Intra-amygdala ZIP injections impair the memory of learned active avoidance responses and attenuate conditioned taste-aversion acquisition in rats

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We have investigated the effect of protein kinase Mzeta (PKMζ) inhibition in the basolateral amygdala (BLA) upon the retention of a nonspatial learned active avoidance response and conditioned taste-aversion (CTA) acquisition in rats. ZIP (10 nmol/μL) injected into the BLA 24 h after training impaired retention of a learned avoidance-jumping response assessed 7 d later when compared with control groups injected with scrambled-ZIP. Nevertheless, a retraining session applied 24 h later indicated no differences between the groups. Additionally, a similar ZIP injection into the BLA during the conditioned stimulus–unconditioned stimulus (CS–US) interval attenuated CTA acquisition. These findings support the BLA PKMζ role in various forms of memory.

Previous findings using the myristoylated -pseudosubstrate inhibitory peptide (ZIP) have demonstrated the essential role of the atypical protein kinase C (PKC), known as protein kinase Mzeta (PKMζ), for maintaining long-term potentiation (LTP) (Sacktor et al. 1993; Ling et al. 2002, 2006; Hernandez et al. 2003; Serrano et al. 2005; Sacktor 2008, 2011) and different types of memory (Pastalkova et al. 2006; Serrano et al. 2008; Cohen et al. 2010; Hardt et al. 2010; Madrorna et al. 2010; von Kraus et al. 2010), including conditioned taste-aversion (CTA) (Shema et al. 2007, 2009, 2011). The role of the basolateral amygdala PKMζ in memory maintenance remains a subject open to some debate. Bilateral ZIP injections into the basolateral amygdala (BLA) induced retrograde amnesia for learned contextual and auditory fear responses, such as freezing and passive avoidance responses (Serrano et al. 2008; Kwapis et al. 2009; Migues et al. 2010). Most of the reported results indicate preserved fear expression (Serrano et al. 2008) and the ability to acquire and maintain new fear memories (Kwapis et al. 2009; Migues et al. 2010). Some recent results, on the other hand, have suggested that ZIP injected into the BLA may disrupt the expression of fear memory instead of erasing memory. In fact, Parsons and Davis (2011) found that ZIP had no effect on fear-potentiated startle if assessed a long time after the injection unless a previous test had been applied shortly afterward. As similar results have been found after amygdala lesions using two-way active avoidance learning tasks (Choi et al. 2010), investigations into the possible role of PKMζ in the long-term maintenance of discrete learned avoidance responses are required.

Previous results have also suggested the relevance of the BLA in CTA acquisition (Gallo et al. 1992; Yasoshima and Yamamoto 1997). This is consistent with the deleterious effect of chelerythrine, a PKC inhibitor that blocks PKMζ activity, on the CTA acquisition processes taking place in the parabrachial area (Sacchetti and Bielavská 1998). Although the absence of CTA savings after post-training ZIP injection in the IC may exclude a role for PKMζ activity in the BLA in maintaining learned taste aversions, other possible roles for PKMζ activity in the BLA during CTA acquisition and early consolidation have not yet been explored.

To understand more fully the role of amygdala PKMζ in memory we have investigated the effect of ZIP microinjections into the BLA upon both CTA acquisition and active avoidance learning retention. In the first experiment, BLA bilateral ZIP microinjections were applied either during the conditioned stimulus–unconditioned stimulus (CS–US) interval or 30 min after training. Additionally, the effect of ZIP infusion before drinking and 24 h after training was assessed. In a second experiment we explored the effect of bilateral ZIP injections into the BLA on the retention of a learned active avoidance response without navigational requirements, consisting of a vertical jump on hearing a tone in order to avoid a shock (Cândido et al. 1988, 1991, 2004; Mannrique et al. 2005).

Seventy adult male Wistar rats (280–390 g) were individually housed in an isolated room at 21°C and a 12:12 h. light–dark cycle. Food and water was available ad libitum except during the CTA protocol. To implant chronic guiding cannulae 22 g (0.7 mm outer diameter [o.d.] and 0.39–0.43 mm inner diameter [i.d.]) in the BLA (anterior-posterior [AP]: 22.5 mm; lateral-medial [LM]: ± 4.8 mm; dorsal-ventral [DV]: 28 mm from Bregma) (Paxinos and Watson 1998), each rat was deeply anesthetized with acapromacine (1–2 mg/kg) and a ketamine and clobutol mixture (IMALGENE) (150 mg/kg) before undergoing stereotactic surgery. The 10-mm-long cannulae were inserted bilaterally 4 mm below the skull and fixed with acrylic. In order to perform the BLA injections, the 30-gauge injection needles (0.3 mm o.d.; 0.13–0.16 mm i.d.) connected to 10-μL Hamilton microsyringes were inserted 14.5 mm deep into the 10-mm-long guiding cannulae to administer 1 μL of ZIP or scrambled-ZIP per hemisphere at a
rate of 0.5 μL/min using an injection pump (Harvard). Two minutes later the injection needle was slowly removed. The procedures were approved by the University of Granada Ethics Committee for Animal Research and were in accordance with the European Communities Council Directive 86/609/EEC.

In the CTA experiment, rats were randomly assigned to four groups: CS-ZIP-US (n = 21), CS-Scram-US (n = 16), CS-US-ZIP (n = 20), and CS-US-Scram (n = 13). Following a 10-day recovery period the rats were adapted for 4 d to a water restriction schedule with two daily drinking sessions at 10:00 a.m. (15 min) and 5:00 p.m. (30 min). Conditioning took place during the morning session of day 5 in which all the rats received an i.p. injection of LiCl (0.15 M; 1% body weight) as the US and 30 min after drinking a 0.1% saccharin solution (CS). ZIP (10 nmol/μL) or scrambled-ZIP were injected bilaterally into the BLA either 15 min after the end of the drinking period (CS-Scram-US and CS-US-ZIP), or 30 min after LiCl injection (CS-US-scram and CS-US-ZIP) (Fig. 1A,B). A one-bottle test was applied 48 h later. Forty-six of these animals were reused with a different taste solution (NaCl 1%) in order to assess the effect of ZIP infusions either before drinking or 24 h after CTA (Fig. 1C,D).

The rest of the animals were subjected to a signaled active avoidance jumping experiment two weeks later. They were assigned to two groups counterbalancing both morning or afternoon sessions and the previous treatment with SCR or ZIP. ZIP (n = 13) and Scram (n = 10). The learning procedure followed was similar to that described in detail elsewhere (Cándido et al. 1988, 1991, 2004; Manrique et al. 2005). In brief, the animals learned to jump upon hearing an 80-dB SPL/1000 Hz warning tone (CS) in order to avoid a 0.8-mA footshock (US) delivered by a LETICA LI 2700 shock-source module. Avoidance and escape latencies were measured by a LETICA LE 130/100 digital chronometer, accurate to 0.1 sec. The temporal sequence of events was controlled by the LI 2700 module connected to a computer. Each daily session consisted of a maximum of 75 trials. An avoidance response was taken to be one that occurred within 5 sec of the onset of the warning signal. Training lasted until the rat reached the acquisition criterion of five consecutive avoidance responses, which is considered to be a medium difficulty task (Manrique et al. 2005). In most cases (65.3%) the rats reached the learning criterion during the first session. The rest of them reached in the second session. No animals had to be eliminated as none of them met the exclusion criterion, i.e., failing to escape the shock for three consecutive trials.

Twenty-four hours after training 1 μL per hemisphere of ZIP or scrambled-ZIP (10 nmol/μL) were injected into the BLA. Seven days later a testing session consisting of 25 trials with no shocks was applied. The number of avoidance-jumping responses was significant.
recorded. To assess the rats’ ability to relearn, a retraining session identical to the one previously conducted was applied 24 h after testing (Fig. 2).

At the end of the behavioral procedure the rats were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and transcardially perfused with saline and formalin solutions. Their brains were removed and processed for histological verification of the injection needle trace location. Figure 3 shows the placement of the injection cannula tips.

In the CTA experiments, the groups differ neither in the water intake during the baseline, nor in taste-solution intake during conditioning. All the conditioned groups exhibited learned aversion (Fig. 1A,B,D), the interaction $2 \times 3$ (group $\times$ day) being significant only when CS-ZIP-US and CS-Scram-US were compared ($F_{(2,70)} = 4.00; P < 0.01$). One-way ANOVA analyses of saccharin-solution intake during the two one-bottle tests indicated significant group effects both in test 1 ($F_{(1,35)} = 6.24; P < 0.01$) and test 2 ($F_{(1,35)} = 7.60; P < 0.01$) (Fig. 1A). Even though the saccharin intake of both groups fell during test 1, only the CS-ZIP-US group showed no differences between conditioning and test 2 ($P > 0.5$), thus returning to the saccharin conditioning level after only one extinction test. No effect of ZIP infusion on tastef solution consumption was evident (Fig. 1C).

As depicted in Figure 2A, in the active avoidance task there were no significant differences between ZIP and Scram control groups in the number of trials required to reach the learning criterion during the acquisition phase ($F_{(1,21)} = 0.22; P > 0.6$). Nevertheless, an ANOVA analysis of the number of avoidance responses during the test applied 1 wk after intracerebral (i.c.) injections revealed that the ZIP group performed fewer avoidance-jumping responses than the Scram group [$F_{(1,21)} = 8.24; P < 0.01$], thus showing that their retention had been impaired (Fig. 2B). Nevertheless, as is shown in Figure 2C, both groups required a similar number of trials to reach the learning criterion during the relearning session ($F_{(1,21)} = 0.4; P > 0.5$). Moreover, a mixed ANOVA $3 \times 2$ (group $\times$ day) of the number of trials required for reaching the learning criterion during the first and second training sessions indicated a significant day effect ($F_{(1,21)} = 24.8; P < 0.001$), but not a group effect ($F_{(1,21)} = 0.54; P > 0.46$) or

![Figure 2](image1.png)

**Figure 2.** (A) Mean (± SEM) number of trials to reach the avoidance jumping learning criterion required by ZIP and SCRAM groups during the acquisition phase before receiving i.c. injections. (B) Mean (± SEM) number of learned avoidance-jumping responses during the test session applied 1 wk after i.c. injections 24 h post-training. Post-training ZIP injection impaired memory maintenance. (C) Mean (± SEM) number of trials to reach the avoidance-jumping learning criterion required by the ZIP and SCRAM groups during the reacquisition phase applied 24 h after the previous testing session.

![Figure 3](image2.png)

**Figure 3.** Placement of the injection cannula tips in the BLA. Infusion sites in a subset of the rats are plotted on schematics of the brain at the indicated distance in millimeters posterior to the bregma (Paxinos and Watson 1998). The hatched area represents the largest footprint of the guide cannula. All the cannula tips were located within the gray region in the BLA.
interaction group \times \text{day} (F_{1,21} = 0.005; P > 0.94). Both groups required fewer trials during relearning, thus indicating savings.

Two findings deriving from our present experiments merit discussion. The main one is that the inhibition of PKMζ activity by ZIP injections into the BLA 24 h after training impaired the retention of a learned nonspatial active avoidance response in rats. It should be stressed that the avoidance tasks applied in other studies have usually included navigational and contextual requirements. This is the first study, to our knowledge, reporting the relevance of PKMζ activity in the BLA to the maintenance of a discrete, non-navigational active avoidance memory.

It seemed to be a long-lasting rather than a temporary effect because the animals were tested 7 d after ZIP injection. Permanent unspecific sensory, motor, or motivational deficits induced by the ZIP injection can be ruled out since the rats were able to relearn the task 24 h after the testing session. These results add to previous data showing the impairment of retention, but not new acquisition, in spatial active avoidance tasks (Pastalkova et al. 2006; Serrano et al. 2008), CTA (Shema et al. 2007, 2009), and fear conditioning (Serrano et al. 2008; Kwapis et al. 2009; Migues et al. 2010). Also, the results allow us to discard explanations based on retrieval or expression deficits (Parsons and Davis 2011), because impairment was still evident 7 d after ZIP infusion without any previous retrieval session. It should be stressed that these investigators used a lower dose than that used previously. This might have contributed to the return of memory under certain conditions, as the effect of ZIP has been reported to be dose-dependent (Shema et al. 2009).

Otherwise, the fact that both the groups injected with ZIP and those with scrambled-ZIP required a similar number of trials to reach the criterion during relearning could be open to different interpretations. First, it might be due to the incomplete erasure of the learned response in question; this would be consistent with previous results showing the attenuation of contextual conditioned freezing (Serrano et al. 2008; Kwapis et al. 2009). Nevertheless, if this were true, differences in savings between the ZIP and Scram groups should still be expected. Thus, the most feasible interpretation relies on the possibility that the post-ZIP savings during relearning may be due to extra-BLA memory processes. In fact, the rats needed an average of 60 trials to reach the learning criterion, which may be considered as extensive training that may have involved additional brain areas. This learning criterion has also been applied in other studies (Maren 1998; Gale et al. 2004; Ponnsamy et al. 2007).

One additional finding concerns the attenuation of CTA acquisition induced by PKMζ inactivation in the BLA during the CS–US interval. However, no effect of post-acquisition PKMζ blockade was found. ZIP infusions either 30 min or 24 h later did not interfere with learned aversions. This is consistent with the results obtained by Yasoshima and Yamamoto (1997) using other PKC inhibitors. Nevertheless, these investigators also reported CTA disruption by post-training infusions applied 30 min after CS–US pairing, but no effect either 4 h after CS–US pairing or 30 min before the retention test (Yasoshima and Yamamoto 1997). This discrepancy might be attributed to a more general effect on cell metabolism by nonspecific PKC inhibitors. Similarly, the impairment of CTA acquisition was lower in our experiments, since all the groups exhibited learned saccharin aversions. In fact, the use of one-bottle tests, which are able to discriminate between aversions of different magnitudes (Batsell and Best 1993; Reilly and Bornovalova 2005), allowed us to detect differences among the groups.

The fact that the BLA is selectively involved in CTA acquisition is also supported both by permanent (for reviews, see Gallo et al. 1999; Reilly and Bornovalova 2005) and reversible lesion studies (Gallo et al. 1992; Roldán and Bures 1994), being CTA attenuation the most common finding. Among the different explanations proposed to account for CTA attenuation by BLA interventions, those related with disruption of the perceived taste novelty (Reilly and Bornovalova 2005; St. Andre and Reilly 2007) do not seem feasible in the present experiment as ZIP injections were applied after drinking the taste solution. Moreover, the results indicated no differences in the amount of novel taste solution drunk after either ZIP or Scram injections (Fig. 1C). A likely explanation for the reported attenuation may well be related to a possible role for PKMζ in processing the attributes of the taste memory trace, such as, familiarity (Moncada and Viola 2008), salience, etc. It is quite conceivable that this memory process might involve the amygdala and other brain areas and so any interference with taste memory during the acquisition session could lead to an attenuation of taste aversion. A role of BLA PKMζ on early CTA consolidation is not supported by our results. Post-training ZIP infusions up to 24 h did not interfere with acquisition. Further research would be needed to explore a potential role of BLA PKMζ in long-term CTA memory maintenance, as well as other amygdala nuclei involvement.

Whatever the specific role of PKMζ in the BLA on CTA learning might be, our findings add support for a modulatory function relevant to the acquisition of new memories. Overall, our results suggest that PKMζ activity in the BLA is required to retain a learned avoidance-jumping response and to facilitate CTA formation to some extent, thus supporting the idea that persistent PKMζ activity plays a wide role in memories that depend on different brain areas.

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