CA) was dissolved in sterile saline (pH  $\sim$  7.0, Teknova, Hollister, CA) to create a final concentration of 10 nmol/ $\mu$ l. This concentration was chosen based on its effectiveness in past studies in our lab (Kwapis et al., 2009) and in other labs (Parsons & Davis, 2011; Pastalkova et al., 2006; Serrano et al., 2008). In Experiment 2, biotinylated ZIP (bioZIP; Myr-SIYRRGARRWRKL-Ahx-(K-Ahx-Biotin)-CONH<sub>2</sub>; Abgent, San Diego, CA) was similarly diluted in sterile saline to 10 nmol/ $\mu$ l. BioZIP was only infused into one hemisphere; the opposite hemisphere was infused with saline for comparison.

All rats received an infusion volume of 0.5  $\mu$ l into the basolateral amygdala over a period of 60 s. The injection cannulae, which were cut to extend approximately 0.5–0.7 mm beyond the guide cannulae, remained in place for an additional 90 s after the infusion to ensure proper diffusion. Previous work with these parameters indicates that they produce coverage throughout the amygdala (Kwapis et al., 2009; Parsons, Gafford, & Helmstetter, 2006). Directly after infusions, rats were returned to their homecages.

### 2.4. Behavioral procedures

#### 2.4.1. Experiment 1

After adaptation to transportation and restraint handling, animals were trained with unsignaled (context-only) fear conditioning ( $n = 22$ ) in which a 2-min baseline period was followed by five unsignaled shocks (1 mA; 1 s) presented with a 60 s ITI. Animals were removed from the conditioning chambers 2 min after the final shock, for a total session length of 8 min. 1 d or 7 d later, animals were infused with either ZIP (1 d,  $n = 5$ ; 7 d,  $n = 6$ ) or saline (1 d,  $n = 6$ ; 7 d,  $n = 5$ ) into the BLA. A context test was given 2 h post-infusion to test the animals' context fear memory. For the context test, animals were transported to the laboratory, where they were exposed to the training context for 8 min. A second context test was conducted 24 h later to determine whether any effects of ZIP were permanent or transient.

#### 2.4.2. Experiment 2

After recovering from surgery, animals were handled for three consecutive days prior to injection as previously described. On the fourth day of handling, all animals received an intra-BLA injection of bioZIP into one hemisphere and vehicle into the opposite hemisphere (0.5  $\mu$ l each). Animals were then sacrificed every 2–3 h post-infusion ( $n = 2$ /timepoint; 0 h, 2 h, 5 h, 8 h, 11 h, 14 h, 17 h, 20 h, 22 h, 24 h, 26 h, 29 h, 3 d, and 1 week post-infusion) and the brain tissue was analyzed to assess the presence of the peptide.

## 2.5. Histology and histochemistry

After behavioral testing was complete, all animals were killed by an overdose of isoflurane and transcardially perfused with saline followed by a 10% buffered formalin solution. Heads were removed and submerged in buffered formalin for at least 24 h. Brains were then removed and soaked in a 30% sucrose formalin solution for a minimum of 24 h. For cannulae placement verification (Experiment 1), frozen 40  $\mu\text{m}$  sections were collected throughout the amygdala, mounted on slides, and stained with cresyl violet. Injection sites were determined with the aid of a rat brain atlas (Paxinos & Watson, 1998). Animals with injection sites outside the basolateral subdivision of the amygdala were not included in the analyses.

For Experiment 2, histochemistry was performed following tissue fixation in order to determine the timecourse of ZIP degradation by visualizing the biotin label attached to the peptide. Tissue fixation was performed in the same manner as in Experiment 1, except that the brains were removed immediately following perfusion and were soaked directly in formalin for the first night followed by 30% sucrose formalin the following day. After at least 24 h in sucrose formalin, brains were rinsed three times for 10 min with 0.1 M PBS and frozen 40  $\mu\text{m}$  coronal sections were collected throughout the amygdala in 24-well plates filled with 500  $\mu\text{l}$  of 0.1 M PBS. Slices were washed on a titer plate with gentle agitation with 1% Triton in PBS (three times, 10 min each) and incubated with the VECTASTAIN Elite ABC reaction (Vector Labs, Burlingame, CA) for 1 h at room temperature. Slices were then rinsed twice with 0.05 M Tris-HCl (pH 7.4) for 10 min and incubated with diaminobenzidine (DAB) reaction for 10 min (0.04% DAB in 0.05 M Tris-HCl buffer). To develop the stain, 1%  $\text{H}_2\text{O}_2$  was added to each well and the development was stopped by rinsing each well with 0.05 M Tris-HCl buffer (five times, 5 min each). Slices were then mounted on slides and allowed to dry for 4 h before being imaged with a microscope (Olympus, Center Valley, PA). The slices were then washed for 5 min in 70%, 95%, and 100% EtOH solutions and Xylene to clear the tissue before coverslipping. Cleared slices were imaged with a computerized image processing system (MCID; Imaging Research, Inc., St. Catherine's, ON, Canada).

## 2.6. Data analysis

The main behavioral dependent variable for Experiment 1 was the amount of time the rats spent engaged in freezing behavior. Freezing was defined as the absence of all bodily movement except that which is required for respiration. All other behavior was scored as general activity. A computer based digital video observation system (FreezeScan 1.0, CleverSys, Inc., Reston, VA, USA) continuously scored each rat as freezing or active throughout each session. Freezing during all behavioral sessions was analyzed as a percentage of each minute. Behavioral data were analyzed using two-way ANOVAs (factors: drug and injection timepoint) and Student's *t*-tests (to test the drug effects for each timepoint separately). In all analyses, an  $\alpha$  value of 0.05 was required for significance.

For Experiment 2, optical density measurements were assessed to determine the timecourse of ZIP degradation. Densitometry software (NIH ImageJ) was used to obtain optical density measurements for the side of the brain injected with biotinylated ZIP and stained with the ABC reagent. For each brain slice, after clearing the tissue with xylene, the optical density on the drug-injected hemisphere was normalized to the background staining, as taken from a parallel position on the vehicle-injected hemisphere. At least three slices were analyzed for each animal. A 60  $\times$  60 pixel box was anchored to the bottom of the cannula tract on the drug-injected hemisphere to determine the optical density of the stain. The sampling box was then dragged to an identical position

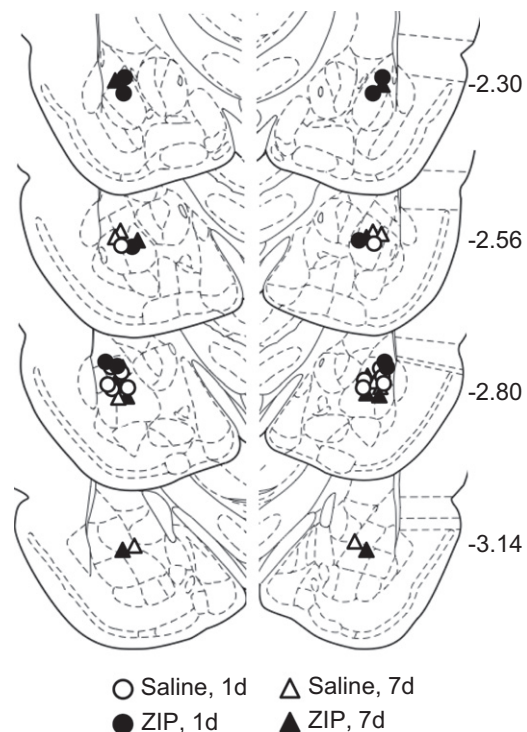
on the vehicle-infused hemisphere to get an accurate sample of the background for each slice. The background value was then subtracted from the optical density value of the stained hemisphere to account for any variance in the background staining between slices. Linear contrast analysis and planned comparisons were conducted on these values to determine whether the drug degradation occurred in a linear fashion and to determine whether the drug was fully degraded by 24 h post-injection, as anticipated (Pastalkova et al., 2006).

## 3. Results

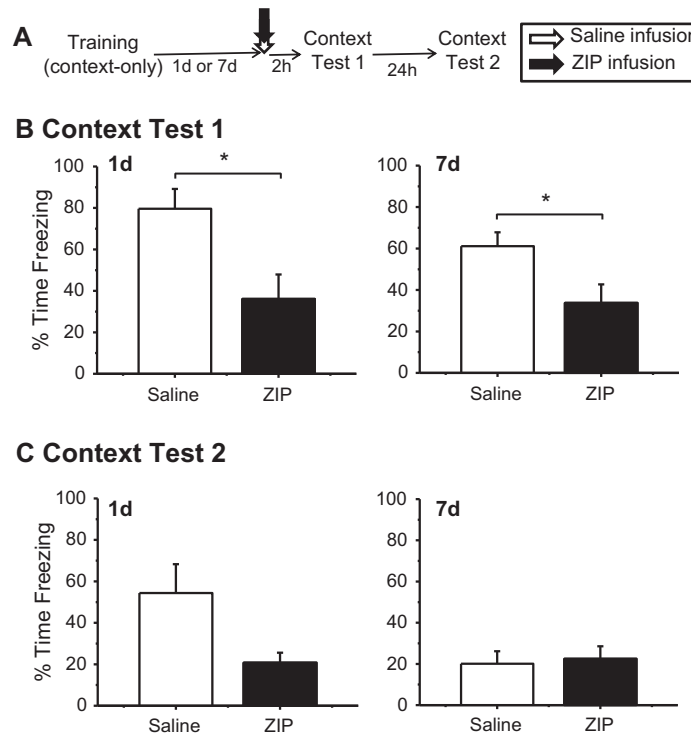
### 3.1. Experiment 1: intra-amygdala ZIP and foreground context memory

Most of the cannulae placements were in or just above the BLA. Four animals were excluded from analysis due to misplaced cannulae (one from the 1 d injection group, and three from the 7 d injection group). The cannulae placements for the remaining 22 animals were deemed acceptable and were included in subsequent analyses (Fig. 1).

The goal of our first experiment was to determine whether the maintenance of foreground context memory requires PKM $\zeta$  activity in the BLA. To this end, we trained animals with context-only fear conditioning and infused ZIP or saline into the BLA 1 or 7 d later (Fig. 2A). All animals showed normal acquisition of context-only fear conditioning before drug infusion (data not shown). Low freezing was observed during the 2-min baseline period (all groups,  $M < 1\%$ ), indicating that the animals did not respond to the training context before the UCS was presented. Importantly, no differences in mean postshock freezing behavior were observed at the end of the training session ( $F_{(3,18)} = 0.854$ ,  $p = 0.482$ ), indicating that the animals showed equivalent freezing levels prior to drug infusion.



**Fig. 1.** Location of cannulae placements for animals infused with either saline (white symbols) or ZIP (black symbols). Animals were infused either 1 d (circles) or 7 d (triangles) after training with context-only fear conditioning. Figures were adapted from Paxinos and Watson (1998).



**Fig. 2.** ZIP infusion in the amygdala disrupts foreground context fear memory. (A) The experimental timeline. (B) ZIP injected into the BLA both 1 d (left panel) and 7 d (right panel) disrupted context fear. Graphs show mean percent time freezing during the entire 8-min context test. (C) Average freezing during context test 2, 24 h after context test 1. No significant differences were observed for either the 1 d post-training (left) or 7 d post-training (right) groups. ZIP animals showed no evidence of memory recovery.

One or seven days after training, animals were infused with ZIP or saline into the BLA. Two hours after the drug infusion, animals were given a context test to determine whether context memory maintenance was disrupted (Fig. 2B). ZIP infusion into the BLA disrupted context memory both 1 d and 7 d after training. Analysis of mean freezing behavior during the context test revealed a main effect of drug ( $F_{(1,18)} = 14.020, p = 0.001$ ), but no main effect of injection time ( $F_{(1,18)} = 1.203, p = 0.287$ ) and no significant drug  $\times$  time interaction ( $F_{(1,18)} = 0.729, p = 0.404$ ). The lack of a significant interaction indicates that ZIP infusion disrupted fear similarly at both 1 and 7 d after training. Student's  $t$ -tests comparing each injection timepoint separately revealed that ZIP significantly impaired freezing at both 1 d post-training ( $t_{(9)} = 2.899, p = 0.018$ ) and at 7 d post-training ( $t_{(9)} = 2.372, p = 0.042$ ).

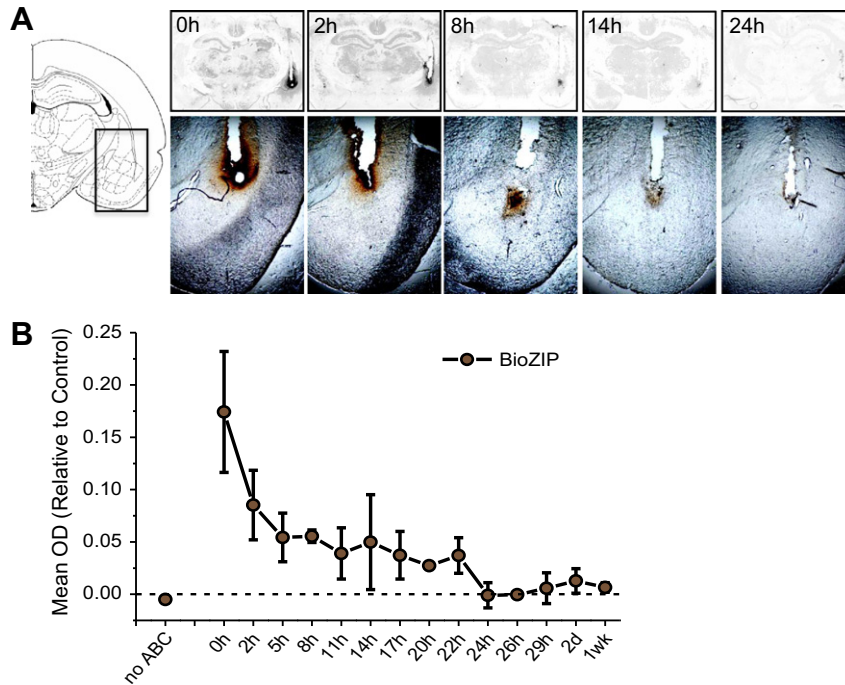
Animals were retested 24 h after the initial test to determine whether the effects of ZIP on context fear were permanent or transient (Fig. 2C). The saline groups both showed reduced freezing relative to the initial test, likely the result of extinction that occurred during the first test, when the animals were exposed to the training context without shock. While the ZIP groups were no longer significantly different from control animals for either the 1 d ( $t_{(9)} = 2.105, p = 0.065$ ) or 1 week ( $t_{(9)} = -0.273, p = 0.791$ ) infusion timepoints, ZIP animals did not show any evidence of memory recovery. Instead, ZIP animals remained freezing at low levels that were similar to the freezing levels observed on the initial test. This indicates that the memory deficit observed in ZIP animals was relatively long-lasting. Thus, our first experiment demonstrates that ZIP infusion into the BLA is able to disrupt the maintenance of both 1- and 7-day-old foreground context fear memories.

### 3.2. Experiment 2: timecourse of ZIP degradation

Experiment 2 was designed to assess the timecourse of ZIP degradation following local microinjection of the peptide into the

amygdala. The findings of past studies on PKM $\zeta$  are based on the assumption that ZIP is fully degraded by the time that long-term tests are conducted, by approximately 24–48 h after infusion. One recent study suggests that ZIP may temporarily disrupt memory expression, rather than permanently disrupting memory maintenance (Parsons & Davis, 2011). The actual timecourse of ZIP degradation, however, has not yet been assessed. To determine whether ZIP effects observed in past studies and in the current set of experiments may be due to memory expression effects, rather than disruption of memory retention, it is important to definitively determine how long ZIP is present in the brain following infusion. We injected a biotinylated version of ZIP (bioZIP) into one hemisphere of the BLA and vehicle into the opposite BLA and sacrificed animals every 2–3 h post-infusion. The brain tissue was then exposed to the ABC reaction to detect the presence of bioZIP to determine the timepoint at which ZIP is fully degraded from the brain after local microinfusion.

BioZIP was consistently detected in brain tissue from animals sacrificed within the first 22 h after drug infusion, however, no bioZIP was detected in tissue taken from animals sacrificed 24 h or more post-infusion. Fig. 3A shows representative slices from 0 h, 2 h, 8 h, 14 h, and 24 h post-infusion. Qualitatively the biotin label is darkest at 0 h and 2 h and fades as the time post-infusion increases until it disappears at 24 h. Further, BioZIP was detected throughout the amygdala region, indicating that our injection parameters produce appropriate coverage of the BLA. Fig. 3B displays the quantification for all animals in this study, in which the optical density (OD) of the stained hemisphere is normalized to background, taken from the control hemisphere. Consistent with the pattern observed in Fig. 3A, the OD intensity is highest immediately after infusion and decreases over time. A one-way ANOVA demonstrated that there was a significant effect of time on OD ( $F_{(13,14)} = 3.24, p = 0.019$ ). Further, these data fit a linear contrast analysis ( $F_{(13,1)} = 28.096, p < 0.001$ ), indicating that the degradation



**Fig. 3.** ZIP is fully eliminated by 24 h after infusion. Representative images taken from slices taken 0 h, 2 h, 8 h, 14 h, and 24 h post-infusion reveal a gradual reduction in the peptide over time, with no staining observed 24 h post-infusion or later. (A) Left panel: schematic illustrates the location of the infusion site; Top panel: cleared images used for quantification; Bottom panel: pre-cleared slices imaged at  $2\times$  magnification to demonstrate the gradual decrease in biotin stain (brown) over time. (B) Quantification of biotin stain in cleared slices. The optical density (OD) of the drug-infused hemisphere was normalized to the background, as taken from a parallel position on the vehicle-injected hemisphere. No ABC = control slice not exposed to ABC reagent. BioZIP = biotinylated  $\zeta$ -pseudosubstrate inhibitory peptide.

of ZIP occurs in a linear fashion, decreasing in intensity as the time after infusion increases. Planned comparisons demonstrated that OD measurements from animals sacrificed 24 h post-infusion and later are significantly lower than the OD from animals sacrificed before 24 h post-infusion ( $t_{(14)} = 4.063$ ,  $p = 0.001$ ), consistent with the observation that no BioZIP was detected 24 h post-infusion or later (Fig. 3A). These data indicate that by 24 h post-infusion, ZIP is fully degraded from the brain.

#### 4. Discussion

The current study had two major findings. First, we demonstrated that inhibiting PKM $\zeta$  in the amygdala with ZIP was sufficient to disrupt both 1-day and 1-week-old foreground context fear memories. This indicates that foreground context associations require PKM $\zeta$  activity for maintenance up to at least 1 week after acquisition. Our second major finding was that ZIP degrades in a linear fashion from brain tissue and is fully eliminated by 24 h after infusion. Together, these results provide new information about PKM $\zeta$ 's role in maintaining context fear and demonstrate, for the first time, the timecourse of ZIP degradation from the brain.

The first objective of this study was to determine whether PKM $\zeta$  activity in the amygdala is necessary to maintain foreground context fear memory. Recent studies have suggested that some memories, specifically contextual, procedural, and background associations, do not require PKM $\zeta$  activity for maintenance in some structures (Kwapis et al., 2009; Serrano et al., 2008). Here, we demonstrate that foreground, context-only fear conditioning, requires PKM $\zeta$  activity in the BLA for maintenance. Infusion of ZIP into the amygdala either 1 or 7 d after acquisition disrupted context fear, indicating that PKM $\zeta$  activity in the amygdala maintains foreground context fear for at least 1 week after training. Foreground

context fear, like background contextual fear, therefore requires PKM $\zeta$  activity in the amygdala.

In our behavioral study, we chose to use saline, rather than a scrambled peptide control, for comparison to ZIP. While an inactive peptide control would provide the ideal comparison, previous results from our lab (Kwapis et al., 2009) and others (Volk, Bachman, Johnson, Yu, & Huganir, 2011) suggest that scrambled ZIP (myr-RLYRKRIWRSAGR-OH) is able to inhibit both PKM $\zeta$  activity and memory maintenance and therefore serves as a poor control. We instead chose to compare ZIP-injected animals to vehicle-injected controls, which showed robust memory typical of animals trained with context-only fear conditioning.

Our second goal was to determine how long ZIP remains in the brain after infusion. Using a biotinylated version of the peptide, we were able to visualize ZIP's degradation timecourse. We found that ZIP degraded in a linear fashion and was fully eliminated from brain tissue by 24 h post-infusion. This is the first demonstration of the timecourse of ZIP degradation, an important piece of information for interpreting the results of long-term memory tests following ZIP infusion. This information indicates that any test 24 h post-infusion or later occurs in the absence of the peptide. Behavioral deficits observed after the 24 h post-infusion timepoint, therefore, cannot be explained as a disruption of memory expression resulting from residual peptide lingering in the infused brain structure.

Although it is clear that the biotinylated peptide is fully cleared from the brain by 24 h post-infusion, the data need to be interpreted cautiously. First, the addition of a biotin label onto the C-terminus of the peptide might have affected the peptide's rate of degradation. Although it was necessary to include this label in order to visualize the peptide, it is possible that the addition of biotin (with a molecular weight of 224.31 g/mol) affected the stability of ZIP or prevented the peptide from being able to cross the cell membrane, preventing it from entering the cell and being

degraded in the same manner as normal ZIP. Further, it is possible that the biotin label was detached from the peptide after infusion, so that the assay for biotinylation was detecting detached biotin molecules, rather than the peptide itself. While these concerns are certainly possible, we believe that they are unlikely for a number of reasons. First, biotin-labeled peptides are commonly used to assess peptide spread and stability (e.g. Blum & Dash, 2004; Dash, Mach, Moody, & Moore, 2004) and studies have shown that peptide biotinylation does not prevent the peptide from being incorporated into cell bodies (Blum & Dash, 2004). Second, research has demonstrated that after incorporation into a peptide, the biotin–lysine bond is cleaved only after the rest of the peptide is degraded (Craft, Goss, Chandramouli, & Wood, 1985; Koivusalo, Elorriaga, Kaziro, & Ochoa, 1963), so it is unlikely that the biotin molecule simply detached from the peptide. These results, therefore, provide initial information on the timecourse of ZIP degradation that is crucial for interpreting the results of many studies using ZIP to inhibit PKM $\zeta$ .

Based on our BioZIP timecourse, in the current study, our initial testing timepoint (2 h after drug infusion) occurred while residual ZIP remains in the brain tissue. A follow-up test conducted the following day (26–28 h post-infusion) demonstrated that the deficits observed in the first test were persistent. This second test occurred after ZIP was fully eliminated from amygdala tissue, so the low freezing levels cannot be explained by residual ZIP disrupting freezing expression. It is important to note that Parsons and Davis (2011) observed lasting disruption of memory if an initial test was conducted shortly (1 or 2 d) after ZIP infusion but failed to observe an effect if the initial test was conducted long after infusion (10 or 15 d later). Gamiz and Gallo (2011), on the other hand, observed disruption of an active avoidance response when the memory was tested 7 d after ZIP infusion without a previous test. Here, our tests occurred both 2 and 26 h after infusion; it is unclear whether we would observe a memory deficit at the 26 h test if separate groups of rats were tested at each timepoint. Indeed, we chose the 2 h post-infusion timepoint to maximize the likelihood that we would observe a ZIP-induced deficit in order to determine whether PKM $\zeta$  plays any role in maintaining foreground context fear in the amygdala. It seems, however, that the effects observed for tests conducted 1 or 2 d after infusion (Parsons & Davis, 2011) or 7 d after infusion (Gamiz & Gallo, 2011) cannot be explained by residual ZIP disrupting memory retrieval, as our results demonstrate that the peptide (injected at the same volume and concentration as Parsons & Davis, 2011) is fully removed by 24 h post-infusion.

In conclusion, our data suggest that foreground context fear memory maintenance requires PKM $\zeta$  activity in the BLA. Further, we have shown that ZIP degrades in a linear fashion and is removed from brain tissue by 24 h after infusion. These data contribute to a growing body of literature that demonstrates that PKM $\zeta$  plays an important (though not fully defined) role in memory maintenance.

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