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Participation of hippocampal cholinergic system in memory persistence for inhibitory avoidance in rats

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ABSTRACT

Memory persistence needs a new event of consolidation 12 h after the acquisition. We investigated the role of the cholinergic activity on the persistence of memory. For this purpose, we performed the treatments 9 or 12 h after acquisition and the memory tested 2 or 7 days after inhibitory avoidance (IA) training. Here we report that activity of medial septum, by transitorily inactivating this structure with lidocaine 12 h after IA training, is essential for memory persistence at the 7th day, but not for the formation at the 2nd day. We also report that muscarinic and nicotinic cholinergic receptors of CA1 area are engaged on memory persistence. Since scopolamine (mAChRs antagonist) and mecamylamine (nAChRs blocker) infusions, 12 h post-training, demonstrated impairment on long term memory (LTM), persistence on the 7th day but no effect on LTM formation was found on the 2nd day in the IA test. The same effects were found with pirenzepine, an M1 antagonist. No effects on the formation and persistence of memory on the 2nd and 7th days were demonstrated after DHβE infusions (nAChRs subtype antagonist α4β2, α3β2). These findings suggest that mAChR and nAChR at the CA1 area, and also MS activation, are required for the persistence of memory.

1. Introduction

Persistence is the most characteristic attribute of long-term memory (LTM) (Bekinschtein et al., 2007). It has been recently reported that pharmacological treatment administered in the hippocampus 12 h after the training episode impairs the persistence of memory (Medina, Bekinschtein, Cammarota, & Izquierdo, 2008). Interestingly, there was no effect of the treatment when the animals were tested 2 days after training, but effects emerge at a much later 7-day retention interval. This observation implies that processing takes place around 12 h following training which is critical to the long-term persistence of the memory trace (Medina et al., 2008).

A new event of protein synthesis on the persistence phase is required for the establishment of remote memory, being the brain-derived neurotrophic factor (BDNF) one of the most important proteins in this event. The studies made by Bekinschtein et al. (2007) and Bekinschtein and collaborators (2008) showed that inhibition of the protein synthesis in rat hippocampus at 12, but not at 9 h after acquisition, hinders the persistence of fear memories (Bekinschtein et al., 2007, 2008). These reports gave rise to a new field of study on the involvement of hippocampus on memory persistence.

The role of cholinergic system on memory acquisition and consolidation is well demonstrated in several works using pharmacological methods (Bancroft & Levin, 2000; Barros, Ramirez, Dos Reis, & Izquierdo, 2004; Barros, Ramirez, & Izquierdo, 2005; Fletcher, Calhoun, Rapp, & Shapiro, 2006; Fletcher, Baxter, Guzowski, Shapiro, & Rapp, 2007; Izquierdo, Barros, et al., 1998; Izquierdo, Medina, et al., 1998b). Nevertheless few works addressed cholinergic influence on the maintenance of memory (Lecourtier et al., 2010).

In this study we investigated the role of the cholinergic activity on the persistence of memory. For this purpose, we performed the treatments 9 or 12 h after the IA training and then had the animals tested 2 or 7 days later. In this case, alterations on memory retention only 7 days after the training session caused by the administration of these cholinergic treatments, together with no alterations of memory formation, as measured 2 days after training, would demonstrate the involvement of cholinergic system on the persistence of memory. To study the influence of muscarinic receptors, as well as the influence of the specific muscarinic M1 receptor subtype on memory persistence, we infused the blockers scopolamine and pirezenpine in the hippocampus. The role of nicotinic cholinergic receptors and the subtypes α4β2, α3β2 was also investigated. We infused the blockers mecamelamine and DHBE in the hippocampus.

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Moreover, we investigated the participation of medial septum on memory persistence, through a transitory inactivation of this structure with lidocaine.

2. Materials and methods

2.1. Subjects and surgery

The animal model used in this study was *Rattus norvegicus*, of the Wistar strain. Male rats $n = 320$ (age 2–3 months; weight 250–280 g) were obtained from the breeding colony of Universidade Federal do Rio Grande (Rio Grande, RS, Brazil). The animals were kept in groups of five in each cage, with a 12 h light/dark cycle, at a temperature of 22 °C ± 1 °C, with food and water *ad libitum*.

After a week of acclimation, the animals were submitted to stereotaxic surgery for the implant of cannulae on the CA1 region of the hippocampus or on the medial septum, under Ketamin (62.5 mg/kg) and Xilazin (13 mg/kg) anesthesia. The guide cannulae were fixed with acrylic resin at 1 mm above of CA1 region of dorsal hippocampus, according to the following coordinates: 4.3 anterior, 3.0 lateral, 1.8 ventral or in the medial septum region, coordinates: −0.6 anterior, 0.0 lateral, 4.7 ventral according to the atlas by Paxinos and Watson (2007) (Fig. 1). At the end of the surgery, to prevent infections, the animals were treated with an antibiotic association (Pentabiótico®, Brazil). The study followed all the ethical recommendations of the Brazilian Society of Aninals Used (SBCAL).

2.2. Drugs and infusion procedures

For experimental procedures, rats were distributed randomly in groups according to the treatments. For the hippocampal infusion on the CA1 region, the groups were divided in mecamylamine 9.82 mM, a general nAChR antagonist; DHβE 18 mM, a specific α4β2/α3β2 mAChR antagonist; scopolamine 9.1 mM, a general mAChR antagonist; Pirenzepine 100 mM, a specific M1 mAChR antagonist, and the control groups were treated with saline solution. For the medial septum treatment, the groups were treated with lidocaine HCl at 40 mg/ml infusion and the control groups were treated with saline solution. In all groups 1 μl/cannulae was infused according to the treatment. For the infusion procedure, an infusion cannula was fitted through the guide cannulae and the infusions were performed with Hamilton microsyringe coupled to the cannulae with a polyethylene tube. At the time of infusion, infusion cannula protruded 1.0 mm beyond the guide cannula and was aimed at the hippocampus CA1 area or medial septum. All the treatments were performed at 9 or 12 h after IA test. All drugs were obtained from Sigma Chemical Co, USA.

2.3. Behavioral procedures

2.3.1. Inhibitory avoidance (IA)

After recovering from surgery, the animals were manipulated for 3 days to avoid neophobia and then submitted to a one trial step-down inhibitory avoidance test. The IA apparatus consists in an acrylic box (50 × 25 × 25 cm), whose floor consists of parallel stainless steel bars spaced 1.0 cm apart and a 5 cm wide platform located beside, slightly above the steel bars. On the training section, each animal was placed over the platform and, immediately after stepping down with its four paws on the steel floor, the animal received three 0.7 mA, foot shock, lasting 1 s each. In the test section no foot shock was applied and the platform step down latencies were measured. Memory retention was tested 2 or 7 days post-training, different groups of animals were used to the tests on the 2nd and 7th day to avoid the extinction of memory (Bekinschtein et al., 2007).

2.3.2. Elevated plus maze

The animals were also submitted to an elevated plus-maze (EPM), 2 or 7 days after the treatments, to determine if the drugs had affected mobility, locomotion or pro- or anti-conflict behaviors (Barros et al., 2004). The EPM apparatus consists of a central platform, two open arms and two enclosed arms. Both open and enclosed arms are disposed in opposition to each other. The maze is located 50 cm above floor level and tests were carried out under a dim red light. In this test the animals are place individually on the central platform and the time spent and the total entries on both open arms are recorded, during 5 min (Aguiar et al., 2006).

2.4. Cannulae placements

Postmortem verification of cannulae placements were performed as previously described (Barros et al., 2004). Briefly, 1 μl of 4% methylene blue in saline solution was infused through the cannulae and the animals were then sacrificed by decapitation and had their brains stored in formalin for at least 48 h. The cannulae placements, as verified by histological examination, were found to be correct (within 1 mm of the intended sites) in 93% of the animals. Only the behavioral data from these animals were analyzed.

2.5. Statistical analysis

Nonparametric statistic was used to analyze the inhibitory avoidance results in test-sessions because a 180 s ceiling was established. Data are expressed as median and interquartile ranges (25/75). Test-session latency in the step-down inhibitory avoidance task Kruskal–Wallis analysis of variance was followed by Mann Whitney U test. On the EPM task parametric statistic was used and statistical differences were tested through analysis of variance ANOVA test followed by Newman–Keuls post-test. In all comparisons, $p < 0.05$ was considered to indicate statistical significance.

3. Results

To determine the role of cholinergic system on LTM persistence, we performed a strong (0.7 mA) inhibitory avoidance training (IA) to induce the formation of a persistent memory and then we tested different cholinergic treatments on the CA1 region of the hippocampus. We also studied the involvement of or the medial septum region, through its inactivation with lidocaine infusion. These regions are implicated in early memory formation (Morris, Garrud, Rawlins, & O'Keefe, 1982; Niewiadomska, Baksalerska-Pazera, & Riedel, 2009). We first analyzed whether cholinergic mAChRs are involved on LTM persistence and therefore we tested the animals 2 or 7 days after training. Scopolamine (9.1 mM) infusion performed at 12 h post-training on the CA1 region (Fig. 2B) caused an impairment on LTM persistence 7 days post-training ($p = 0.0008$, $n = 10$–12, vs. control, Mann Whitney U test), but no impairment on LTM formation was found 2 days after training in the IA test. We next analyzed the effect of mAChR M1 subtype on LTM persistence with a 12 h post-training infusion of M1 antagonist pirenzepine (100 mM) in the CA1 region (Fig. 2B). Similarly to the treatment with scopolamine, no alterations of LTM formation at 2 days were observed and a hindered LTM persistence at 7 days in IA test was found ($p = 0.0003$, $n = 10$–12, vs. control, Mann Whitney U test).

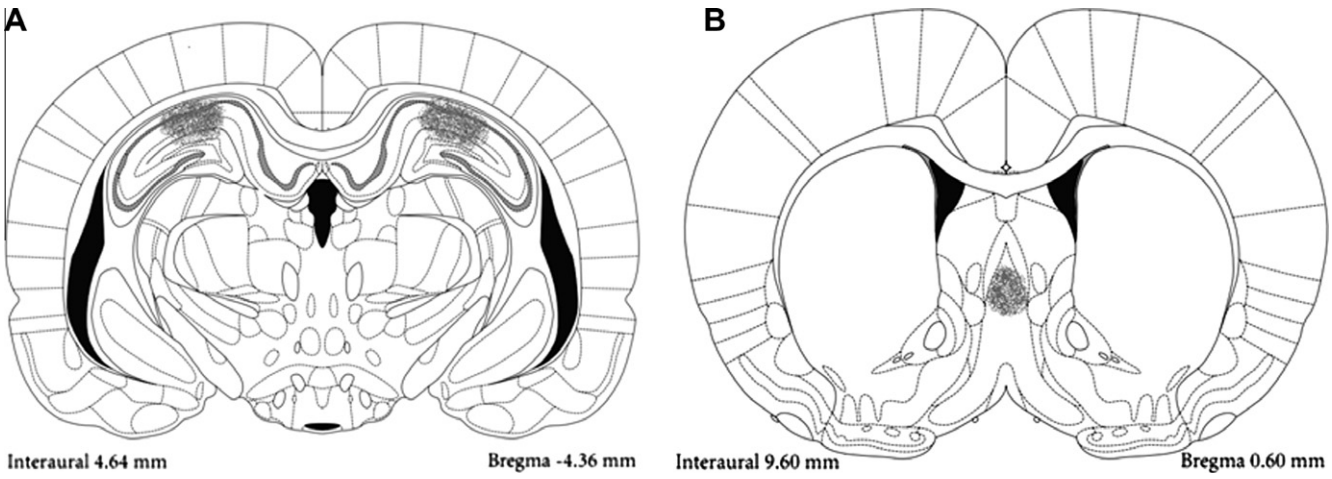

Fig. 1. Figure representing AP plane – 4.3 mm or 0.6 mm from bregma adapted from the Atlas of Paxinos and Watson (2007) indicating cannulae placements in the dorsal hippocampus (A) or medial septum (B) (marked area represents average region of acceptance, dyed with 1 μl of 4% methylene blue).

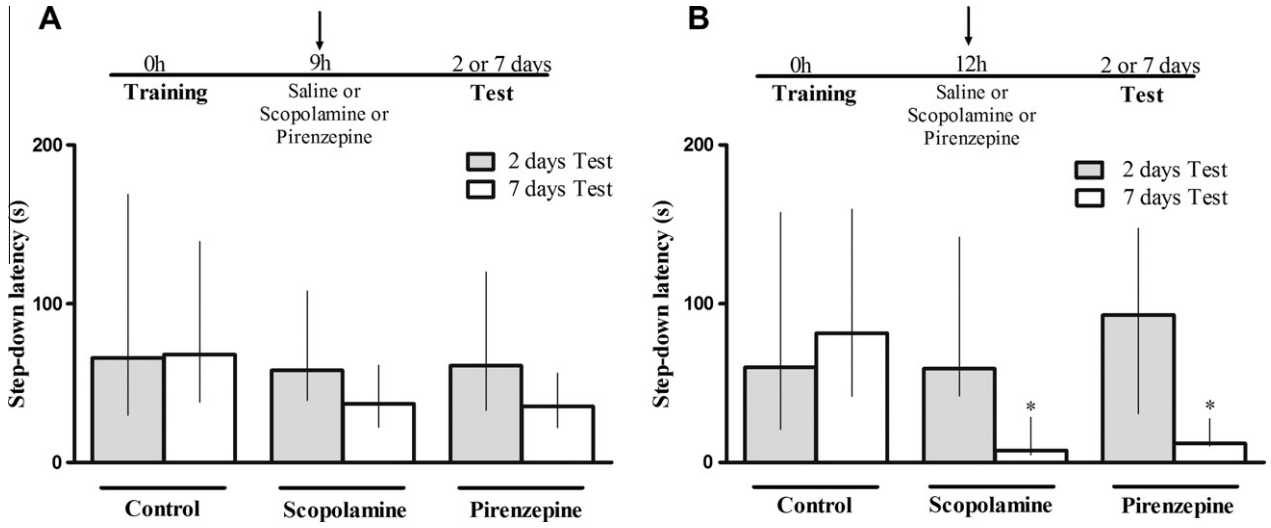

Fig. 2. Cholinergic muscarinic receptors participate in memory persistence 12 h post-training. (A) Scopolamine or pirenzepine infusion into dorsal hippocampus 9 h after training does not interfere on memory formation at 2 days test or memory persistence at 7 days test. (B) Scopolamine intrahippocampal infusion 12 h after IA training impairs memory persistence at 7 days test, but spares memory formation 2 days after training. Moreover Intrahippocampal infusion of pirenzepine causes an impairment of memory persistence 7 days, but does not alter memory 2 days after training. *, $p < 0.05$, $n = 10–12$; vs. control 7 days. Data are expressed as median and interquartile ranges (25/75).

The same treatments were performed 9 h after the training in IA, however memory formation and persistence remained unaltered ($p = 0.2039$, $n = 9–11$, Kruskal–Wallis test) (Fig. 2A).

Furthermore, nAChRs participation on memory persistence was tested. nAChRs blocker, mecamylamine (9.82 mM) was infused 12 h post-training in the CA1 area (Fig. 3B), and it seems to have blocked LTM persistence at the 7th day of the IA test ($p = 0.0076$, $n = 10–13$, vs. control Mann Whitney U test). However LTM at 2nd day of the test remained unaffected. We also investigated the participation of α4β2/α3β2 nAChR in memory persistence with DHβE (18 mM) infusions, 12 h post-training, at the CA1 area (Fig. 3B). However no effects on the formation and persistence of memory at the 2nd and 7th days respectively were demonstrated after DHβE infusions. According to the mAChR blockade experiments, the infusion of nAChR antagonists, mecamylamine and DhβE, performed 9 h after training had no effects on LTM formation and persistence ($p = 0.5316$, $n = 9–11$, Kruskal–Wallis test) (Fig. 3A).

We next analyzed memory persistence for 9 or 12 h post-training infusion of lidocaine in an attempt to inactivate the medial septum. We found that lidocaine infusion 12 h post-training impairs LTM persistence, as observed 7 days after training in IA task ($p = 0.0019$, $n = 10–11$, vs. control Mann Whitney U test), but leaves memory intact on 2 days test (Fig. 4B). Lidocaine infusion 9 h post-training showed no effects on both 2nd and 7th day tests ($p = 0.3930$, $n = 7–9$, Kruskal–Wallis test) (Fig. 4A). On the data regarding training session, the step-down latencies among the groups showed no differences (data not shown) ($p = 0.5628$, Kruskal–Wallis test).

To verify the absence of any interference on locomotion or anxiety that the treatments might have caused on the subjects, we performed the elevated plus maze test, 2 or 7 days after the infusions. No alterations in the percentage of time spent in open arms among the groups were found ($p = 0.3930$, One-way analysis of variance) however the group tested with scopolamine 2 days after the injection showed an increase of the total entries, suggesting a slight locomotion alteration ($p < 0.05$, vs. control, Newman–Keuls Test) (Table 1).

4. Discussion

The present study attempts to correlate the cholinergic system with the modulation of memory persistence, at the crucial time

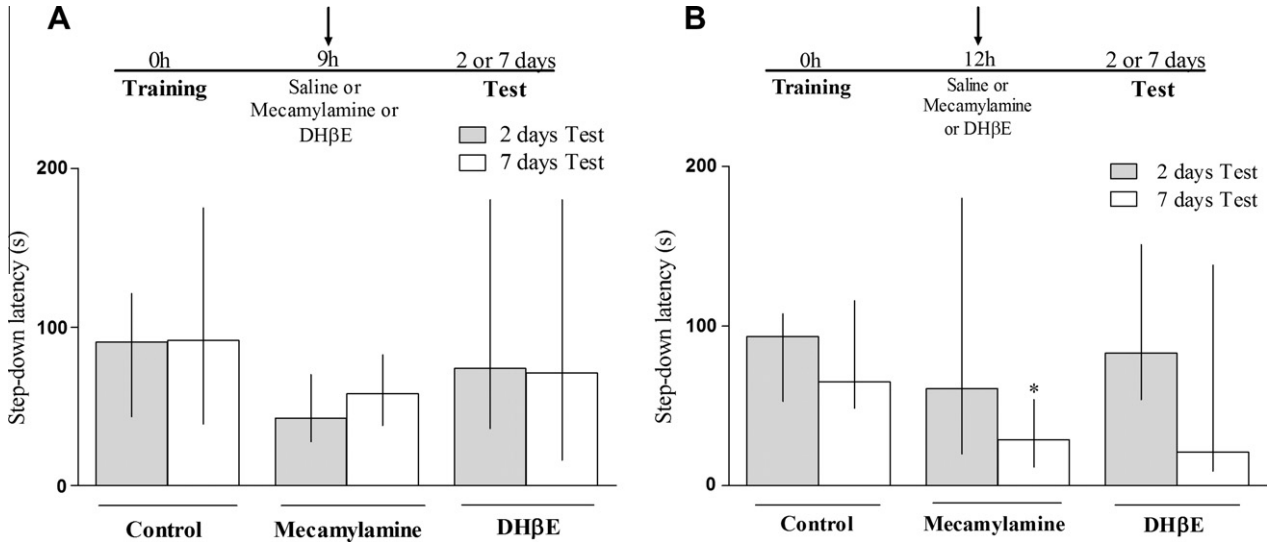

Fig. 3. *Cholinergic* nicotinic receptors participate on memory persistence 12 h post-training in IA. (A) Mecamylamine or DHβE intrahippocampal infusion 9 h post-training in IA does not interfere with memory formation on 2 days IA test or memory persistence on 7 days IA test. (B) Mecamylamine infusion on dorsal hippocampus 12 h post-training blocks memory persistence at 7 days test in IA, but does not interfere in memory formation on 2 days post-training test. Infusions of DHβE 12 h post-training does not interfere in memory retention on 2 or 7 days test. *, $p < 0.05$, $n = 10–13$; vs. control 7 days. Data are expressed as median and interquartile ranges (25/75).

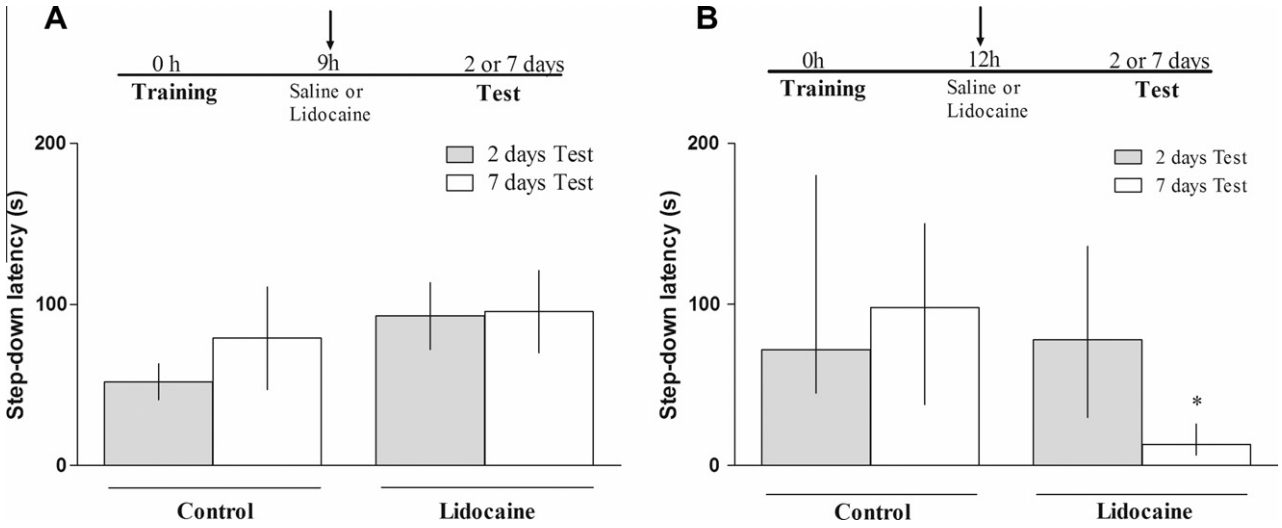

Fig. 4. Medial septum activation is required for memory persistence at 12 h post-training. (A) Infusion of lidocaine on medial septum 9 h post-training on IA does not interfere in memory retention, as tested 2 or 7 days after training. (B) Lidocaine infusion on medial septum 12 h post-training impairs memory persistence 7 days after IA training, but leaves memory retention intact on animals tested 2 days after IA training. *, $p < 0.05$, $n = 10–11$; vs. control 7 days. Data are expressed as median and interquartile ranges (25/75).

point of 12 h post-training. The data shows a decrease of remote memory retention, with no interference on its formation at 2 days, when mAChR or the M1 subtype are blocked at 12 h post acquisition. Moreover, similar results are found when nAChR were also blocked at this aforementioned time, the animals are able to form the memory, but the retention decays over time. That demonstrates that the activation of these receptors at this time is essential for the participation of the hippocampus on memory persistence. Surprisingly, both α4β2 and α3β2 nAChR subtypes did not interfere in LTM persistence, since the blockade of these receptors 12 h after the acquisition does not impair memory formation or its retention for several days. We then performed a transitory inactivation of medial septum nucleus (MS) 12 h post-training. Normal memory formation was observed 2 days after the acquisition, but its retention was impaired at 7 days. This shows that the activation of this area, which contains a population of cholinergic neurons projected towards the hippocampus, is, at this critical period, essential for memory persistence. (Niewiadomska et al., 2009). Therewith we demonstrated that the cholinergic system is engaged in hippocampal activity for the maintenance of fear memories.

Our results show that the treatments carried out 12 h after acquisition did not interfere on memory formation. This data corroborates with Bekinschtein et al. (2007), which states that this time point of 12 h after training in IA is crucial for the persistence of memory, not causing any interference whatsoever on memory formation (Bekinschtein et al., 2007). Moreover, no inference, on either formation or persistence of memory, was observed when the anticholinergic treatments were administered 9 h post-training. This reaffirms the findings of Rossato, Bevilaqua, Izquierdo, Medina, and Cammarota (2009) about the dopaminergic system, which is only implicated on the persistence of LTM at

Table 1
Results from the elevated plus-maze task performed 2 or 7 days after treatments.

Treatment	Total entries	% Time open arms
Control 2 days	10.3 ± 0.9	43.4 ± 4.7
Control 7 days	10.6 ± 0.9	33.9 ± 6.6
Scopolamine 2 days	17.0 ± 1.3*	51.8 ± 4.9
Scopolamine 7 days	11.7 ± 1.4	39.7 ± 4.0
Pirenzepine 2 days	14.1 ± 1.3	49.7 ± 5.0
Pirenzepine 7 days	10.7 ± 0.9	36.8 ± 4.6
Mecamylamine 2 days	12.2 ± 2.1	49.2 ± 6.1
Mecamylamine 7 days	13.8 ± 1.7	40.5 ± 5.4
DHβE 2 days	14.7 ± 1.1	36.6 ± 4.1
DHβE 7 days	11.7 ± 2.8	41.3 ± 3.9
Control MS 2 days	13.4 ± 1.2	37.9 ± 3.6
Control MS 7 days	11.2 ± 1.3	35.0 ± 4.8
Lidocaine MS 2 days	10.7 ± 1.6	41.5 ± 2.9
Lidocaine MS 7 days	11.8 ± 1.1	36.0 ± 6.5

* $p < 0{,}05$ $n = 7–8$; vs. control 2 days. Data are expressed as mean and SEM.

the time point of 12 h, but not at 9 h after the training in IA (Rossato et al., 2009). One aspect that should be considered is that the treatments carried out 9 h after the IA training could also interfere at the time point of 12 h, as the interval between the two time points is relatively short. Still, in all treatments, no alterations to the persistence of memory were observed. Hence, if this putative interference did occur, it did not suffice to alter the persistence of memory.

The role of muscarinic receptors in synaptic plasticity and behavior has been widely studied. The stimulation of these receptors enhances LTP induction *in vitro* and also enhances LTP maintenance *in vivo* in the CA1 region of hippocampus (Blitzer, Gil, & Landau, 1990; Iga, Arisawa, Ise, Yasuda, & Takeshita, 1996). Also, behavioral experiments have showed impairments on memory consolidation with post-training infusions of muscarinic antagonists, while the administration of muscarinic agonists renders an enhancement of memory formation (Jerusalinsky, Cerveñansky, Walz, Bianchin, & Izquierdo, 1993). Studies have shown that M1 receptors facilitated LTP in the CA1 region, probably through PKC induced potassium SK channels inhibition (Buchanan, Petrovic, Chamberlain, Marrion, & Mellor, 2010). In addition, M1 and M3 mAChRs in the hippocampus, as well as PKC, are involved in fear memory formation (Ferreira et al., 2003; Izquierdo et al., 2008). Moreover, Berzaghi and collaborators' (1993) findings show that regulation of the expression of BDNF on hippocampus is highly regulated by mAChR (Berzaghi et al. 1993).

Nicotinic receptors are widely expressed in the hippocampus, being both pre and post-synaptic α7 subunits and post-synaptic β2 subunits the most expressed ones (Fabian-Fine et al., 2001; Zarei, Radcliffe, Chen, Patrick, & Dani, 1999). They participate on neuronal plasticity in the hippocampus by boosting LTP and by modifying neuronal protein transcription through CREB activation and related cFOS induction (McKay, Placzek, & Dani, 2007). They also participate in memory processes, as impairments on acquisition, consolidation and retrieval of LTM as well as working memory, after nonspecific and α4β2, α3β2 and α7 subtypes blockade, have been reported (Barros et al., 2004; Barros et al., 2005; Nott & Levin, 2006). Memory persistence impairment due to nonspecific nAChR blockade on CA1 could be explained by the plasticity of the neuronal modulation through these receptors. However, no significant effects of α4β2, α3β2 antagonist DHβE on memory persistence could be observed. Further works concerning hippocampus dependent eyeblink conditioning reported that α7 but no α4β2, α3β2 "Knockouts" showed impairments on memory acquisition (Brown, Comalli, Biasi, & Woodruff-Pak, 2010). Since the nAChR subtypes α4β2, α3β2, and α7 show a distinct subcellular distribution and physiological roles, a α7 modulation of memory persistence may be proposed, but further investigations are necessary to prove this hypothesis (McKay et al., 2007; Zarei et al., 1999).

We also demonstrated the role of the medial septum on memory persistence, as most of the cholinergic projections to the hippocampus originate in the medial septal nucleus and the vertical limb nucleus of the diagonal band. Lesions or the inactivation of this region impairs memory (Fletcher, Calhoun, et al., 2006; Fletcher, Baxter, et al., 2007; Janis, Glasier, Fulop, & Stein, 1998; Mizumori, Perez, Alvarado, Barnes, & Mcnaughton, 1990; Niewiadomska et al., 2009; Pang & Nocera, 1999; Pang, Nocera, Secor, & Yoder, 2001; Rashidy-Pour et al., 1996). Furthermore, Lecourtier and collaborators (2010) demonstrated that lesions made prior to the training session on the septal cholinergic and GABAergic neurons can hinder remote memory in animals trained in the Water Maze Task. It was also observed that the formation of memory was not affected 1 day after the training session, but was hindered after 5 or 25 days. Together with the data presented in this work this information suggests a possible role for the medial septum on the maintenance of the memories, not interfering in its formation and thus reinforcing a possible activation of this area 12 h after the acquisition (Lecourtier et al., 2010).

The uniformity on both anxiety levels and motor activity observed in most of the treatments reinforces the reliability of the results obtained from the IA task, since alterations in such task could interfere on the parameters involved in the evaluation of memory retrieval. Though an increased motor activity was observed in the group scopolamine tested 2 days after the training session, the retrieval of the memory was not affected, as the learning among the animals was unaltered.

In conclusion, our findings suggest that mAChR and nAChR at the CA1 area are required for the persistence of memory. In addition, the cholinergic medial septal nucleus, as well as M1 mAChR subtype, but not nAChR α4β2, α3β2, participate in the memory persistence.

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