Persistence of Spatial Memory Induced by Spaced Training Involves a Behavioral-Tagging Process

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Abstract—Spaced training, which involves long inter-trial intervals, has positive effects on memories. One of the main attributes of long-term memories (LTM) is persistence. Here, to identify the process that promotes LTM persistence by spaced learning, we used the spatial object recognition (SOR) task in rats. The protocol consisted of a first strong training session that induced LTM formation (tested 1 day after training), but not LTM persistence (tested 7 or 14 days after training); and a second weak training session that promoted memory persistence when applied 1 day, but not 7 days, after the first training. We propose that the promotion of memory persistence is based on the Behavioral Tagging (BT) mechanism operating when the memory trace is retrieved. BT involves the setting of a tag induced by learning which gives rise to input selectivity, and the use of plasticity-related proteins (PRPs) to establish the mnemonic trace. We postulate that retraining will mainly retag the sites initially activated by the original learning, where the PRPs needed for memory expression and/or induced by retrieval would be used to maintain a persistent mnemonic trace. Our results suggest that the mechanism of memory expression, but not those of memory reinforcement or reconsolidation, is necessary to promote memory persistence after retraining. The molecular mechanisms involve ERKs1/2 activity to set the SOR learning tag, and the availability of GluA2-containing AMPA receptor. In conclusion, both the synthesis of PRPs and the setting of learning tags are key processes triggered by retraining that allow SOR memory persistence.

Key words: spatial object recognition, spaced learning, long-term memory persistence, memory retrieval, hippocampus, learning tag.

INTRODUCTION

Persistence, which refers to the durability of memories after their formation, is the main attribute of long-term memory (LTM). In the last two decades, the dominant idea about the stabilization and maintenance of the learned information has established the special dependence on two different continuous and dynamic processes: (1) the synaptic or cellular consolidation and (2) the system consolidation. The former involves molecular and cellular events occurring early after training and lasting several hours up to a few days in particular brain regions engaged in acquisition (McGaugh, 2000), whereas the latter is an additional slow process that entails the participation of neocortical regions and their interactions with the medial temporal lobe structures that organize the recently learned material. It has been suggested that this latter process allows storing remote memories, operationally defined as the information that lasts for weeks after learning (Frankland and Bontempi, 2005).

It has been observed that beyond LTM formation, the hippocampus is also involved in memory processing. Previous studies have reported the existence of a cellular consolidation window in the hippocampus around 12 h after training for the persistence of aversive or appetitive memories for at least one week (Bekinschtein et al., 2007; Rossato et al., 2009; Kramar et al., 2014). Thus, a way to improve memory persistence is to act over specific mechanisms several hours after learning. By contrast, if there is interference in these

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Abbreviations: BT, Behavioral Tagging; CaMKII, calcium/calmodulin-dependent kinase II; ITIs, inter-trial intervals; LTM, long-term memories; NMDA, N-methyl-d-aspartate; PRPs, plasticity-related proteins; SOR, spatial object recognition.

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

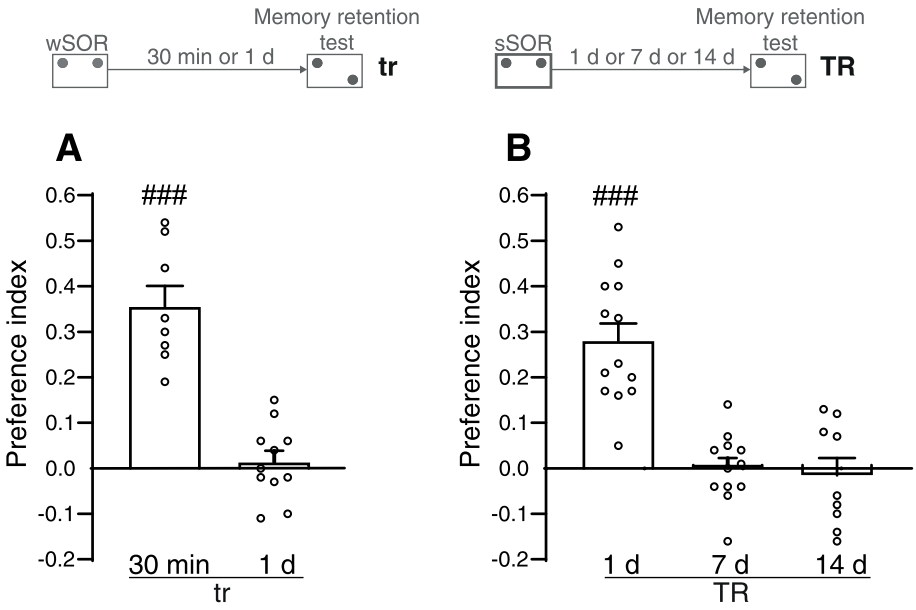


Fig. 1. Time course of memory expression after a single weak or strong spatial object recognition (SOR) training session. The top diagrams show the experimental designs. **(A)** Animals were exposed to a 4-min weak SOR (wSOR) training session (tr). Independent groups were tested at 30 min ($n = 8$), 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.

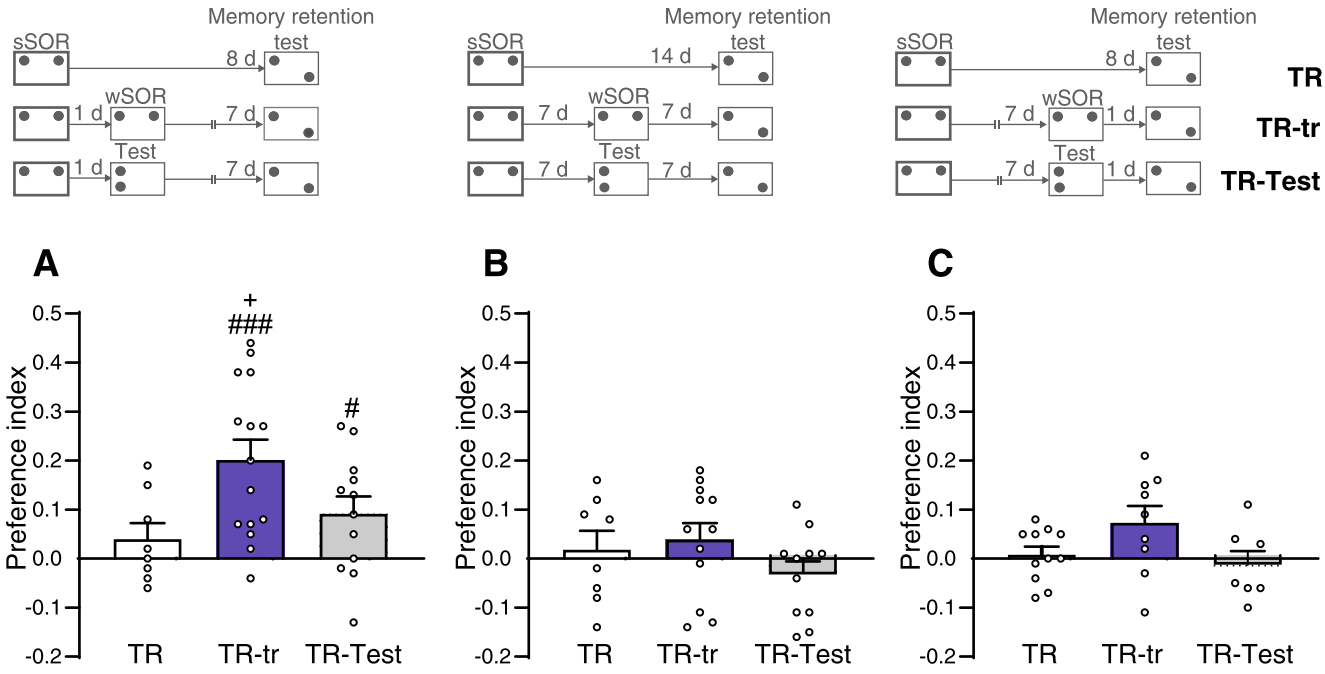


Fig. 2. A wSOR retraining session performed 1 day after a sSOR session promotes SOR-LTM persistence. The top diagrams show the experimental designs. **(A)** Control animals (TR) received a single sSOR training ($n = 8$). Retrained animals (TR-tr, $n = 15$) received a subsequent wSOR training spaced from 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

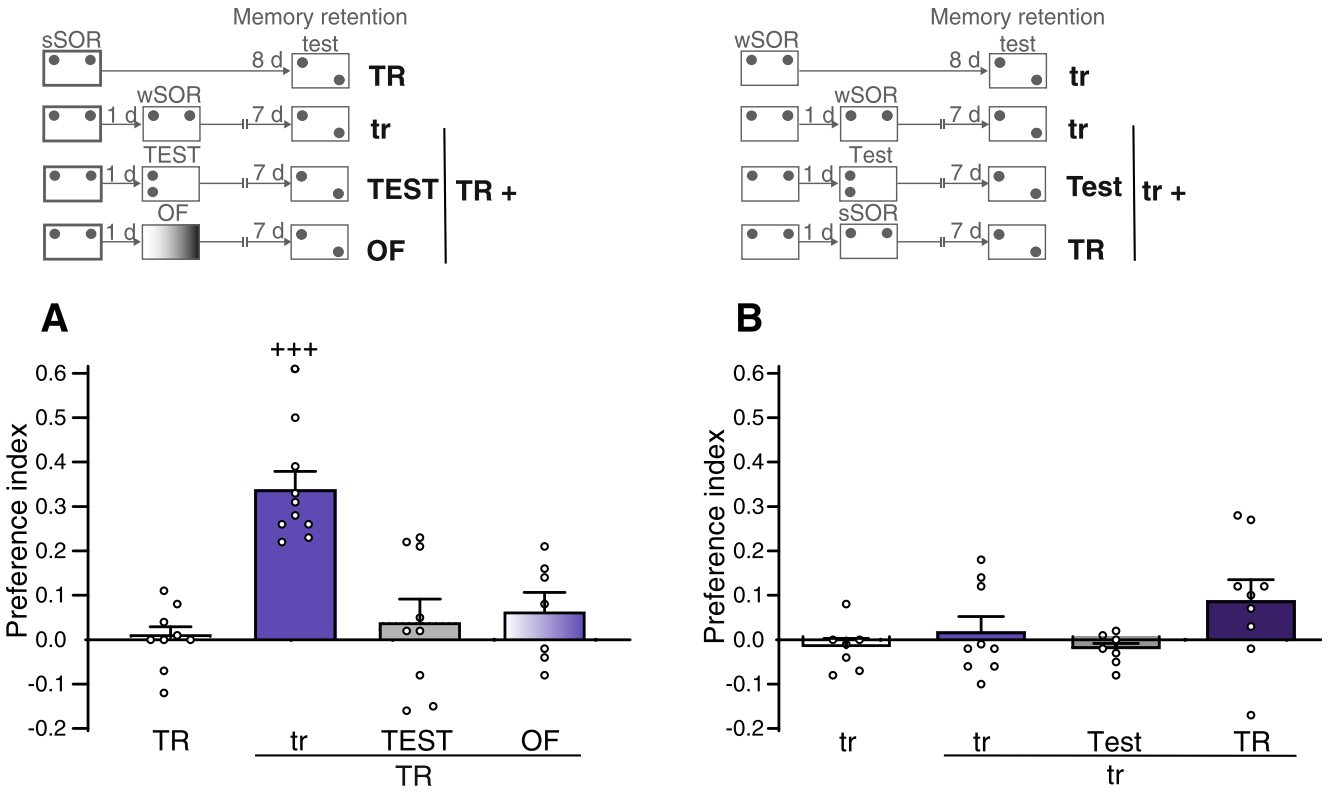


Fig. 3. A SOR retraining specifically promotes SOR-LTM persistence after a sSOR but not after a wSOR training. The top diagrams show the experimental designs. **(A)** Control animals (TR) received a single sSOR training session (n = 9). Independent groups of rats were exposed to a wSOR retraining session (TR-tr, n = 10), a 4-min test session (TR + TEST, n = 9) or a novel open field (OF) session (TR + OF, n = 7) one day after a sSOR. LTM was tested 8 days after the first training. Data are expressed as preference index mean ± SEM. + + +P < 0.001 vs all groups, Newman–Keuls after one-way ANOVA. **(B)** Control animals (tr) were trained with a single wSOR training session (n = 7). Retrained rats received a second session of weak or strong SOR 1 day after a wSOR training (tr + tr, n = 9 or tr + TR, n = 9). Animals in the tr + Test group (n = 7) were exposed to a 2-min test session 1 day after a wSOR training. LTM was tested 8 days after the first training. Data are expressed as preference index mean ± SEM. P > 0.05 one-way ANOVA.

induced SOR-LTM persistence does not labilize the original memory trace, and then a reconsolidation process would not be involved in extending the duration of the trace in this case.

Overall, our results rule out processes of reinforcement or reconsolidation of the trace triggered by retraining, while suggesting that the persistence of the SOR memory requires that the mechanisms of memory expression are available. To test this hypothesis, we impaired the mechanism of LTM expression by using drugs with proven effectiveness in blocking memory retrieval (Zhang et al., 2004; Lopez et al., 2015; Pereyra et al., 2018; Zamorano et al., 2018). Rats were trained with a sSOR session and tested one day later. As expected, the local administration of EME (Fig. 5A), RAPA (Fig. 5B) or U0126 (MEK inhibitor, Fig. 5C) 15 min before the test session induced amnesia in all three groups (EME $t_{(6)}$ = 0.35, P > 0.05 vs 0, RAPA $t_{(4)}$ = 0.45, P > 0.05 vs 0, U0126 $t_{(12)}$ = 2.16, P > 0.05 vs 0), in contrast to vehicle-infused animals, which exhibited SOR-LTM (unpaired Student's Veh vs EME $t_{(11)}$ = 6.49, P < 0.001, Veh vs RAPA $t_{(8)}$ = 3.32, P < 0.05, Veh vs U0126 $t_{(22)}$ = 3.13, P < 0.01). We next performed the retraining protocol, but infused these drugs 15 min before the wSOR to ensure that the mechanisms of memory expression were not available at the moment of retraining. Fig. 5D–F shows that these three drugs impaired SOR-LTM persistence measured a week after retraining (EME $t_{(8)}$ = 0.08, P > 0.05 vs 0, RAPA $t_{(12)}$ = 0.61, P > 0.05 vs 0, U0126 $t_{(5)}$ = 0.25, P > 0.05 vs 0). The groups infused with vehicle solution showed the expected positive effect over the persistence of the trace (Fig. 5D One-way ANOVA $F_{(2,\ 21)}$ = 5.48, P < 0.05, Newman–Keuls multiple comparisons test Veh vs EME and TR P < 0.05. Fig. 5E One-way ANOVA $F_{(2,\ 29)}$ = 22.13, P < 0.001, Newman–Keuls multiple comparisons test Veh vs RAPA and TR P < 0.001. Fig. 5F One-way ANOVA $F_{(2,\ 15)}$ = 5.48P < 0.05, Newman–Keuls multiple comparisons test Veh vs U0126 and TR P < 0.05). These results demonstrate that a single sSOR is capable of expressing LTM 1 day later, and that memory retrieval could be blocked with specific drugs. In addition, this suggests that the mechanism involved in memory expression should work at the time of retraining to prolong the durability of the trace.

It has been described that memory retrieval requires ongoing protein synthesis. Since we used wSOR as retraining, which by itself does not induce sufficient protein synthesis to trigger the formation of LTM, then the proteins derived from the process of memory

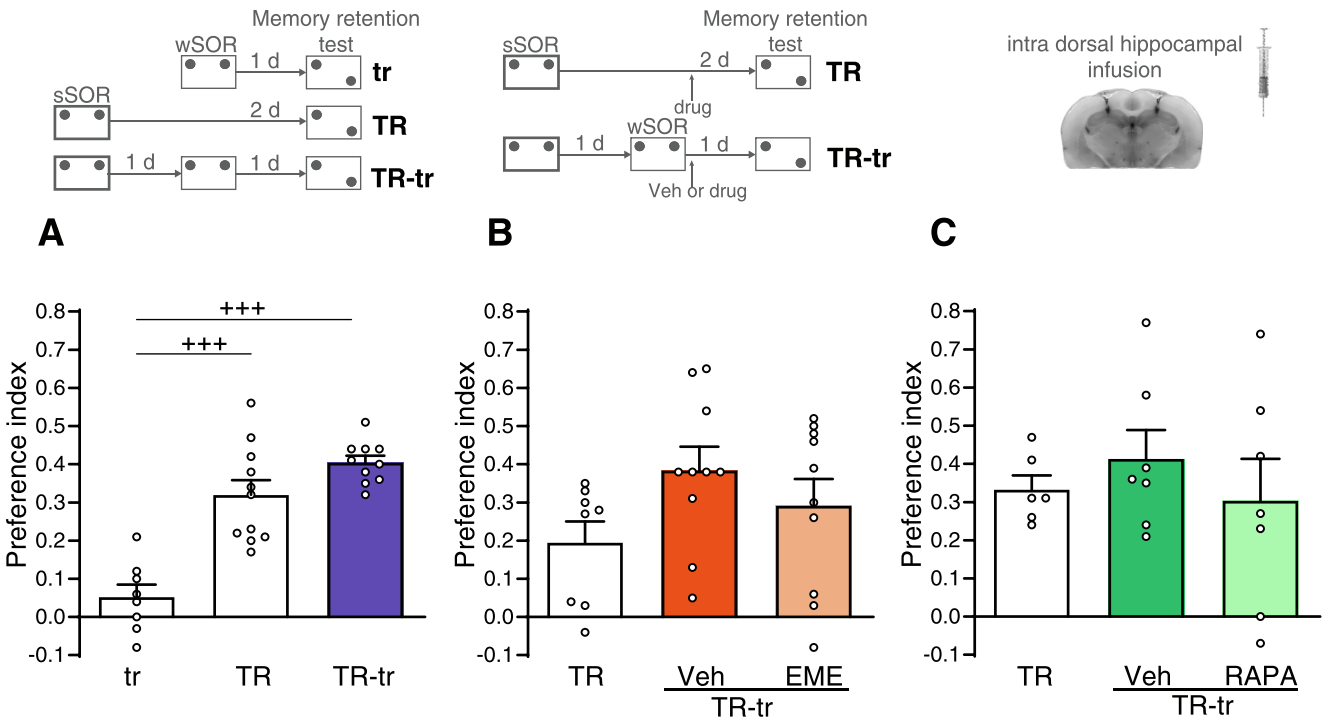
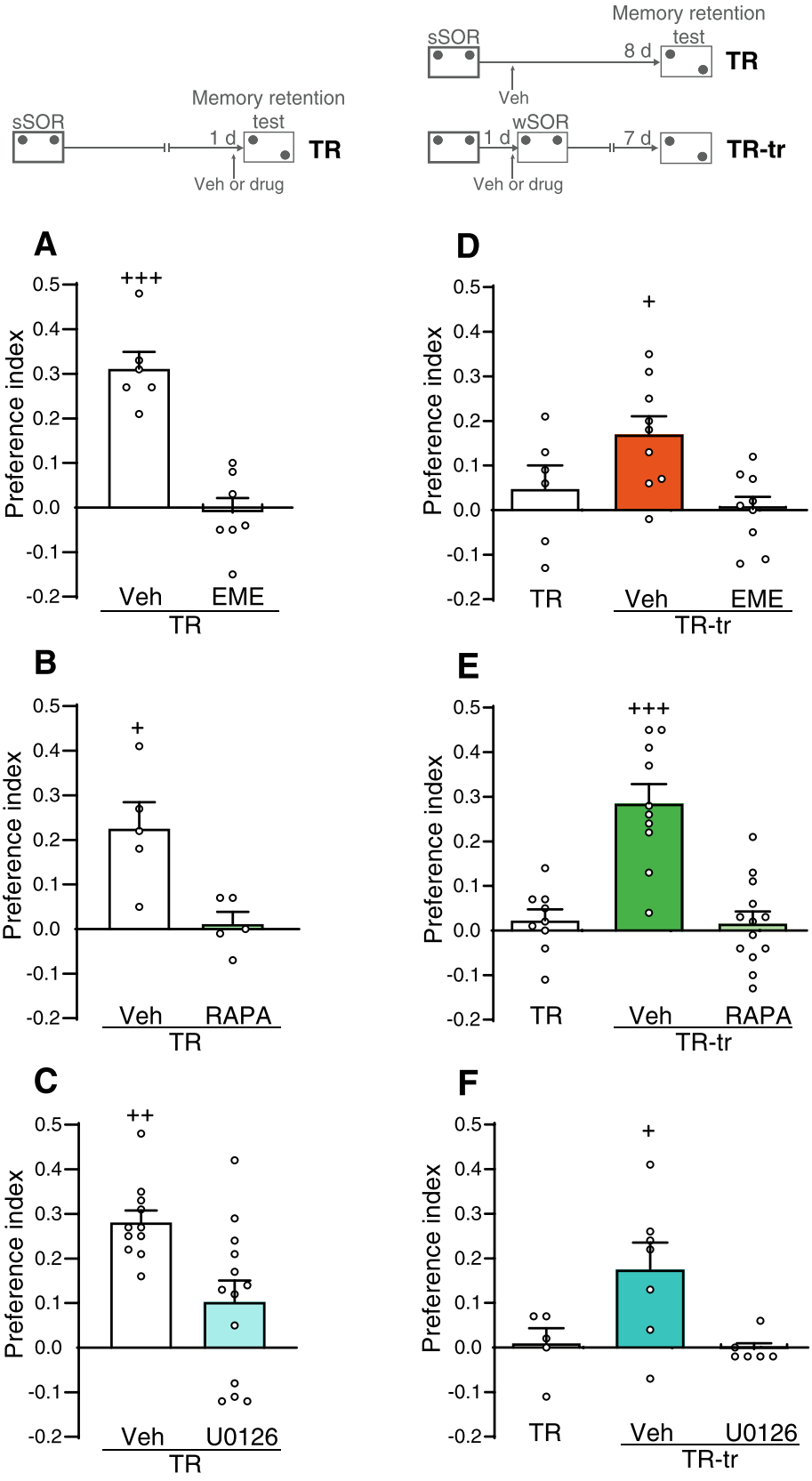


Fig. 4. The retraining protocol that induces SOR-LTM persistence does not reinforce consolidation or induce labilization of the original SOR trace. The top diagrams show the experimental designs and the image on the right shows the infusion site. **(A)** Independent animals were exposed to a single wSOR (tr, $n = 8$) or sSOR (TR, $n = 11$) training session and tested 1 or 2 days later, respectively. Retrained animals (TR-tr, $n = 10$) were subjected to a wSOR retraining session 1 day after a sSOR training, and SOR-LTM was tested 1 day after the second training session. Data are expressed as preference index mean ± SEM. $+++P < 0.001$, Newman–Keuls after one-way ANOVA. **(B)** The experimental protocol is similar to **(A)**, except that retrained animals (TR-tr) received intra-dorsal hippocampal infusions of vehicle (Veh, $n = 10$) or Emetine (EME, $n = 10$) immediately after the second training and LTM was tested 1 day after retraining. Control animals (TR, $n = 8$) received an intra-dorsal hippocampal infusion of EME 1 day after a sSOR training and tested the following day. Data are expressed as preference index mean ± SEM. $P > 0.05$ one-way ANOVA. **(C)** Rats received an intra-dorsal hippocampal infusion of rapamycin 1 day after a sSOR training (TR, $n = 6$) and tested the following day. Retrained animals (TR-tr) received intra-dorsal hippocampal infusions of vehicle (Veh, $n = 7$) or rapamycin (RAPA, $n = 7$) immediately after the wSOR retraining. They were tested one day later. Data are expressed as preference index mean ± SEM. $P > 0.05$ one-way ANOVA.

expression acquire a relevant role in the maintenance of the trace. Previous works have demonstrated that the provision of PRPs induced by a novel OF exposure induce promnesic effects by acting on the learning tag established in a wSOR session, which constitutes two key factors in the BT process (Ballarini et al., 2009). Then, rats were trained with a sSOR and, one day later, subjected to a wSOR with a previous local infusion of Veh or EME (Fig. 6A TR-tr). SOR-LTM was tested a week later. As previously seen, EME impaired the SOR-LTM persistence observed in animals infused with vehicle (One-way ANOVA $F_{(2,\ 19)} = 12.66$, $P < 0.001$, Newman–Keuls multiple comparisons test Veh vs EME $P < 0.001$); however, this amnesia was reversed when the rats were exposed to a novel OF 1 h post-retraining (EME + OF vs EME $P < 0.001$). In contrast, the amnesia induced by U0126 infusion could not be rescued by OF exploration (Fig. 6B, One-way ANOVA $F_{(2,\ 20)} = 5.85$, $P < 0.01$, Newman–Keuls multiple comparisons test Veh vs U0126 and U0126 + OF $P < 0.05$). These results suggest that the effect of inhibition of the activity of ERKs1/2 at the moment of retraining on the persistence of memory could not be reversed by the provision of PRPs; so, we propose that U0126 disrupts the learning tag induced by retraining. In that sense, previous reports have endorsed the participation of ERKs1/2 in the setting/maintenance of the SOR-learning tag (Tintorelli et al., 2020).

Finally, considering that the synthesis of the GluA2 AMPA receptor subunit is required for LTM expression (Pereyra et al., 2021), we wanted to test whether its presence at the synaptic membrane is enough to promote SOR-LTM persistence after spaced learning. Thus, animals were trained with a sSOR and, 7 days later, when the LTM of that single trial is not expressed, they received a wSOR training session (TR-tr). Before the wSOR retraining, we infused the dorsal hippocampus with GluR23ɣ, a peptide that selectively interferes with GluA2-containing AMPA receptor endocytosis. We observed that these rats expressed LTM 7 days after retraining (Fig. 7A, $t_{(10)} = 4.14$, $P < 0.01$ vs 0). GluR23ɣ had no effect in the group of rats subjected to a 2-min test session instead of retraining, and they did not express LTM 7 days later (TR-Test, $t_{(6)} = 0.0$, $P > 0.05$ vs 0). The retraining group treated with the inhibitor exhibited significantly higher LTM than the other groups (One-way ANOVA $F_{(5,\ 38)} = 6.59$, $P < 0.001$, Newman–Keuls multiple comparisons test TR-tr GluR23ɣ vs all groups, $P < 0.05$–0.001) (Fig. 7A). These results suggest that both retraining and the presence of AMPA receptors con-



taining the GluA2 subunit in the membrane are required for the memory trace to be durable after spaced SOR relearning. In accordance, spaced learning formed a persistent memory when the rats were exposed to a novel OF 1 h after retraining (Fig. 7B, One-way ANOVA $F_{(2,\ 23)} = 24.48$, $P < 0.001$, Newman–Keuls multiple comparisons test TR-tr + OF, $P < 0.001$ vs all groups). This finding suggests that the OF experience could provide PRPs to the tag established by the wSOR at the retraining, performed 7 days after the first training.

DISCUSSION

The aim of this study was to describe the mechanisms and conditions that improve the persistence of SOR memory through spaced learning. The main findings of this work suggest that if a SOR memory trace is liable to be expressed, a wSOR retraining session will promote its persistence. However, when the

Fig. 5. Inhibition of protein synthesis and ERKs1/2 activity impairs SOR-LTM expression and SOR-LTM persistence after retraining. The top diagrams show the experimental designs. **(A–C)** Inhibition of protein synthesis and ERKs1/2 activity impairs SOR memory expression. Rats were trained with a sSOR session and received an intra-dorsal hippocampal infusion of **(A)** vehicle (Veh, $n = 6$) or emetine (EME, $n = 7$), **(B)** Veh ($n = 5$) or rapamycin (RAPA, $n = 5$), or **(C)** Veh ($n = 11$) or U0126 ($n = 13$) 15 min before LTM test, performed 1 day after training. Data are expressed as preference index mean ± SEM. $+P < 0.05$, $++P < 0.01$, $+++P < 0.001$ Student's *t*-test. **(D–F)** Rats were subjected to a wSOR training session 1 day after a sSOR training (TR-tr), and received intra-dorsal hippocampal infusions either of **(D)** Veh ($n = 9$) or EME ($n = 9$), or **(E)** Veh ($n = 10$) or RAPA ($n = 13$), or **(F)** Veh ($n = 7$) or U0126 ($n = 6$) 15 min before the second training. Animals exposed to a single sSOR training session (TR, $n = 5$–9) received a Veh infusion 1 day after that. LTM was tested 8 days after the sSOR training. Data are expressed as preference index mean ± SEM. $+P < 0.05$, $+++P < 0.001$ vs all groups, Newman–Keuls after one-way ANOVA.

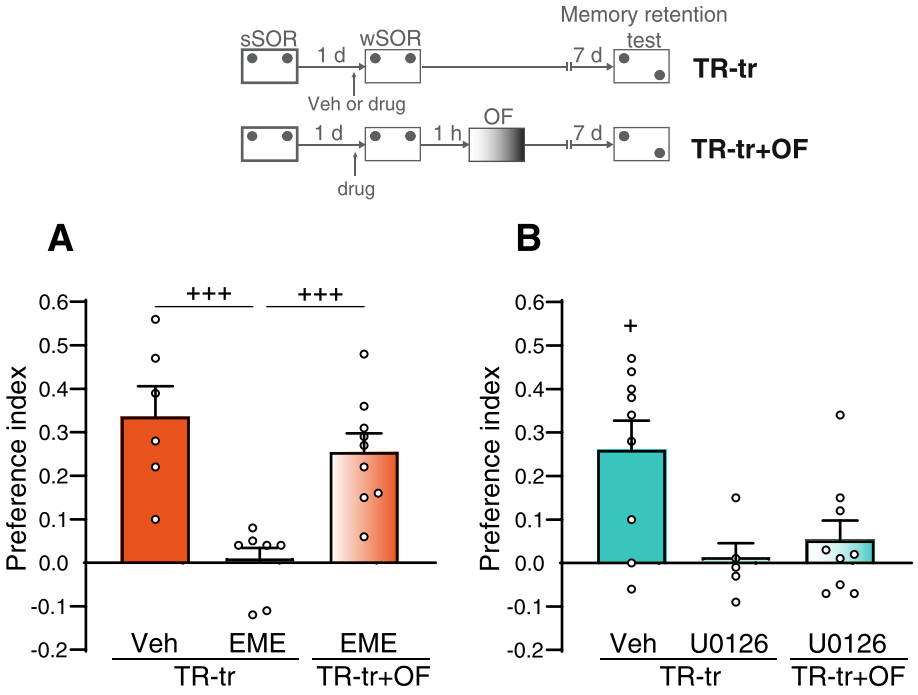


Fig. 6. Activation of ERKs1/2 is necessary for the setting of a wSOR-induced learning tag in the retraining session. The top diagram shows the experimental design. **(A)** Retraining groups (TR-tr) received intra-dorsal hippocampal infusions either of vehicle (TR-tr, Veh $n = 6$) or emetine (TR-tr EME, $n = 7$) 15 min before the second training; and one group (TR-tr EME + OF, $n = 9$) was also exposed to a novel OF session 1 h after the retraining session. SOR-LTM was tested 7 days after the second training session. Data are expressed as mean ± SEM. $+++P < 0.001$, Newman–Keuls after one-way ANOVA. **(B)** Other groups of rats were retrained as in A, but infused with vehicle (TR-tr Veh, $n = 9$) or U0126 (TR-tr U0126, $n = 5$), or U0126 plus an OF session (TR-tr U0126 + OF, $n = 9$). Data are expressed as preference index mean ± SEM. $+P < 0.05$, Newman–Keuls after one-way ANOVA.

wSOR is replaced by a test session, this is no longer effective in maintaining the memory trace. Also, our results suggest that neither memory strengthening nor labilization are required for this phenomenon. We propose that the re-activation of the specific neural sites induced by the first training session and the use of PRPs (probably those needed for memory expression and/or those induced by retrieval) are necessary, at the moment of the weak retraining, to maintain the SOR memory over time. This cellular mechanism, which involves site-specific tagging induced by learning and the use of PRPs, operates in the BT process. Our results suggest that the tagging process depends on the activity of ERKs1/2 and that GluA2-containing AMPA receptors are some of the proteins needed to promote LTM persistence.

We also observed that a test session performed 24 h after a sSOR training session is not effective in inducing SOR-LTM persistence (Fig. 2), although it can labilize this memory trace (Orlandi et al., 2020). However, a wSOR retraining session, which does not trigger a reconsolidation process (Fig. 4), is effective in inducing the persistence of the trace (Fig. 2). Thus, it is not essential to labilize the memory trace to promote its persistence, but the reactivation of neural substrates similar to those of the initial training would be relevant. However, the neural overlap induced by a test session is insufficient to cause this effect. There is evidence that representations of disrupted spatial memory re-emerge in the same form after relearning (Gridchyn et al., 2020). In contrast, although most observational studies have revealed that the overlap between populations of active neurons during training and testing exceeds chance levels, the general correspondence between these two populations is relatively low (roughly 10 to 40%, depending on the study) (Richards and Frankland, 2013; Rubin et al., 2015). Moreover, we observed that the exploration of an OF 1 day after the sSOR training was not able to induce the persistence of SOR-LTM (Fig. 3A). This could be due to a low overlap between the sites activated by the original training and those activated by OF, being insufficient to promote memory persistence. This is in contrast to what would happen with a retraining session, where there would be a high overlap with the originally activated sites, providing them with a renewed tagging process where the PRPs could be used to maintain the trace.

It has been proposed that retrieval involves two instances: one of execution (memory expression) and one of integration (reconsolidation) (Rodriguez-Ortiz and Bermúdez-Rattoni, 2017). Moreover, although a phase of integration is required to destabilize and modify the memory trace, memory reconsolidation can be achieved without expression (Mamou et al., 2006; García-DeLaTorre et al., 2009; Rodriguez-Ortiz et al., 2012; Balderas et al., 2013; Barreiro et al., 2013; Santoyo-Zedillo et al., 2014). Here, we showed that retraining-induced memory persistence is not based on reconsolidation mechanisms, but rather on those of memory expression. We observed that a wSOR retraining promotes SOR-LTM persistence if it is performed 1 day but not 7 days after the first sSOR session (when memory is no longer expressible). Although the behavioral expression of the memory at the time of the second trial cannot be recorded in the retraining protocol, the LTM shown by the animals tested 24 h after the sSOR confirms that the molecular mechanism of expression is available at that moment. Previous studies have reported that the mechanism of memory retrieval requires the activity of NMDA receptor, mTOR, and ongoing protein synthesis (Lopez et al., 2015). Infusion of RAPA or anisomycin prior to memory testing for multiple behavioral tasks such as inhibitory avoidance, contextual fear conditioning, SOR or Morris water maze has been shown to impair LTM expression (Rodriguez-Ortiz et al.,

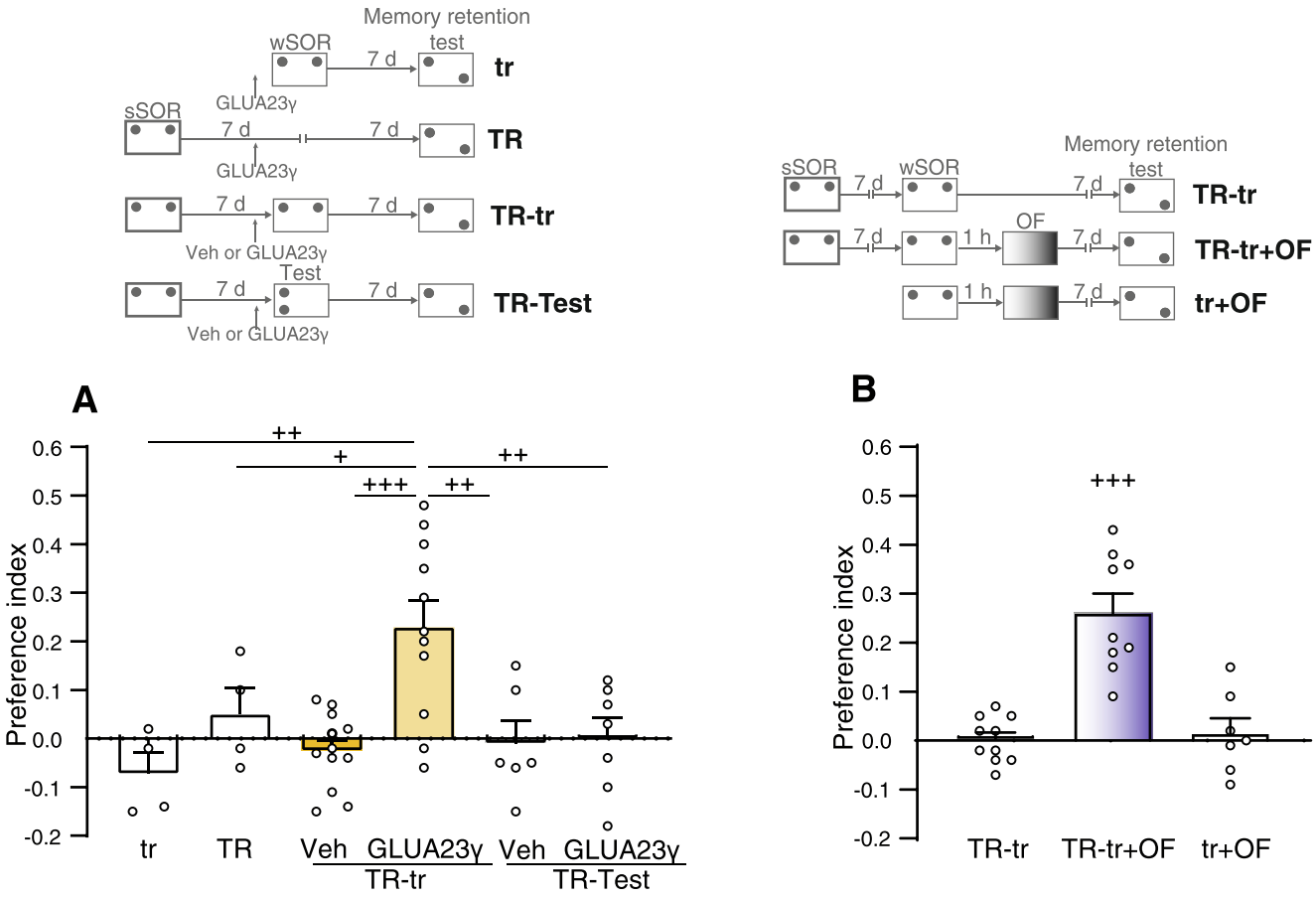


Fig. 7. GluA2-containing-AMPA receptor endocytosis blockade and exploration of a novel open field promote SOR-LTM persistence induced by retraining with an ITI of 7 days. The top diagrams show the experimental designs. **(A)** Control groups were injected with GluR23γ 1 h before a wSOR (tr, $n = 4$) or 7 days after a sSOR (TR, $n = 4$) training session. Rats were trained with a sSOR session and, 7 days later, independent animals received intra-dorsal hippocampal infusions either of Veh ($n = 12$) or GluR23γ ($n = 11$) 1 h before a wSOR retraining session (TR-tr); other groups were infused with Veh ($n = 6$) or GluR23γ ($n = 7$) 1 h before a 2-min test session (TR-Test). SOR-LTM was tested 14 days after the first training session. Data are expressed as mean ± SEM. $+P < 0.05$, $++P < 0.01$, $+++P < 0.001$, Newman–Keuls after one-way ANOVA. **(B)** Animals were retrained with a wSOR session 7 days after a sSOR training session (TR-tr, $n = 10$). Independent animals were also exposed to a novel OF session 1 h after retraining (TR-tr-OF, $n = 9$). The control group was exposed to an OF session 1 h after a wSOR training session (tr + OF, $n = 7$). SOR-LTM was tested 14 days after the sSOR training session. Data are expressed as mean ± SEM. $+++P < 0.001$ vs all groups, Newman–Keuls after one-way ANOVA.

2008; Lopez et al., 2015; Pereyra et al., 2018). Here, we confirmed that administration of RAPA or EME 15 min before testing inhibits SOR-LTM expression, adding that these treatments before the retraining session impair SOR-memory persistence (Fig. 5). Also, we observed the absence of persistence of the trace when retraining was performed 24 h after a wSOR training session, at the time when this memory is no longer expressed (Fig. 3). Together, these results strongly suggest that the mechanism of LTM expression is necessary to elicit the retraining-induced persistence of the memory trace.

The reconsolidation process has been proposed as a reinforcer of the memory trace tested 24 h after the reminder. Previous studies have observed this effect by using two trials separated by one day in a fear-conditioning task (Lee, 2008), as well as by a retrieval session followed by a conditioning in the same task (Tay et al., 2019). Our present results show that our retraining protocol does not strengthen memory formation or destabilize the trace (Fig. 4). This may be because the first training is strong and the second is weak, so the information in the original memory is not updated or reinforced (Rodriguez-Ortiz and Bermúdez-Rattoni, 2017). This result is similar to that found by Levitan et al. (2010) using the *Aplysia californica* model responding to inedible-food training. In that work, a strong training induced 24-h memory, but not 48-h memory, and a weak training did not induce LTM. These authors observed that a weak training presented 24 h after a strong training was able to cause the memory trace to persist for 48 h, and this effect was abolished by the administration of anisomycin before weak retraining. The authors concluded that anisomycin blocked the reconsolidation process. However, according to our findings, its role in the impairment of memory-expression mechanisms should be considered.

As explained earlier, not all reminders destabilize the memory trace. Merlo et al. (2018) suggested that different types of reminders, given one day after a fear training, are key to trigger reconsolidation, extinction or a limbo memory state (new phase of retrieval insensitive to recon-

solidation or extinction). These authors observed that while interventions that induced memory reconsolidation resulted in increased pERKs1/2 levels, other interventions that did not induce this process did not alter pERKs1/2 levels either. In previous studies, we demonstrated that the activity of ERKs1/2 is necessary for the setting of the learning tag in the sites activated by the SOR session (Tintorelli et al., 2020). These results are in accordance with the fact that ERKs1/2 are specifically required for the setting of synaptic-tags associated with long-term depression (Sajikumar et al., 2007), a cellular plasticity model associated with the acquisition of spatial memory for object location in rodents (Kemp and Manahan-Vaughan, 2004). Here, we found that infusion of the ERKs1/2 inhibitor U0126 into the dorsal hippocampus prior to the retraining session impairs the induction of memory persistence (Fig. 5F) and also blocks SOR-LTM expression when administered prior to a test session (Fig. 5C). However, the amnesia induced by U0126 could not be reversed by a novel OF exposure (PRPs donor) after retraining (Fig. 6B). Therefore, without ruling out a possible role of ERKs1/2 in PRPs synthesis, this result suggests that ERKs1/2 activity is relevant for tagging sites during retraining. In contrast, local injection of protein synthesis inhibitors before retraining impaired the persistence of LTM, but this effect was reversed by exposure to an OF after retraining. This result is consistent with the findings showing that protein synthesis inhibitors do not disrupt the learning tag (Moncada and Viola, 2007; Ballarini et al., 2009).

Beyond the process of retagging the learning sites, the proteins involved in the expression of memory would be required for the persistence of the trace. Some of those proteins could be the GluA1 and GluA2 subunits of the AMPA receptor (Lopez et al., 2015). Lopez et al. (2015) described that memory retrieval requires ongoing protein synthesis and NMDA receptor activity-mediated AMPA receptor trafficking. These authors showed that RAPA administration prior to a cue fear conditioning test impaired the traffic of GluA1 to the postsynaptic density and memory expression. Activation of mTORC1 is known to increase GluA1 levels in memory formation (Slipczuk et al., 2009). In this sense, Pedroso et al. (2013) observed that retrieval of inhibitory avoidance memory can lead to memory strengthening, an effect dependent on mTORC1 activity after reactivation. This allowed us to think that mTORC1 signaling could still be active after retrieval, and could be involved in reactivation-induced protein synthesis. However, we cannot rule out that mTORC1 activity is required for the tagging mechanism. Evidence for this is found in a study by Sosanya et al. (2015), who showed that the NMDA receptor activates mTORC1 and that HuD (an mRNA stabilizer protein) targets CAMKIIα mRNA and mediates its branch-specific expression.

In the present study, we did not observe SOR memory persistence when the animals were retrained with a wSOR one week after a sSOR session. However, we found that infusion of the GluA2-endocytosis inhibitor prior to wSOR retraining promoted the persistence of SOR memory (Fig. 7A). This effect was not observed when the animals received a test session instead of a retraining session. These results suggest that part of the machinery of memory retrieval of the original sSOR is still active one week after training. The results also suggest that it is important to reactivate the originally stimulated sites and that the availability of GluA2-containing AMPA receptors is sufficient to promote the persistence of SOR-LTM. Our study supports that the mechanism is no longer available one week after sSOR training, but, if we artificially block the GluA2 endocytosis, then the persistence of the trace is promoted. We also showed that an exploration of a novel OF 1 h after retraining also promotes the persistence of the trace, probably through the provision of PRPs (Fig. 7B). Finally, our results are also consistent with the phenomenon of metaplasticity, a term used to describe the way in which synaptic plasticity can be regulated by prior synaptic activity (Abraham and Bear, 1996; Schmidt et al., 2013). Thus, the effect of the history of the animal on synaptic plasticity could impact on subsequent learning and memory abilities (Parsons, 2018). In this framework, the amount of AMPA receptor subunits and other proteins in the synapse at the moment of retraining could be considered a metaplastic change derived from the first training. It is also important to highlight that *in vitro* studies have reported that tag duration can be extended based on metaplasticity induced by previous ryanodine or mGluR activation receptors (Sajikumar et al., 2009; Li et al., 2014). Thus, since the establishment of the tag can be altered by metaplasticity processes, we cannot rule out that the first training could modify the characteristics of the tag induced by retraining.

In this work, we studied the mechanism underlying the persistence of SOR memory after spaced retraining. We propose that memory fate is affected by the nature of events that occur many hours after the original training. We suggest that several processes operate in this effect, and predicted that: (a) the event will promote the persistence of the original trace depending on the proportion of original sites that can be reactivated; and thus, in a retraining session, the retagging would be greater than in a test session or in a novel OF exposure, where the retagging would be partial, and therefore retraining is more efficient to promote persistence; (b) the event will promote the memory persistence while the original trace is still expressible; (c) it is not necessary for the event to reinforce the memory formation or labilize the original trace; and d) if the retagging process or the availability of PRPs are impaired on retraining, the memory will not persist.

The results of this study suggest that the most effective protocol for memory persistence involves a retraining session at a time when the original trace is still retrievable and expressible. Our results demonstrate the requirement for ERKs1/2 activity and availability of GluA2-containing AMPA receptors in the retraining session, suggesting their roles in the tagging process at originally activated sites, where diverse PRPs can be used to maintain plasticity. We proposed that BT is a cellular mechanism that explains our findings, and that the setting of a learning tag and protein synthesis are two processes required to ensure the persistence of SOR memory.

ACKNOWLEDGEMENTS

We thank Dr. Jorge H. Medina for his helpful comments and discussion of the manuscript. This work was supported by funding from grants from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, PICT 2016-0172) and the University of Buenos Aires (UBACyT 20020170100501BA), Argentina, to Haydée Viola. Author contributions: H.V. was responsible for conceiving and designing the study. J.C., R.T and P.B. carried out behavioral experiments. J.C. and H.V. analyzed the data and interpreted the findings. H.V and J.C. drafted the manuscript. R.T and P.B. revised the manuscript. All authors have approved the final version for submission.

COMPETING INTERESTS

The authors declare no competing interests.

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