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Original Article

PKMζ contributes in consolidation, retrieval and maintenance of amygdala dependent fear memory in rats

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Abstract

Introduction: Protein kinase M zeta (PKMζ) is assumed to be actively involved in retainig long-term potentiation. The goal of this study was to investigate the role of PKMζ in basolateral amygdala (BLA) upon acquisition, consolidation, retention and retrieval of memory using a specific inhibitor of PKMζ.

Methods: Sixty male wistar rats underwent stereotaxic surgery and were cannulated bilaterally at the BLA nucleus. Then animals were divided into 4 groups of receiving BLA microinjection of zeta inhibitory peptide (ZIP) in different time courses: 30 min before and after training, 30 min before the testing (on the day after the learning) and 30 min after testing (but testing 10 days later). Memory was assessed using step through passive avoidance.

Results: ZIP infusion in BLA had no significant change on acquisition (P=0.06), however significantly impaired consolidation, retrieval and maintenance of passive avoidance memory (P=0.012).

Conclusion: Findings indicate that PKMζ activity in the BLA plays an important role in retaining amygdala dependent avoidance memory interfering the process of consolidation, retrieval and maintenance of learned task.

Keywords:
Protein kinase Mζ (PKMζ);
Learning;
Fear memory;
zeta inhibitory peptide (ZIP)

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Introduction

Amygdala is a component of the limbic system that plays a critical role in emotion, learning and memory, and complex behavior (McDonald and Mott, 2017). This brain region exhibits atrophy and accumulation of neurofibrillary tangles in Alzheimer disease (AD), which leads to impairment in memories of emotional events (Poulin et al., 2011). These findings show the importance of amygdala in the formation of fear memory. On the other hand, post-traumatic stress disorder (PTSD) is assumed to result from impairments in fear, extinction and/or over-consolidation of fear memories due to amygdala dysfunction (Shin and Liberzon, 2010).

It has been known that the amygdala receives different sensory information via cortex, hippocampus and thalamus (McDonald, 1998). Among different nucleus of the amygdala, the anterior portion of the

basolateral nucleus (BLA), modulates consolidation of memories of emotionally arousing experiences through its projections to the hippocampal regions (McGaugh, 2004). These projection neurons of the BLA are glutamatergic pyramidal or GABAergic nonpyramidal neurons (McDonald, 1992).

Learning and memory were known to be mediated by different mechanisms such as strengthening of long term potentiation (LTP) (Giese, 2012), synaptogenesis (Stewart and Popov, 2012) and modulation of intrinsic excitability properties (Papoutsi et al., 2012). Studies of synaptic plasticity in the rat have shown that LTP is a candidate mechanism for memory formation in BLA (Ikegaya et al., 1996). Despite numerous theories regarding molecular pathways of memory formation and consolidation, the mechanisms underlying storage of established memory and retrieval have not been understood yet. It has been known that different kinds of synaptic plasticity require different protein kinases. In the adult mammalian brain, at least 250 protein kinases are expressed, but only a few of them participate in the mechanism underlying learning and memory (Giese and Mizuno, 2013). A number of kinases such as cAMP-dependent protein kinase A (PKA), protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent protein kinases (CaMKII) have been shown to have the type of persistent activity following synaptic stimulation (Schwartz and Greenberg, 1987) and blocking these proteins affected the development of intermediate-term facilitation, rather than the maintenance of long-term potentiation (Sutton and Carew, 2000). protein kinase M zeta (PKMζ) which is expressed only in the nervous system (Hernandez et al., 2003), is an atypical isoform of PKC due to the lack of inhibitory domain, sustains constantly active to preserve LTP and memory (Madroñal et al., 2010). A study in rats has shown a high level of PKMζ expression in the prefrontal cortex and hippocampus after training in a delayed non match-to-sample task (Wang et al., 2014). Hara and his colleagues found higher level of PKMζ in the hippocampal dentate gyrus of fast learner monkeys (Hara et al., 2012). Also, it has been shown that over-expression of PKMζ in the prelimbic cortex selectively enhanced the formation of long-term fear memory (Xue et al., 2015). In contrast, aged monkeys with lower recognition function expressed a lower level of PKMζ in the hippocampal dentate gyrus (Hara et al., 2012).

PKMζ has been known to be translated within only 10 min in response to LTP-inducing stimuli (Hernandez et al., 2003), not only in cell bodies, but also locally in the dendritic area (Muslimov et al., 2004). Previous results have also suggested the role of the BLA in associative memories such as conditioned taste aversion (CTA) acquisition (Barot et al., 2008) which may be mediated by PKC activity (Yasoshima and Yamamoto, 1997). The role of the basolateral amygdala PKMζ in memory formation and maintenance has not been fully understood. Administration of bilateral inhibitor of PKMζ, ZIP, into the BLA induced retrograde amnesia for learned contextual and auditory fear responses, such as freezing and passive avoidance responses (Migues et al., 2010; Gámiz and Gallo, 2011).

Also, Tiunova and colleagues (2015) showed that PKMζ involves in the maintenance of long term memory (LTM) for taste aversion learning in young chicks. In addition, PKMζ has recently been observed to be down-regulated during memory extinction, reconsolidation blockade (Xue et al., 2012) and spatial familiarity in hippocampus (Moncada and Viola, 2008). Abnormal aggregations of PKMζ was observed in and near neurofibrillary tangles in the brains of individuals with AD (Crary et al., 2006). Despite all these facts, whether or not PKMζ, plays a role in BLA dependent fear memory remains to be clarified.

Therefore, to clarify the role of amygdala PKMζ in the process of fear memory formation, this experiment was designed to investigate the effect of ZIP (myr-SIYRRGARRWRKL-OH) microinjections into the BLA upon acquisition, consolidation, retention and retrieval of memory.

Materials and methods

Animals

Sixty four male Wistar rats (3 months age and 180-200 g weight) were housed four per cage and fed rat chow and tap water and libitum. Room temperature was maintained at 22±2 °C with a 12/12 h light/dark cycle (light on 7:00 am). All experiments were performed in accordance with the guidelines provided by the Experimental Animal Laboratory prepared by ethical committee of Guilan University of Medical Sciences, Rasht, Iran (Ethic code: IR.GUMS.REC.1394.425).

Surgery

Subjects were handled for three consecutive days in experimental room before surgery. Each rat was anesthetized with an intraperitoneal injection of ketamine hydrochloride (100 mg/kg) and xylazine (10 mg/kg), then were cannulated bilaterally with stainless steel 22-gauge cannula at the BLA using stereotaxic coordinates of (2.8 mm posterior, ±5.0 mm lateral, 7.2 mm ventral) relative to Bregma (Paxinos and Watson, 1986). Each rat was given a recovery period of at least one week before behavioral testing.

Drug

The PKMζ inhibitor, ZIP (Tocris Bioscience, USA) (Serrano et al., 2005) was diluted in sterile saline to a concentration of 10 nmol/µl (Pastalkova et al., 2006). Animals received 1µl of ZIP per side of BLA according to the design of the experiment.

Experimental design

After recovery, animals were divided into 8 groups of receiving either BLA microinjection of ZIP or saline in different time courses as follows: 1) Animals received microinjection of ZIP into the BLA, 30 min before the training in step through passive avoidance task, and then were tested 24 h later in the same task. Control group received the same volume of saline into the BLA, 30 min before training and were tested 24 h later. 2) Animals received microinjection of ZIP into the BLA, 30 min after the training, time for consolidation of memory, and then were tested 24 h later. Control group received the same volume of saline into the BLA, 30 min after the training and were tested 24 h later. 3) Animals were trained in step through passive avoidance task and received either ZIP or saline microinjection 30 min before testing, on the day after training. 4) Animals were trained, ZIP or saline were injected 30 min after testing, then animals were tested 10 days later to assess the maintenance of memory (Parsons and Davis, 2011b).

Training

Memory traces are formed over time and includes number of phases including acquisition, or learning, consolidation (the labile phase during which the memory trace will be physically stored); Recall, or memory retrieval, which takes place during the re-exposition to the learning context, and finally storage or maintenance of established memory (Quillfeldt, 2016). Acquisition in our experiment took place in one trial, when the animals were exposed to a context with delivery of electric foot shock. Here we injected ZIP 30 min before the learning in order to study the effect of PKMζ inhibitor on acquisition processes.

In the second part of the experiment thirty minutes after learning, we injected ZIP to interfere consolidation phase; when the memory trace undertakes a labile phase and the animal is susceptible to disturbances (Dudai, 2000). Recall test was done after a 24h interval and animals received ZIP before exposing to the same context in which they acquired the task. Finally maintenance of LTM was assessed after 10 days in our experiment. It has been know that LTM can be assessed after minimum period of 6 hours, preferentially 24h interval, but larger time spans from several days, to months are possible according to the memory intensity, forgetting, extinction and reconsolidation (Quillfeldt, 2016).

Step through passive avoidance apparatus consisted of two-compartment dark/light shuttle box separating by a guillotine door. Floor of the dark compartment consisted of a stainless steel shock grid floor. Before initial of the experiment, animals were handled for three days in order to familiarize to the context and experimenter as well. Our protocol was single trial, placing rat individually in the light room for 20 seconds in order to explore the context. Then the door was opened and the rat was allowed to move freely into the dark room. Upon entry into the dark room, the door was closed and the rat was given 1mA electrical shock for 5 seconds and immediately was returned to its home cage. After 24 hours the rat was placed in the light room for testing the memory retrieval without delivery of any shock for 180 seconds. Latency to enter to the dark chamber and total time spent in the dark room were chosen as indicators of memory.

Statistics

Normality of variables was evaluated by Kolmogrov-Seminorov (KS) test in SPSS ver 22 and non-parametric mann-whitney test was used to compare means of ZIP and saline receiving groups based on the results of KS. *P*-value less than 0.05 was considered to be statistically significant and results are expressed as the means±SEM.

Results

Experiment 1: Effect of BLA injection of ZIP on acquisition of memory

In the first experiment, ZIP or saline was infused into the BLA, 30 minutes before training and the rats were tested 24 h later. As Figure 1 illustrates, animals receiving ZIP before training session showed no significant change in step through latency after 24 h, compared to saline receiving group (Fig. 1A), (166.25 ± 6.1 vs. 178.75 ± 1.2 sec *P*=0.06), or total time spent in the dark room (TTS), (8 ± 2.7 vs. 1.8 ± 1.3, *P* = 0.1, Fig. 1B).

Experiment 2: Effect of BLA injection of ZIP on consolidation of memory

ZIP or saline were infused into the BLA, 30 minutes after training and the rats were tested 24 h later. Mann-Whitney test showed less step through latency (24.1 ± 7.5 vs. 178 ± 1.25, *P* = 0.012, Fig. 2A), but more total time spent in the dark room (97.87 ± 18.1, vs. 3 ± 1.51 sec, *P* =0.012, Fig. 2B), in the animals receiving ZIP.

Experiment 3: Effect of BLA injection of ZIP on retrieval of memory

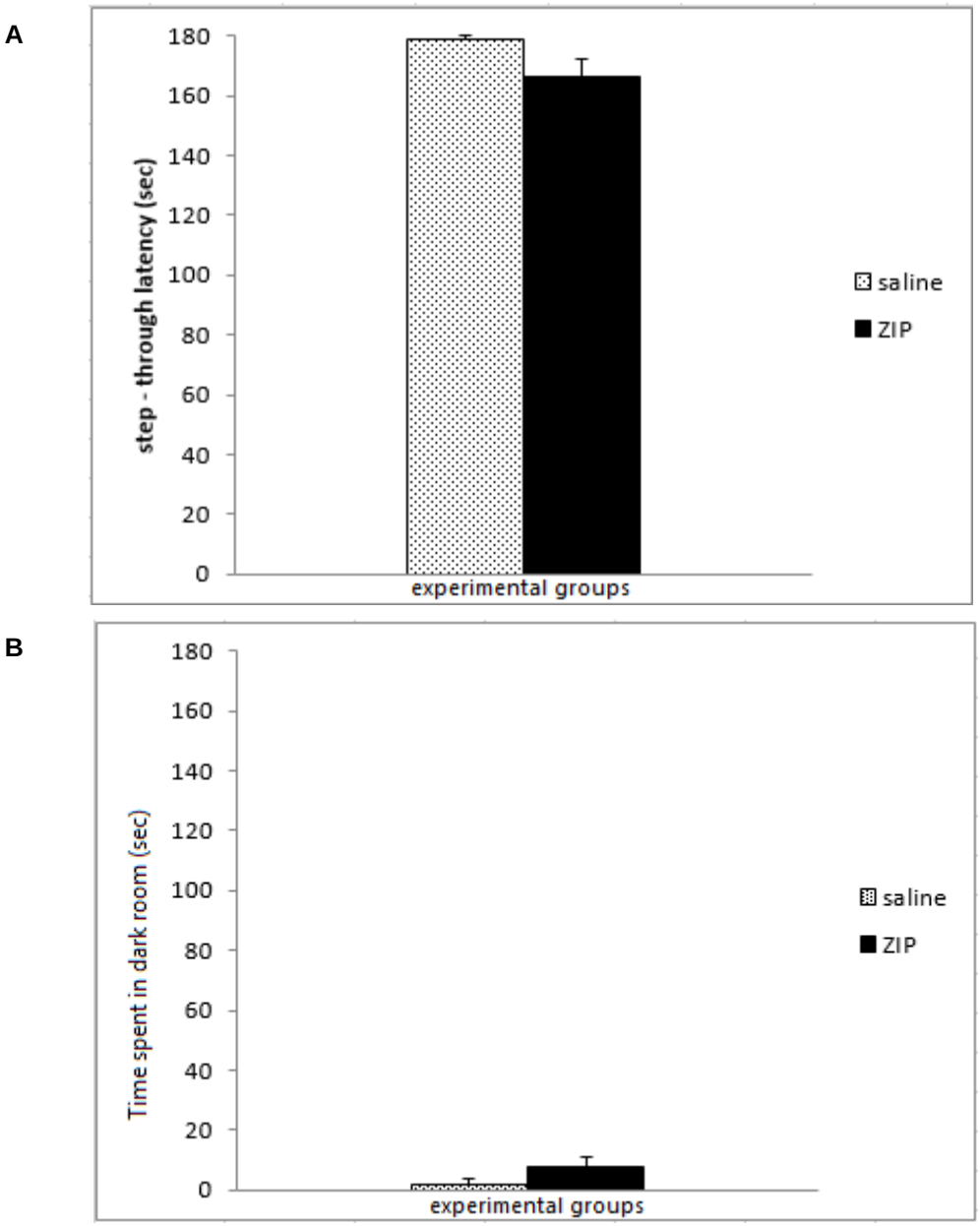

Fig.1. The effect of ZIP administration 30 min before training on acquisition of memory in step through passive avoidance learning. A) First latency to enter to the dark room, data is expressed as mean±SEM, *P*=0.1 vs. control group. B) Columns show mean±SEM of total time spent on dark room of retention. No significant difference was found between ZIP and saline groups (*P*=0.06, n=8 per each group).

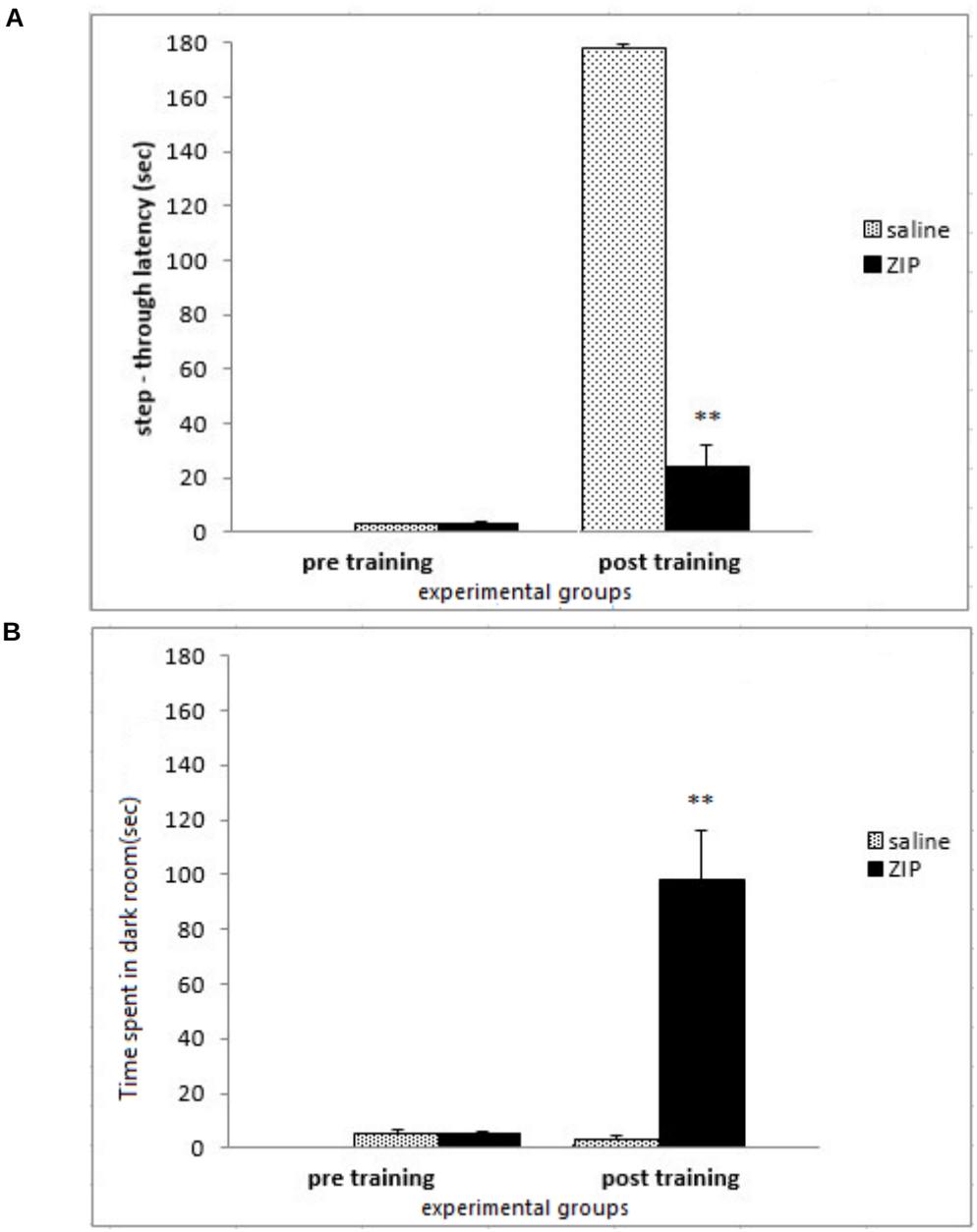

Fig.2. The effect of ZIP administration 30 min after training on consolidation of memory in step through passive avoidance learning. A) First latency to enter to the dark room, data is expressed as mean±SEM (n=8) *P*=0.012 vs. control group. B) Columns show mean±SEM of total time spent on dark room of retention. *P*=0.012 indicates significant difference vs. control group. Data are expressed as mean±SD (** *P*=0.012, n=8 per each group).

Animals of the third group were trained in the first day and received ZIP, 30 minutes before testing on the day later, when molecular memory consolidation was completed. Mann-Whitney test analysis showed less step through latency (9.6 ± 3.1 vs. 176.25 ± 1.62 sec, *P* = 0.012, Fig. 3A) but more total time spent in the dark room (97.37 ± 20.4 vs. 6.6 ± 2.7, *P* = 0.012, Fig. 3B) in the animals receiving ZIP 30 minutes before testing.

Experiment 4: Effect of BLA injection of ZIP on maintenance of memory

The PKMζ inhibitor, ZIP was injected 30 min after retrieval test, 24 h after training (when molecular memory consolidation was completed and successfully recalled), but animals were tested 10 days later. Mann-Whitney test analysis showed less step through latency (9.6 ± 3.2 vs. 176.25 ± 1.62, *P* = 0.012, Fig. 4A) but more TTS (74.5 ± 15.9 vs. 4 ± 1.5 sec, *P* = 0.012, Fig. 4B) in the animals receiving ZIP compared with saline receiving ones**.**

Discussion

The results of the present study in line with study carried out by (Shema et al., 2007; Crespo et al.,

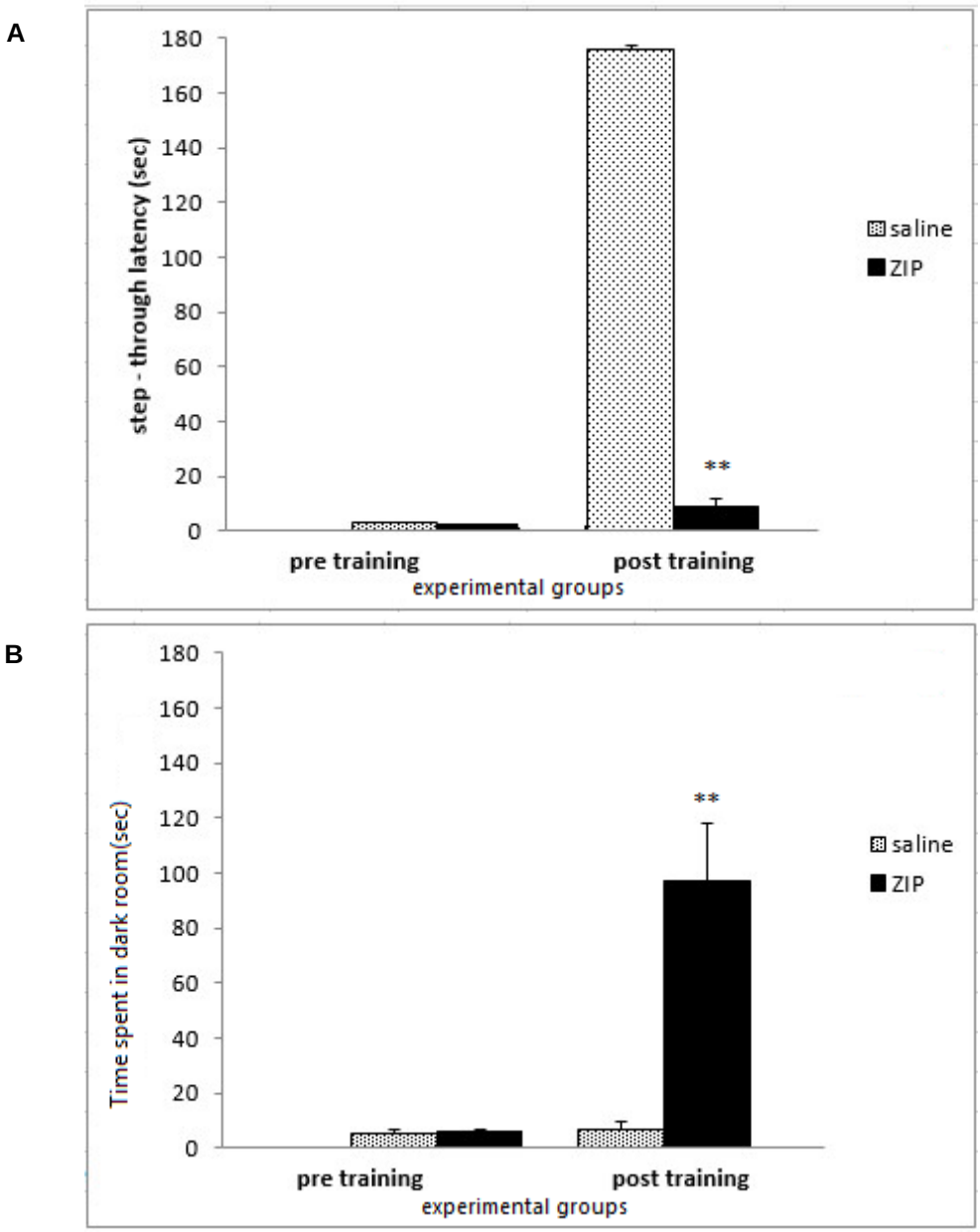

Fig.3. The effects of ZIP infusion 24h after training on memory retrieval in step through passive avoidance learning. A) Columns show mean±SEM of step-down first latency of retention test. Mann-Whitney test analysis showed less step through latency, *P*=0.012. B) Total time spent in the dark room showed significant difference (*P*=0.012) in the animals receiving ZIP 30 minutes before testing compared with saline. Data are expressed as mean±SD (** *P*=0.012, n=8 per each group).

2012) on cortex, showed that injection of ZIP into the BLA, 30 minutes before training had no significant effect on the acquisition of CTA. As it has been known, acquisition of learning involves NMDA receptor activation and influx of calcium (Morris et al., 1986) to induce early phase of long term memory (E-LTP) (Bliss and Collingridge, 1993). E-LTP lasts 1–3 hours and does not require protein synthesis (Stanton et al., 2006), however memory acquisition or learned associations are transferred from a labile short-term memory state to a stable form, through memory consolidation (McGaugh, 2000). Blocking BLA PKMζ, 30 mintes after the training in the present study, significantly inhibited the consolidation of passive avoidance learning similar to the previous study on CTA in insular cortex of rats (Shema et al., 2007) and also in conditioning response (Serrano et al., 2008). To explain the mechanisms underlying this finding, it is important to notice that late phase of long term potentiation (L–LTP) is induced by a cascade of cellular signaling events following increase in intracellular cAMP concentration (Edelman et al., 1987), activation of PKA and transcription factors such as cAMP response element binding protein

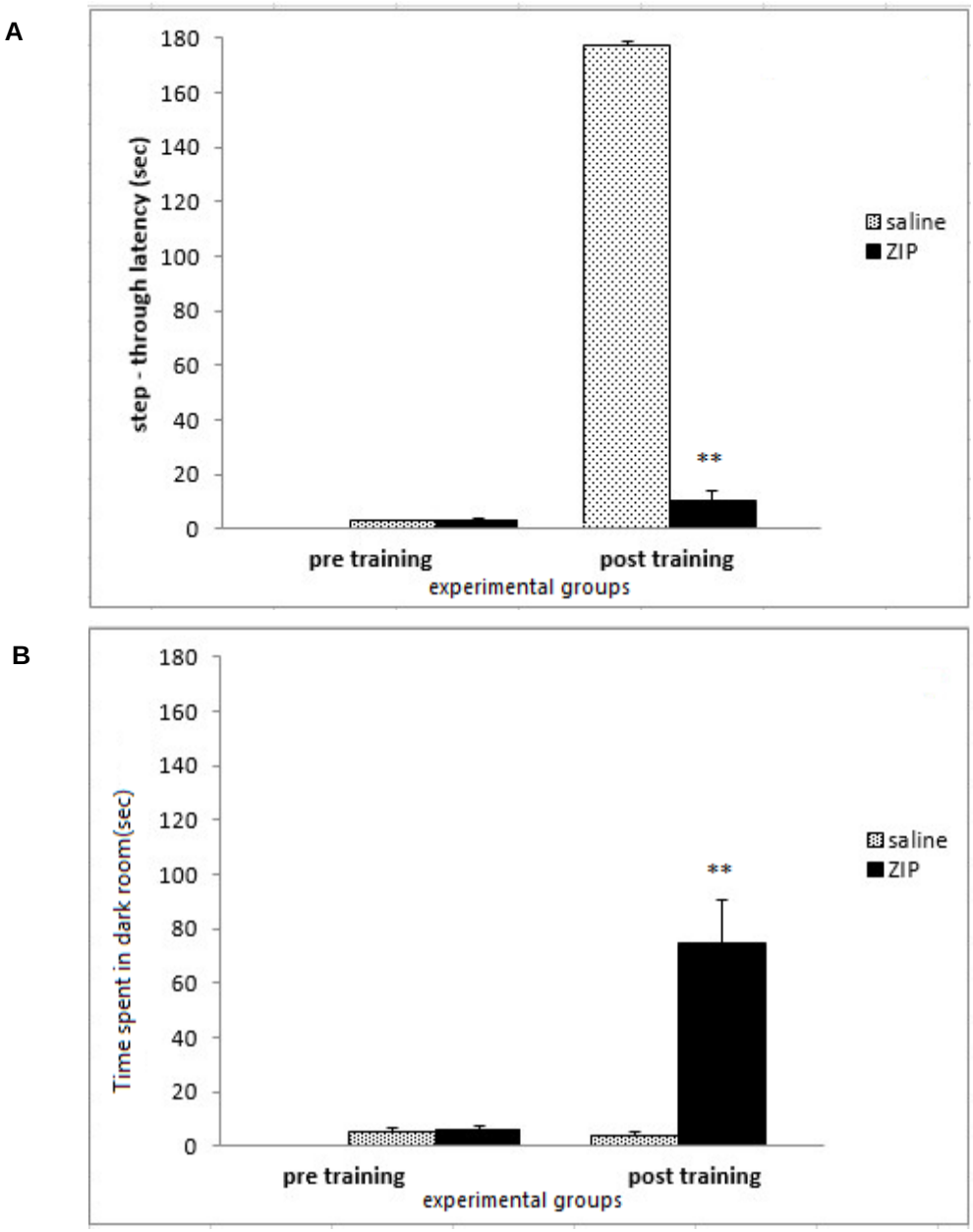

Fig.4. The effects of ZIP infusion 24h after training on memory maintenance in step through passive avoidance learning. A) Columns show mean±SEM of step-down first latency of retention test which was performed 10 days after training. Mann-Whitney test showed significant difference vs. control group, *P*=0.012. B) Columns show mean±SEM of total time spent dark room of retention test. Mann-Whitney test showed less TTS, *P*=0.012. Data are expressed as means±SD (** *P*=0.012, n=8 per each group).

(CREB) as well (Abel et al., 1997). Finally, CREB-dependent transcription leads to the expression of a variety of downstream effector molecules that help to stabilize synaptic changes and memory consolidation (Alberini, 2005). Also L-LTP appears to be mediated by translation of PKMζ which stabilizes AMPA receptors containing GluA2 (Sacktor, 2011). In rodents, LTP maintenance has been shown to be reversed applying PKMζ inhibitor even one day after LTP induction (Serrano et al., 2005).

After consolidation, memory storage requires alterations in gene transcription and protein synthesis (Reis et al., 2013). Our data in the experiment 3 showed that blocking the PKMζ activity, 30 minutes before the memory retriaval successfully attenuated this process similar to the previous report (Serrano et al., 2008). Retrieval is generally thought to be involved in the reactivation or reconstruction of components of the neural ensembles activated during the initial learning. Indeed, proteins associated with consolidation have been implicated in memory retrieval (Murchison et al., 2004) and that AMPA

receptors activation in the amygdala during memory retrieval is necessary for expression of the conditioned response (Mamou et al., 2006).

In addition, injection of ZIP into the BLA after succsessfull retrival, erased avoidance memory tested on 10 days later. Similar to theses findings have been reported by Tiunova et al. (2015) and Shema et al. (2007) on CTA and also Pastalkova et al on storage of spatial information. Also our findings are in agreement with data obtained from amygdala dependent fear conditioning (Migues et al., 2010; Kwapis et al., 2012) and avoidance learning (Gamiz and Gallo, 2011) indicate PKMζ role in maintenance of emotional memory. Despite the existing litreatures pointing to the role of PKMζ in the process of memory formation and maintenance, there are some controversial data showing that some forms of memory could not be erased by inhibiting PKMζ. These include short-term memories mediated by the hippocampus (Pastalkova et al., 2006), neocortex (Shema et al., 2009), spatial reference memory in a water maze task (Serrano et al., 2008) and olfactory fear memory (Parsons and Davis, 2011a).

Therefore, PKMζ may be involved in the process of consolidation (Crespo et al., 2012), reestablishment (Zhang et al., 2016) and maintenance (Kwapis et al., 2012) of long term memory. To explain the mechanisms by which PKMζ interfere these process we can address the issue to the fact that PKMζ is located in the nucleus of neural cells as well as postsynaptic sites (Hernández et al., 2013) and subsequently leads to AMPA trafficking and Ca^{2+} influx. Then Ca^{2+} influx trigger an early and transient phase of CREB phosphorylation (Wu et al., 2001).

In addition, PKMζ prevents internalization of GluR2-dependent AMPA receptors (Balaban et al., 2015) through interaction with proteins such as “kidney and brain expressed protein” (Yoshihama et al., 2009) and PKC1 (PICK1) (Yao et al., 2008). PKMζ also helps to sustain histone acetylation levels during memory maintenance and using PKMζ inhibitor erases long-term memory in part by reducing histone acetylation level (Ko et al., 2016). Future investigations with a design of reconsolidation and reminder in different times window of memory formation are needed to clarify the exact role of PKMζ in learning and memory.

Finally, in clinical importance, it is suggested that inhibiting PKMζ could provide a strategy for treatment of drugs addictions, substance abuse, fear and PTSD and enhancing PKMζ level might be a candidate to prevnet cognition deficit in Alzheimer diseases.

Conclusion

The results indicate the contribution of PKMζ in consolidation, retrieval and maintenance, but not acquisation of amygdala – dependent long-term fear memory in rat.

Acknowledgments

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Conflict of interest

Authors declare no conflict of interest

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