mechanisms, LTM is formed but is unable to persist; thereby, memory expression can be noticed 24 h but not 7 days after training. Other strategies to maintain the memory trace include exposing the animal to a reminder event like a test session or a retraining session.

Some reminders induce memory reconsolidation, which refers to the process of destabilization/re-stabilization of a memory after its activation (Rodriguez-Ortiz et al., 2012). This makes the reactivated LTM transiently sensitive to amnesic agents that are effective during the consolidation process (Haubrich and Nader, 2016). In general, these reminders are performed one day after learning and can strengthen or preserve the memory trace, when tested 24 h after the reactivation session (Tronson and Taylor, 2007). However, it has not yet been elucidated whether the phenomenon of memory reconsolidation mediates LTM persistence measured several days after reactivation. Moreover, not all reminders induce reconsolidation; this is only observed under conditions in which the original memory is updated or reinforced (Rodriguez-Ortiz and Bermúdez-Rattoni, 2017). Thus, sometimes, test or retraining sessions do not induce the memory reconsolidation process.

The formation and persistence of memories can be facilitated by spaced training, which involves long inter-trial intervals (ITIs), and is superior to massed training, which involves short or no ITIs. This superiority of spaced training has been explained by means of three cognitive theories, based on: the encoding, the processing, or the retrieval of learned information (Smolen et al., 2016). In particular, the study-phase retrieval theory posits that each spaced trial elicits retrieval of a memory trace that was formed by the preceding trial, and therefore the memory can be reinforced. This theory led us to test whether molecular mechanisms of memory expression triggered by retrieval are required to promote memory persistence through spaced learning.

Little is known about the molecular mechanism of memory expression. It has been described that memory retrieval requires the activity of protein kinase A (PKA) and the activation of extracellular regulated kinases 1/2 (ERKs1/2), but not that of the calcium/calmodulin-dependent kinase II (CaMKII) (Szapiro et al., 2000, 2002). Recent evidence has reported that the N-methyl-d-aspartate (NMDA) activity-mediated trafficking of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors that takes place during memory retrieval involves an ongoing protein synthesis (Lopez et al., 2015). In agreement, Pereyra et al. (2018) showed that aversive and spatial memory expression is controlled by mammalian target of rapamycin complex 1 (mTORC1), a protein complex that is implicated in a variety of fundamental cell functions such as metabolism and synaptic plasticity via the regulation of protein synthesis. In the present study, we aimed to investigate whether the molecular mechanisms involved in memory expression could affect memory persistence induced by retraining. We propose that the promotion of memory persistence is based on the Behavioral Tagging (BT) mechanism that operates when the memory trace is retrieved.

The BT hypothesis proposes a cellular model for memory establishment (Moncada and Viola, 2007). This conceptual framework involves the setting of a tag induced by learning and the synthesis of plasticity-related proteins (PRPs). Both processes were originally postulated by the Synaptic Tagging and Capture hypothesis using synaptic plasticity models (Frey and Morris, 1997). The learning tags determine the input selectivity and have a transient temporal course that lasts around 2 h depending on the type of task (Redondo and Morris, 2011; Moncada et al., 2015a). PRPs are required to consolidate the mnemonic trace and can be provided either by the same learning experience (if strong enough) that sets the learning tag or by an independent associated event. Besides, for this consolidation to occur, the tags and the PRPs must be present at the same time and on the same neuronal substrate (Ballarini et al., 2009; Moncada et al., 2015b). The BT phenomenon has been demonstrated by several research groups in diverse memory paradigms and tasks based on aversive, spatial and appetitive learning (Ballarini et al., 2009; Wang et al., 2010; Dong et al., 2012; Cassini et al., 2013). This theory also provides a wide framework to explain diverse memory processes like formation and maintenance, retrograde interference, and reconsolidation (Moncada et al., 2011; Lu et al., 2011; Almaguer-Melian et al., 2012; Martínez et al., 2014; Dong et al., 2012; Martínez et al., 2012; Salvetti et al., 2014; Viola et al., 2014; de Carvalho Myskiw et al., 2014; Tomaiuolo et al., 2015; Liu et al., 2015; Bae and Richardson, 2018; Gros and Wang, 2018; Naseem et al., 2019; Lopes da Cunha et al., 2019, 2021; Orlandi et al., 2020; Tintorelli et al., 2020).

Here, to identify the process that promotes LTM persistence by spaced learning, we used the Spatial Object Recognition (SOR) task. Our results suggest that the promotion of memory persistence after retraining requires the mechanisms of memory expression, but not the mechanisms of memory reinforcement or reconsolidation. The promoting effect of retraining involves ERKs1/2 activity, to set the learning tag, and the availability of GluA2-containing AMPA receptors in the dorsal hippocampus. In summary, the persistence of SOR memory involves a BT process that requires the synthesis of PRPs and the setting of a learning tag.

EXPERIMENTAL PROCEDURES

Animals

Five hundred and twenty-five male adult Wistar rats between 2 and 3 months of age (weight, 200–350 g) obtained from the Faculty of Exact and Natural Sciences of the University of Buenos Aires (Buenos Aires, Argentina) were used in this study. Animals were housed in groups of three with water and food *ad libitum* under a 12-h light/dark cycle at a constant temperature of 21–23 °C. The behavioral testing took place during the light phase of the cycle. Rats were handled for 2 min for two consecutive days before each experiment to avoid emotional stress. During behavioral procedures,

animals were individually moved from their home cages to the arena and returned immediately after each trial session. All experiments were conducted in accordance with the National Institutes of Health Guides for Care and Use of Laboratory Animals (Publication No. 80-23, revised 1996) and approved by the Animal Care and Use Committee of the University of Buenos Aires (CICUAL), Buenos Aires, Argentina.

Drugs

The protein synthesis inhibitors used were emetine (EME, 50 μg/side) and rapamycin (RAPA, 60 nM/side, a specific mTORC1 inhibitor), both dissolved in saline solution. For RAPA, the volume infused was 0.5 μl/side, whereas for EME, the volume infused was 1 μl/side. U0126 (0.4 μg diluted in 10% DMSO in saline and infused in a volume of 0.8 μl per side) was used as an ERKs1/2 inhibitor given that it blocks the kinase activity of MEK1/2, thus preventing the activation of MAP kinases p42 and p44. These drugs were purchased from Sigma (St. Louis, MO, USA). For GluR23ɣ, the GluA2-containing AMPA receptor endocytosis inhibitor (Tat-GluR32ɣ, H-YGR KKR RQR KEG YNV YG-OH, Eurogentec, Anaspec), the dose was 15 pmol/side dissolved in saline solution, and infused in a volume of 0.5 μl per side. The doses were chosen based on published studies (Moncada et al., 2011; Lopez et al., 2015; Migues et al., 2016; Tintorelli et al., 2020; Pereyra et al., 2021).

Surgery and drug infusion

For cannulae implantation, rats were deeply anesthetized (70 mg/kg ketamine and 7 mg/kg xylazine), and then 22-G cannulae were stereotaxically aimed at the CA1 region of the dorsal hippocampus at coordinates A: −3.9 mm, L: ±3.0 mm, and D: −3.0 mm, from Bregma (Paxinos and Watson, 2007), and then cemented to the skull with dental acrylic. Animals received a subdermal application of analgesics and antibiotics during surgery (Meloxicam 0.2 mg/kg, gentamicin 3 mg/kg) and then allowed to recover from surgery for at least four days. Drugs were infused using a 30-G needle with its tip protruding 1.0 mm beyond the guide. The infusion needles were linked by an acrylic tube to a Hamilton microsyringe and the entire bilateral infusion procedure lasted about 3 min. Needles were left in place for one additional minute after infusion to minimize backflow. Histological examination of cannulae placements was performed after the end of the behavioral procedures by the infusion of 0.5 μl of 4% methylene blue in saline solution. Animals were killed by decapitation 15 min after the infusion and their brains were sliced to verify the infusion area (Villar et al., 2017). Only data from animals with correct cannulae implants (95%) were included in statistical analyses.

Behavioral procedures

The memory performance of animals was evaluated in a SOR task. SOR memory represents the ability to detect the spatial displacement of previously encountered objects. In this task, an animal reveals its learning of the spatial configuration of two identical objects, when it spends more time exploring the spatially displaced familiar object relative to a stationary familiar object in a test (Dere et al., 2005). Depending on the experiment, animals were exposed to single or double SOR training sessions spaced by different ITIs. Also, the exposure to an open field (OF) was used as a novel event able to induce the synthesis of PRPs (Moncada and Viola, 2007). The experiments were carried out almost in the same sequence exposed in the results section, and the experimental designs are presented at the top diagrams of each figure. Each figure was generally composed of two sets of experiments that included all the experimental groups/conditions. These sets of experiments were performed in the same season of the year.

SOR task

The SOR arena was a 60 cm wide × 40 cm long × 50 cm high acrylic box, with different visual clues in its lateral white walls. The floor was white, the front wall was transparent and the back wall was hatched. For habituation to the context, all subjects explored the arena without objects for a 20-min daily session for two consecutive days before the training day. In the training session, two identical plastic or glass objects were included in the arena in two adjacent corners and animals were left to explore it for 4 min in a weak training (wSOR) or 8 min in the case of a strong training (sSOR). In the test session, one of the objects was moved to a new position and animals were allowed to explore this context for 2 min. The exploration time for each object, defined as sniffing or touching it with the nose or forepaws, was measured using a hand stopwatch. Rats were excluded from the analysis when they explored one object more than 65% of the total object-exploration time during training sessions or when they did not reach 10 s in the total object-exploration time during the 2-min test session. Results are expressed as a preference index: [Exploration time of the object in a new location (Tn) − Exploration time of the object in the familiar location (Tf)]/[Tn + Tf]. A positive preference index in the test session, differing significantly to zero, indicates the presence of memory. A representative mean ± SEM of the total object-exploration time during the first sSOR training session was 131.2 ± 7.8 s. It was 51.24 ± 3.40 s during the wSOR retraining session and 23.96 ± 1.80 s during the test session.

OF task

The OF task consists in placing an animal within an arena to record its locomotor and exploratory behavior in this novel spatial context. The arena was a 50 cm wide × 50 cm long × 39 cm high square box, with black plywood walls and floor divided into nine squares by white lines. The number of line crossings and rearings was measured in blocks of 1 min for 5 min under normal room lighting (Moncada and Viola, 2007).

Data analysis

Results are expressed as preference index mean ± SEM. The bar graphs in the figures also show the individual data points. One-sample *t*-test was used to determine whether the preference index differed from zero and thus the animal expressed SOR memory. The index differences between groups were analyzed with unpaired Student's *t* test when comparing two groups, and one-way ANOVA Test followed by Newman–Keuls post-hoc Comparison Test when comparing three or more groups. Analyses were performed in GraphPad Prism ® version 8.00 (GraphPad Software, La Jolla, CA, USA). Effects were considered significant when $P < 0.05$.

RESULTS

To study the spaced learning effects on the persistence of a SOR memory, we used a first training session that induced LTM formation, but not LTM persistence (tested at 7 or 14 days after training), and performed a second training session temporarily spaced to promote long-lasting LTM retention. This second training session was a weak session unable to induce SOR-LTM formation *per se*, but able to reactivate the neural sites originally activated by the first strong training session. In the SOR paradigm, spatial memory was evidenced by a higher exploration rate of the object that moved to a new location in a test session, expressed as an increase in the preference index. Fig. 1A shows that the group of rats trained with a 4-min SOR session and tested 30 min later exhibited SOR-short term memory (STM), determined by a preference index different from zero (tr, $t_{(7)} = 7.74$, $P < 0.001$), whereas a parallel group of rats tested 1 day after training did not show SOR-LTM ($t_{(10)} = 0.55$, $P > 0.05$ vs 0). In contrast, an 8-min SOR session induced LTM formation when tested 1 day later (Fig. 1B, TR, $t_{(12)} = 7.26$, $P < 0.001$ vs 0), but did not show SOR-LTM persistence when tested at 7 ($t_{(11)} = 0.04$, $P > 0.05$ vs 0) or 14 days post-training ($t_{(8)} = 0.4$, $P > 0.05$ vs 0) in independent groups of rats. Thus, we considered an 8-min SOR session as a strong training (sSOR) and a 4-min SOR session as a weak training (wSOR).

Next, we performed a spaced learning protocol, in which a sSOR training was followed 1 day later by a wSOR training, identical to the first one, except for the shorter duration of the session (Fig. 2A). In the memory retention test performed 7 days post-retraining, SOR-LTM persistence was observed (TR-tr, $t_{(14)} = 4.93$, $P < 0.001$ vs 0). A control group of rats confirmed that a single sSOR training session was unable to express LTM 8 days post-training (TR, $t_{(7)} = 1.24$, $P > 0.05$ vs 0). A third group of animals, instead of being exposed to a retraining session, was subjected to a 2-min test session 1 day after the sSOR training. In this session, one of the objects changed its location relative to the training, and the memory of the original position of the objects was measured 1 week later. We found that, although the preference index for this group was different from zero (TR-Test, $t_{(11)} = 2.60$, $P < 0.05$), it was not different from that of the control group (One-way ANOVA $F_{(2, 32)} = 4.47$, $P < 0.05$, Newman–Keuls multiple comparisons test TR-Test vs TR $P > 0.05$), and significantly lower than in the retraining group (TR-Test vs TR-tr $P < 0.05$). Then, we performed a similar experiment changing the ITI to 7 days, and also testing memory retention 7 days post-retraining. In this case, none of the three groups showed SOR-LTM persistence (Fig. 2B, TR $t_{(7)} = 0.49$, $P > 0.05$ vs 0, TR-tr $t_{(11)} = 1.18$, $P > 0.05$ vs 0, TR-test $t_{(10)} = 1.21$, $P > 0.05$ vs 0). With this protocol, no persistence promotion was observed even when the animals were tested 1 day post-retraining (Fig. 2C, TR $t_{(10)} = 0.50$, $P > 0.05$ vs 0, TR-tr $t_{(8)} = 2.15$, $P > 0.05$ vs 0, TR-test $t_{(6)} = 0.45$, $P > 0.05$ vs 0), thus suggesting that the ITI is a key variable of the retraining effect.

Then, we studied which features of the second session, performed 1 day after a sSOR training, are required to promote SOR-LTM persistence. The exposure to a test session longer

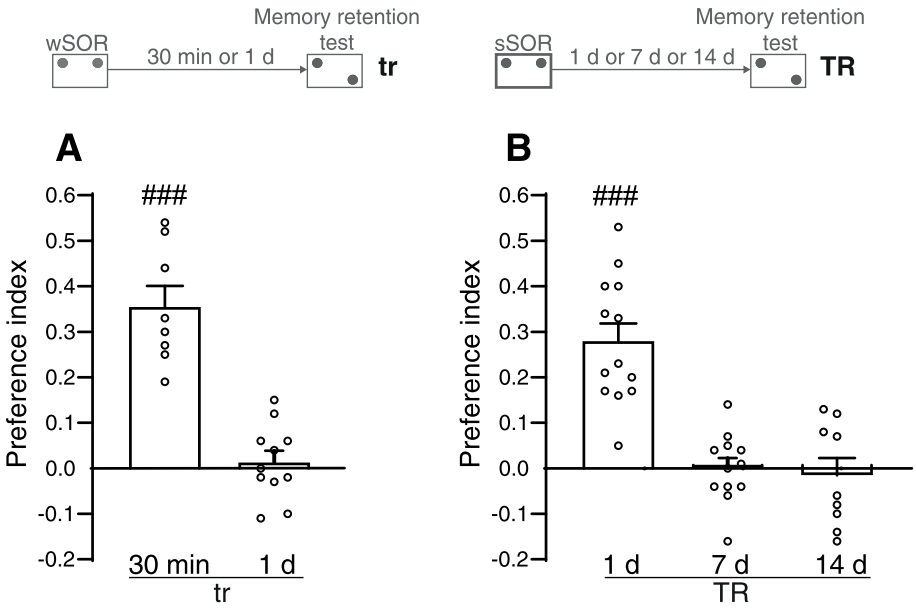

Fig. 1. Time course of memory expression after a single weak or strong spatial object recognition (SOR) training session. The top diagrams show the experimental designs. **(A)** Animals were exposed to a 4-min weak SOR (wSOR) training session (tr). Independent groups were tested at 30 min ($n = 8$), or 1 day ($n = 11$) after training, to record short term memory (STM) and long term memory (LTM) respectively. **(B)** Animals were exposed to an 8-min strong SOR (sSOR) training session (TR). Independent groups were tested 1 day later to record LTM ($n = 13$); or at 7 ($n = 12$), or 14 ($n = 9$) days after training to evaluate the persistence of LTM. Data are expressed as preference index mean ± SEM. ###$P < 0.001$ vs 0, One sample *t* test.

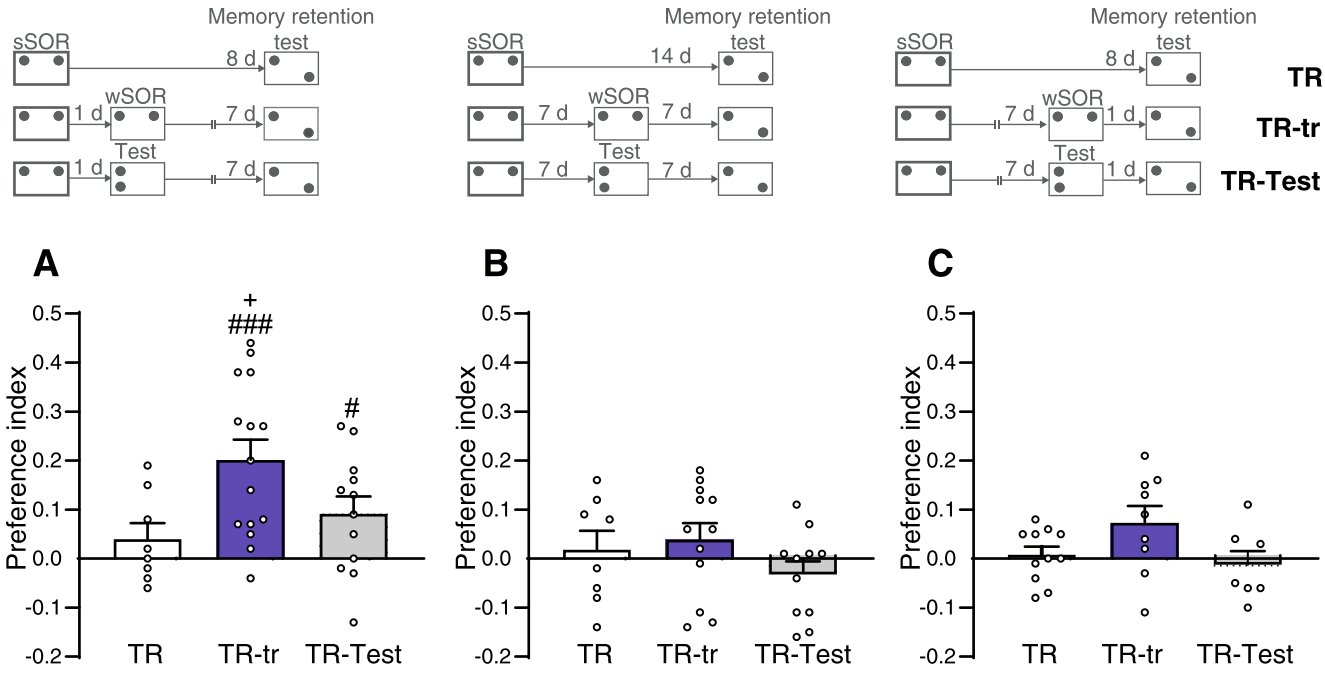

Fig. 2. A wSOR retraining session performed 1 day after a sSOR session promotes SOR-LTM persistence. The top diagrams show the experimental designs. **(A)** Control animals (TR) received a single sSOR training ($n = 8$). Retrained animals (TR-tr, $n = 15$) received a subsequent wSOR training spaced from the sSOR session by a 1-day inter-trial interval (ITI). Animals in the TR-Test ($n = 12$) group were exposed to a test session 1 day after the sSOR training. SOR-LTM was tested 8 days after the sSOR training. Data are expressed as preference index mean ± SEM. #$P < 0.05$, ###$P < 0.001$ vs 0, One sample t test. +$P < 0.05$ vs all groups, Newman–Keuls after one-way ANOVA. **(B)** The ITI between sessions was 7 days, independent animals were subjected to a wSOR retraining (TR-tr, $n = 12$) or a test session (TR-Test, $n = 11$) after the sSOR training session. Control animals (TR) were trained only with a sSOR session ($n = 8$). LTM was tested 14 days after the sSOR training. Data are expressed as preference index mean ± SEM. $P > 0.05$ one-way ANOVA. **(C)** The ITI was also 7 days, but the LTM was tested 8 days after the sSOR training. Rats were exposed or not (TR, $n = 11$) to a wSOR retraining (TR-tr, $n = 9$) or a test session (TR-Test, $n = 7$) after the sSOR training session. Data are expressed as preference index mean ± SEM. $P > 0.05$ one-way ANOVA.

than the one used in Fig. 2A (TEST, lasting 4 min) or to a novel OF session for 5 min did not have effects over SOR-LTM persistence (Fig. 3A, TEST $t_{(8)} = 0.78$, $P > 0.05$ vs 0, OF $t_{(6)} = 1.52$, $P > 0.05$ vs 0). As a positive control, we observed that memory persisted at 8 days post-training when the animals were subjected to a wSOR retraining session (One-way ANOVA $F_{(3, 31)} = 15.61$, $P < 0.001$, Newman–Keuls multiple comparisons test TR-tr, $P < 0.001$ vs all groups). These results suggest that it is necessary to repeat the same original experience (to be retrained) to induce the persistence of that memory, and that a similar experience is not enough to achieve it. However, neither a 2-min test session nor a wSOR or sSOR retraining session were effective in promoting SOR-LTM persistence when the initial learning was a wSOR training session (Fig. 3B, tr-Test $t_{(6)} = 1.60$, $P > 0.05$ vs 0, tr-tr $t_{(8)} = 0.56$, $P > 0.05$ vs 0, tr-TR $t_{(8)} = 1.92$, $P > 0.05$ vs 0). These results indicate that the retraining session is effective when the memory of the initial training is liable to be expressed, and this condition is not fulfilled when the original training is weak.

The persistence of the trace could be due to reinforcement of the mechanisms of SOR-LTM formation. In other words, the retraining protocol could induce a stronger LTM than with just one sSOR session and then take longer to decay, explaining its persistence over time. To study this possibility, rats were subjected to a wSOR 1 day after a sSOR (TR-tr), and memory retention was tested one day later. In parallel, another group of animals were exposed to a single sSOR and then tested for retention 2 days post-training (TR). Both groups of animals expressed SOR-LTM (Fig. 4A, TR-tr $t_{(9)} = 23.11$, $P < 0.001$ vs 0, TR $t_{(10)} = 8.35$, $P < 0.001$ vs 0), and no significant differences between them were observed (One-way ANOVA $F_{(2, 26)} = 30.81$, $P < 0.001$, Newman–Keuls multiple comparisons test TR vs TR-tr $P > 0.05$). This result suggests that SOR-LTM persistence induced by spaced learning is not based on a reinforcement mechanism of the original trace consolidation. On the other hand, we also studied whether a mechanism of reconsolidation is involved in the prolonged maintenance of the SOR trace induced by the retraining protocol. To this end, we infused the dorsal hippocampus with either vehicle solution (Veh) or the protein-synthesis inhibitor emetine (EME), capable of destabilizing the trace if retraining induced its labilization. We included a control group of rats infused with EME 1 day after a single sSOR training (TR). No significant differences were observed between these three groups of rats when SOR-LTM was tested 2 days after the sSOR (Fig. 4B, One-way ANOVA $F_{(2, 25)} = 2.10$, $P > 0.05$). A similar result was found with the intra-hippocampal administration of rapamycin (RAPA), a local protein-synthesis inhibitor (Fig. 4C, One-way ANOVA $F_{(2, 17)} = 0.49$, $P > 0.05$). These results suggest that the spaced-learning protocol that