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Spermidine improves fear memory persistence

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ABSTRACT

Persistence is the most characteristic attribute of long-term memory (LTM). For memory persistence, a second late event of consolidation, that occurs around 12 h after the acquisition, is necessary. Although the N-methyl-D-aspartate (NMDA) receptor has been involved in the persistence of memory, whether endogenous modulators of the NMDA receptor actually modulate memory persistence is unknown. In the current study we investigated whether spermidine and arcaine, respectively agonist and antagonist of polyamine binding site at NMDA receptor, alter the persistence of the memory of contextual fear conditioning task in rats. While 12 h post-training administration of spermidine (10 and 30 mg/kg, i.p.) facilitated, arcaine (10 mg/kg, i.p.) impaired the memory of fear assessed 2 and 7 days after training. Arcaine (0.1 mg/kg) prevented the facilitatory effect of spermidine (10 mg/kg, i.p.), and spermidine (1 mg/kg), prevented the memory impairment induced by arcaine (10 mg/kg, i.p.) when tested 2 and 7 days after training. These results suggest that endogenous polyamines improve the persistence of fear memory.

1. Introduction

Current evidence suggests that memories are initially fragile, but become more stable with time (McGaugh., 1966, 2000). Such an observation has led to the concept of memory consolidation, which refers to the stabilization of memory over time by neural processes activated by recently learned information (Roesler and McGaugh, 2010). Memories that last for several hours, days and weeks are termed long-term memories (LTM).

Depending on the strength and/or saliency of the information to be remembered, consolidated LTMs can persist for just 24–48 h, or for many days or weeks (Countryman and Gold, 2007; Wang et al., 2004). Persistence of LTM depends on a late consolidation process, which occurs around 12 h after acquisition. The synthesis of selected proteins, such as brain-derived neurotrophic factor (BDNF), Homer 1a, Akt, CamKII, and ERK2 in the hippocampus (Bekinschtein et al., 2007, 2010) increases during this phase. Pharmacological manipulations that increase mitogen activated kinases (MAPKs) and extracellular signal-regulated kinase (ERK) phosphorylation, as well as synthesis of the transcription factors Zif268 and c-Fos also facilitate memory persistence (Bekinschtein et al., 2007, 2008, 2010). Recent studies have unveiled some of the neurotransmitters and neuromodulators involved in this late consolidation process (Medina et al., 2008). Accordingly, the activity of muscarinic and nicotinic receptors at the CA1 region 12 h post-training is needed for memory persistence (Parfitt et al., 2012), as well as the activation of dopamine and N-methyl-D-aspartate (NMDA) receptors, and of cAMP-dependent protein kinase (PKA) (Rossato et al., 2009). On the other hand, systemic corticosterone or stress exposure during this time window impairs memory persistence (Yang et al., 2013).

Polyamines are biologically active polycations that activate NMDA receptor containing the GluN2B subunit by binding at a dimer interface between GluN1 and GluN2B N-terminal domains (Mony et al., 2011).

Accumulating evidence suggests that polyamines modulate learning and memory (Kishi et al., 1998a, 1998b; Rubin et al., 2000, 2001, 2004). Accordingly, systemic (Berlese et al., 2005; Camera et al., 2007; Gomes et al., 2010; Guerra et al., 2006; Rubin et al., 2000), and intra-amygdalar (Rubin et al., 2004) spermidine improves the performance of rats in the inhibitory avoidance, fear conditioning and social recognition tasks (Camera et al., 2007; Mikolajczak et al., 2002) and facilitates the extinction (Gomes et al., 2010) and the reconsolidation of the memory of fear (Ribeiro

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et al., 2013). Moreover, the administration of the polyamine antagonist arcaine impairs the memory of inhibitory avoidance (Ceretta et al., 2008; Rubin et al., 2001) and fear conditioning tasks (Camera et al., 2007; Rubin et al., 2004), suggesting that endogenous polyamines may physiologically modulate memory processing.

Despite the growing evidence suggesting a role for polyamines in learning and memory processes, no study has addressed whether polyamines modulate late consolidation processes. Therefore, in the current study we investigated whether spermidine and arcaine alter the persistence of the memory of fear in rats.

2. Materials and methods

2.1. Animals

A total of 190 experimentally naive male Wistar rats (200–280 g), from the animal house of the Federal University of Santa Maria were used. The animals were housed four to a cage and maintained on a 12 h light/dark cycle (lights on at 7:00 A.M.) at a temperature of 21 °C with water and standard laboratory chow (Guabi, Santa Maria, Rio Grande do Sul, Brazil) *ad libitum*. All experimental procedures were conducted in accordance with the policies on the use of animals and humans in neuroscience research, revised and approved by the Society for Neuroscience Research in January 1995 and with the institutional and national regulations for animal research (process 068/2011).

2.2. Drugs

Animals were injected with saline (0.9% NaCl), 1,4-diguanidinobutane sulfate (arcaine; Pfaltz& Bauer, Waterbury, CT, USA), or N-(3-aminopropyl)-1.4-butanediamine trihydrochloride (spermidine; Sigma, St. Louis, MO). All drug solutions were prepared daily in saline and injections were performed intraperitoneally (i.p.) in a 1 ml/kg injection volume. Doses were selected based on previous studies (Camera et al., 2007) and pilot experiments.

2.3. Apparatus

Contextual fear training and testing took place in an identical observation chamber ($30 \times 25 \times 25$ cm^3), located in a well-lit room. The front and ceiling walls of the chamber were made of clear acrylic plastic, whereas the lateral and rear walls were made of opaque plastic. The floor of the chamber consisted of 32 stainless steel rods (3 mm diameter), spaced 1 cm apart and wired to a shock generator. The cage was cleaned with 30% ethyl alcohol before and after each rat occupied it.

2.4. Contextual fear conditioning

Each animal was subjected to a single fear-conditioning training session, as described by Rubin et al. (2004), with some modifications. In brief, the rat was placed in the conditioning chamber (conditioned stimulus, CS) and habituated to the apparatus for 3 min. Immediately after habituation, three 1 s, 0.4 mA footshocks (unconditioned stimulus, US) were delivered. The shocks were 40 s apart. After the last CS/US pairing, rats were allowed to stay in the chamber for additional 60 s before returning to their home cages.

Two or seven days after training, each rat was placed back in the conditioning chamber and an 8 min test session was performed. During this time, no shock was given, and every 4 s an instantaneous observation of the rat was made to assess whether it was in freezing, or not. Behavior was judged as freezing if there was an absence of any visible movement, except for that required for breathing. The percentage of samples scored as freezing during this 8 min was taken as a contextual fear conditioning measure.

2.5. Statistical analyses

Data were analyzed by one- or two-way analysis of variance (ANOVA) depending on the experimental design. *Post hoc* analyses were carried out by the Student–Newman–Keuls test, when indicated. A $P < 0.05$ was considered significant.

2.6. Experimental groups

2.6.1. Experiment 1

This experiment was designed to investigate the effect of spermidine on the persistence of contextual fear memory. Animals were trained in the fear conditioning apparatus, as described above. Twelve hours post-training the animals received an intraperitoneal injection of vehicle (saline) or spermidine (0.1–30 mg/kg) and, two or seven days after training, were tested in the fear conditioning apparatus, as described above.

2.6.2. Experiment 2

This experiment was designed to investigate the effect of arcaine on the persistence of contextual fear memory. Animals were trained in the fear conditioning apparatus, as described above. Twelve hours post-training the animals received an intraperitoneal injection of vehicle (saline) or arcaine (0.1–10 mg/kg) and, two or seven days after training, were tested in the fear conditioning apparatus as described above.

2.6.3. Experiment 3

This experiment was designed to investigate the involvement of polyamine-binding sites at the NMDA receptor in the facilitatory effect of spermidine on the persistence of contextual fear memory. The animals were trained in the fear conditioning apparatus and 11:30 h post-training the animals received an intraperitoneal injection of vehicle (saline) or of the polyaminergic antagonist arcaine (at doses that have no effect *per se* on memory, 0.1 mg/kg i.p., as determined by the dose-effect curve shown in Fig. 2). Twelve hours after training the animals received an intraperitoneal injection of vehicle (saline) or spermidine (10 mg/kg), in different flanks. Two or seven days after training, the animals were tested in the fear conditioning apparatus and their freezing responses were scored, as described above.

2.6.4. Experiment 4

In order to confirm the involvement of polyamine-binding sites at the NMDA receptor in the effect of polyamines, we investigated whether spermidine prevents the deleterious effect of arcaine on the persistence of contextual fear memory. The animals were trained in the fear conditioning apparatus and, 11:30 h post-training, the animals received an intraperitoneal injection of vehicle (saline) or the polyaminergic agonist, spermidine (at doses that have no effect *per se* on memory, 1 mg/kg i.p., determined by the dose-effect curve shown in Fig. 1). Twelve hours after training, the animals received an intraperitoneal injection of vehicle (saline) or arcaine (10 mg/kg), in different flanks. Two or seven days after training, the animals were tested in the fear conditioning apparatus and their freezing responses were scored, as described above.

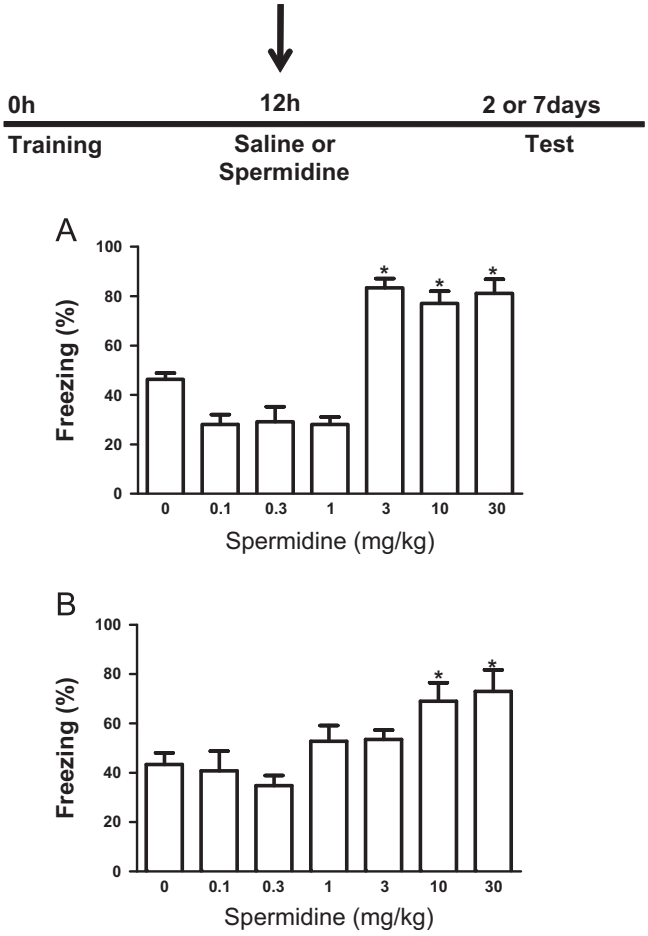

Fig. 1. Effect of 12 h post-training intraperitoneal administration of spermidine on the memory of fear, assessed 2 (A) or 7 (B) days after training. *$P < 0.05$ compared with vehicle by the SNK. Data are the means + S.E.M. of percentage of freezing for 5 animals in each group.

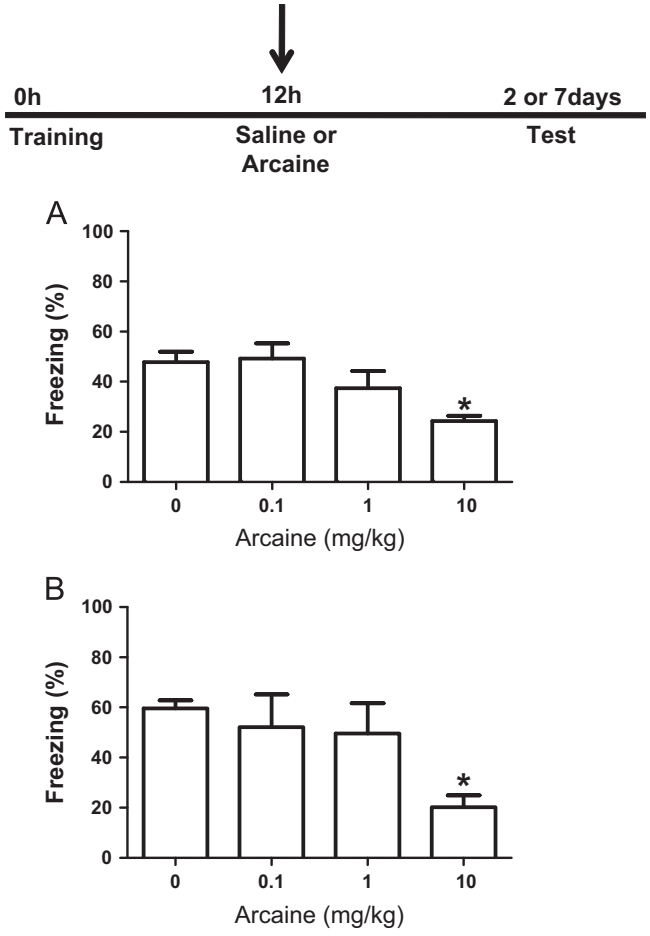

Fig. 2. Effect of 12 h post-training arcaine on the memory of fear, assessed 2 (A) or 7 (B) days after training. *$P < 0.05$ compared with vehicle by the SNK. Data are the means + S.E.M. of percentage freezing for 5 animals in each group.

3. Results

Fig. 1A and B shows the effect of the administration of spermidine (0.1–30 mg/kg, i.p., 12 h post-training) on the memory of fear assessed 2 or 7 days after training, respectively. Statistical analysis (one-way ANOVA) revealed that spermidine, at the doses of 3, 10 and 30 mg/kg, increased the freezing scores of animals tested at 2 days [$F_{6,28} = 34.48$, $P < 0.05$, Fig. 1A] and the doses of 10 and 30 mg/kg, increased the freezing scores of animals tested at 7 days after training [$F_{6,28} = 4.907$, $P < 0.05$, Fig. 1B].

Fig. 2A and B shows the effect of the administration of arcaine (0.1–10 mg/kg, i.p., 12 h post-training) on the memory of fear assessed 2 or 7 days after training, respectively. Statistical analysis (one-way ANOVA) revealed that arcaine, at the dose of 10 mg/kg, decreased the freezing scores of animals tested at 2 days [$F_{3,16} = 4{,}943$, $P < 0.05$, Fig. 2A] and 7 days after training [$F_{3,16} = 3.461$, $P < 0.05$, Fig. 2B].

Fig. 3A and B shows the effect of arcaine (0.1 mg/kg) on spermidine-induced improvement of the memory of fear, assessed 2 or 7 days after training, respectively. Statistical analysis (two-way ANOVA) revealed a significant pretreatment (saline or arcaine) *versus* treatment (saline or spermidine) interaction at 2 days [$F_{1,16} = 22.29$, $P < 0.05$, Fig. 3A] and 7 days after training [$F_{1,16} = 14.66$, $P < 0.05$, Fig. 3B], indicating that arcaine prevented spermidine-induced increase of freezing scores.

Fig. 4A and B shows the effect of spermidine (1 mg/kg) on arcaine-induced impairment of the memory of fear assessed 2 or 7 days after training, respectively. Statistical analysis (two-way ANOVA) showed a significant pretreatment (saline or spermidine) *versus* treatment (saline or arcaine) at 2 days [$F_{1,16} = 25.26$, $P < 0.05$, Fig. 4A] and 7 days after training [$F_{1,16} = 33.75$, $P < 0.05$, Fig. 4B], revealing that spermidine prevented arcaine-induced decrease of freezing scores.

4. Discussion

The current study showed that while the 12 h after training administration of the polyamine spermidine improves (Fig. 1), the injection of arcaine, an antagonist of the polyamine binding site at the NMDA receptor, impairs (Fig. 2) the persistence of memory of contextual fear conditioning, assessed 2 and 7 days after training. We also showed that arcaine, doses that have no effect *per se* on memory, prevented the facilitatory effect of spermidine on memory of fear assessed 2 and 7 days after training (Fig. 3). Accordingly, spermidine prevented the detrimental effect of arcaine on memory persistence (Fig. 4) assessed 2 and 7 days after training.

Current evidence suggests that systemic administration of spermidine immediately after training improves the context memory of fear (Camera et al., 2007). Moreover, the early post-training administration of arcaine impairs the memory of inhibitory avoidance task (Rubin et al., 2001) and contextual fear conditioning (Camera et al., 2007; Rubin et al., 2004). Since the administration of spermidine 6 h post-training does not alter the memory of inhibitory avoidance task (Berlese et al., 2005), it has

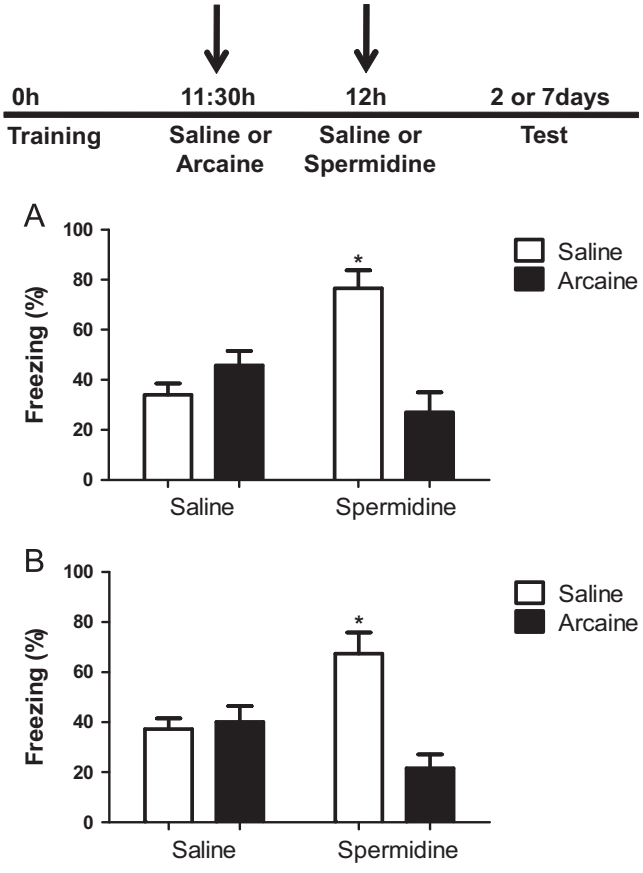

Fig. 3. Effect of 11:30 h post-training arcaine (0.1 mg/kg) and 12 h post-training spermidine (10 mg/kg) on the memory of fear, assessed 2 (A) or 7 (B) days after training. *$P < 0.05$ compared with vehicle by the SNK. Data are the means + S.E.M. of percentage of freezing for 5 animals in each group.

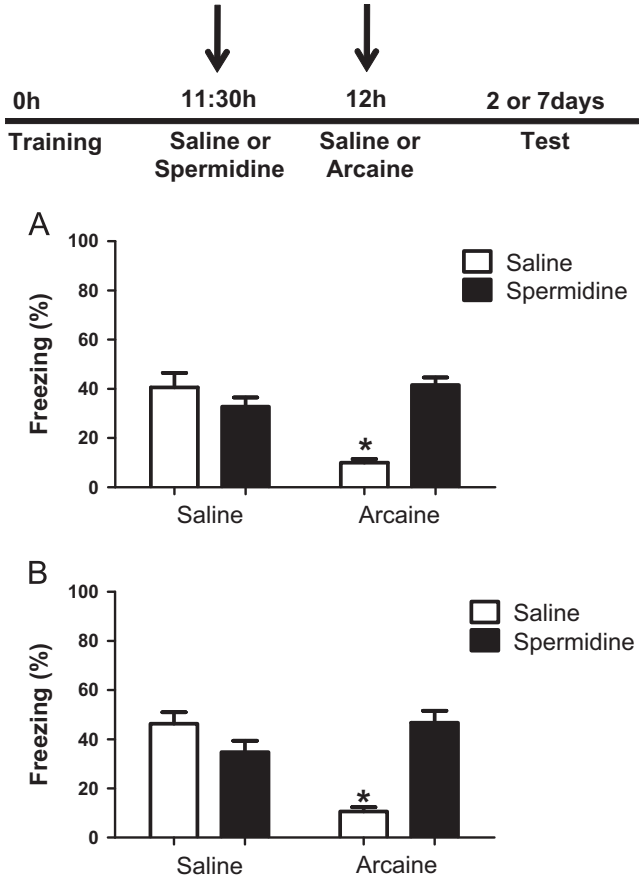

Fig. 4. Effect of 11:30 h post-training spermidine (1 mg/kg) and 12 h post-training arcaine (10 mg/kg) on the memory of fear, assessed 2 (A) or 7 (B) days after training. *$P < 0.05$ compared with vehicle by the SNK. Data are the means + S.E.M. of percentage of freezing, for 5 animals in each group.

been suggested that polyamines play a role only in the early stages of memory consolidation. However, whether polyamines alter late memory consolidation, related to persistence, it is still unknown. This question is particularly relevant, considering that existing evidence suggests that polyamines endogenously modulate memory, presumably by activating NMDA receptors (Rubin et al., 2001, 2004).

Transport of polyamines across the blood–brain does exist but it is limited, with a brain uptake index of approximately 5% (Shin et al., 1985; Diler et al., 2002). However, previous studies have shown that only minute amounts of polyamines are necessary to improve memory when injected into the amygdala and hippocampus (within the picomole range, Rubin et al., 2000, 2004). Therefore, it is plausible that the systemic pharmacologic doses of polyamines may cause biological relevant increases in the concentration of polyamines in the brain.

In this context, Shimizu et al. (2000) have shown that reactivation of NMDA receptors of the CA1 region in the following days and week(s) after learning is crucial for the formation of the long term contextual fear memory (Shimizu et al., 2000). These findings have suggested that persistent NMDA receptor activation in the first several days after training is important to establish remote memories, and are in agreement with the findings that LTM persistence requires NMDA receptor activation in the ventral tegmental area (VTA), late after training. Accordingly, the intra-VTA infusion of the NMDA receptor antagonist AP5, 12 h after training, impairs long term memory assessed on day 14, but not on day 2 post-training. Moreover, the intra-VTA infusion of NMDA 12 h after training enhances long-term retention on day 14, but not on day 2 after training (Rossato et al., 2009). In line with this view, the administration of nicotinic and muscarinic antagonists (Parfitt et al., 2012), and protein inhibitors (Bekinschtein et al., 2007, 2008) 12 h after training impairs inhibitory avoidance performance on day 7, but not on day 2 post-training. These findings have led investigators to propose that, as a general rule, late molecular events that take place in the rat hippocampus 12 h after acquisition affect memory persistence at 7–14 days but not at 2 days after training, in the inhibitory avoidance task. Our experiments, however, revealed that 12 h post-training injections of polyamine ligands alter memory on both days 2 and 7 after training. A possible explanation for these results could be that spermidine and arcaine respectively improves and impairs the formation of LTM (Berlese et al., 2005; Camera et al., 2007; Ceretta et al., 2008; Gomes et al., 2010; Guerra et al., 2006, 2011, 2012; Rosa et al., 2012; Rubin et al., 2000, 2001, 2004). Notwithstanding, spermidine administration 6 h after training does not alter the performance of rats at testing, performed 24 h after training, in the inhibitory avoidance task (Berlese et al., 2005). Therefore, it seems unlikely that a delayed injection, such as the performed in the current study, improves the formation of LTM. As a consequence, our results do not seem to fit in the current behavioral models and interpretations that injections performed 12 h after training do not alter the performance of the animals when they are tested 48 h after training. While one might conclude, based on the current results, that polyamines may have a broader temporal effect than anisomycin (Bekinschtein et al., 2007) on the stabilization of the memory of fear, further studies have to be performed in order to clarify the relative importance of the polyaminergic modulation of fear memory and its full temporal window. This is particularly

relevant because in the current study spermidine and arcaine were administered systemically, while in the study of Bekinstein and colleagues anisomicyn was injected into the hippocampus. Such a difference, that may cause an action of polyaminergic agents in cerebral structures other than the hippocampus may have contributed for this apparent disparity.

Transport of polyamines across the blood–brain does exist but it is limited, with a brain uptake index of approximately 5% (Shin et al., 1985; Diler et al., 2002). However, previous studies have shown that only minute amounts of polyamines are necessary to improve memory when injected into the amygdala and hippocampus (within the picomole range, Rubin et al., 2000, 2004). Therefore, it is plausible that the systemic pharmacologic doses of polyamines may cause biological relevant increases in the concentration of polyamines in the brain.

In summary, this study shows that spermidine and arcaine, polyamine binding site ligands at the NMDA receptor, respectively improves and impairs the persistence of memory of contextual fear conditioning. These findings suggest that polyamines modulate memory persistence, opening up arrange of possibilities for further studies on the functions of polyamines, including the mechanisms by which spermidine facilitates memory persistence, that seems to involve the NMDA receptor.

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