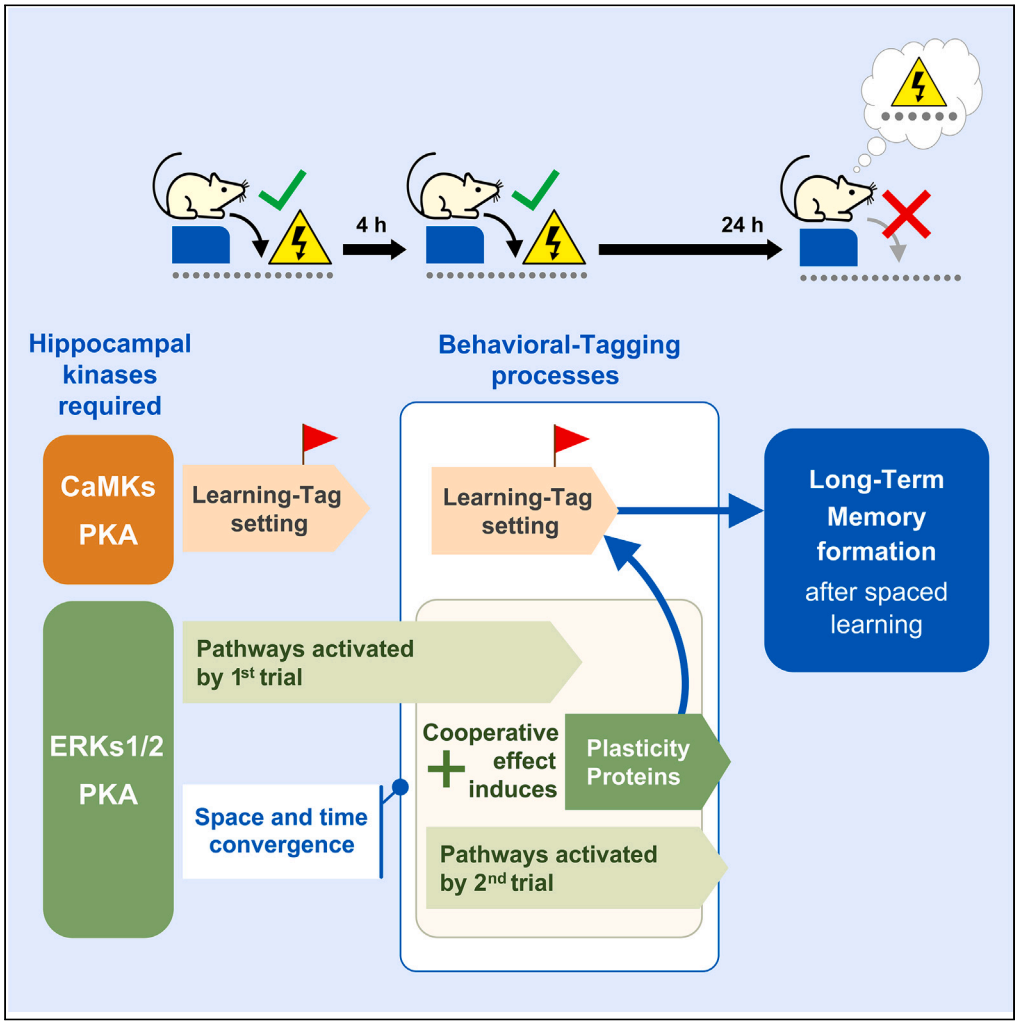
iScience

CellPress
OPEN ACCESS

Article

A behavioral tagging account of kinase contribution to memory formation after spaced aversive training



Pablo Budriesi, Ramiro Tintorelli, Julieta Correa, Maria Eugenia Villar, Paul Marchal, Martin Giurfa, Haydee Viola

hviola@fmed.uba.ar

Highlights

Two spaced, weak inhibitory-avoidance sessions induce long-term memory in rodents

CaMKs and PKA signaling are required for tag setting in memory formation

ERKs1/2 and PKA signaling are required for protein synthesis in memory formation

Behavioral tagging accounts for memory formation during rodent spaced learning

Budriesi et al., iScience 26, 107278
August 18, 2023 © 2023
https://doi.org/10.1016/j.isci.2023.107278

iScience

CellPress
OPEN ACCESS

Article

A behavioral tagging account of kinase contribution to memory formation after spaced aversive training

Pablo Budriesi,[1] Ramiro Tintorelli,[1] Julieta Correa,[1] Maria Eugenia Villar,[1,2] Paul Marchal,[1,3] Martin Giurfa,[4,5] and Haydee Viola[1,6,7,8,*]

SUMMARY

Long-term memory (LTM) can be induced by repeated spaced training trials. Using the weak inhibitory avoidance (wIA) task, we showed that one wIA session does not lead to a 24-h LTM, whereas two identical wIA sessions spaced by 15 min to 6 h induce a 24-h LTM. This LTM promotion depends both on hippocampal protein synthesis and the activity of several kinases. In agreement with the behavioral tagging (BT) hypothesis, our results suggest that the two training sessions induce transient learning tags and lead, via a cooperative effect, to the synthesis of plasticity-related proteins (PRPs) that become available and captured by the tag from the second session. Although ERKs1/2 are needed for PRPs synthesis and CaMKs are required for tag setting, PKA participates in both processes. We conclude that the BT mechanism accounts for the molecular constraints underlying the classic effect of spaced learning on LTM formation.

INTRODUCTION

Strong, unique experiences can generate lasting memories that may persist for a lifetime. On the contrary, weak experiences need to be repeated to induce durable memories. The time lapse between experiences is known to be crucial for the formation of a long-term memory (LTM). More than a century of memory research has led to the conclusion that spaced training sessions are more efficient than massed training sessions to generate LTM.[1] Several theories have been proposed to explain this phenomenon based on the mechanisms triggered by spaced training and their effects on memory formation (see Smolen et al.[2]).

The storage of an LTM goes through a period of consolidation dependent on protein synthesis triggered by learning.[3] Synaptic plasticity models indicate that the storage of information is a site-specific process.[4] Among these models, the synaptic-tagging and capture hypothesis (STC) postulates that plasticity-related proteins (PRPs) are captured and used specifically at neural sites tagged by stimulation to induce long-lasting plasticity phenomena.[5] For instance, a weak tetanic stimulation may tag activated neural sites without inducing protein synthesis whereas a further strong stimulation may result in the synthesis of PRPs that can be captured/used by the tag induced by the weak stimulation. This association is possible if both phenomena, tagging and PRP synthesis, occur within the same temporal window and share a common neural substrate. Through this process, a weak stimulation may achieve a stabilized long-term plasticity.

In analogy to synaptic plasticity models, the behavioral tagging (BT) model proposed that learning session induces tags in specific activated sites where PRPs can be captured to consolidate memory into LTM.[6,7] PRPs can be induced by the learning itself, if it is sufficiently relevant, or by another event temporarily associated. A key feature of this process is that the learning tags are transient and have a short half-life. Therefore, to promote LTM, PRPs must be available at tagged sites while tags are still functional.[8,9]

The BT mechanism has been demonstrated for a broad spectrum of memory types, animal species, and ages of individuals.[8,10–19] PRPs can be provided by either novel events,[6,17,20–24] reactivation or extinction sessions,[25,26] rewarding events,[27] exposure to acute stress,[28,29] memory expression of enough similar experiences[30] or retraining sessions.[31] To what extent BT provides a valid framework to understand LTM formation on repeated aversive spaced training trials remains unexplored.

[1]Instituto de Biología Celular y Neurociencia "Prof. E. De Robertis" (IBCN), Facultad de Medicina, UBA-CONICET, Ciudad Autónoma de Buenos Aires, Argentina

[2]Departamento de Biología y Geología, Física y Química Inorgánica, Área de Biodiversidad y Conservación, Universidad Rey Juan Carlos, Madrid, Spain

[3]Poe Lab, Integrative Biology and Physiology department, University of California Los Angeles, Los Angeles, CA, USA

[4]Centre de Recherches sur la Cognition Animale, Centre de Biologie Intégrative (CBI), University of Toulouse, CNRS, UPS, 31062 Toulouse cedex 9, France

[5]Institut Universitaire de France (IUF), Paris, France

[6]Departamento de Fisiología, Biología Molecular y Celular "Dr. Héctor Maldonado" (FBMC), Facultad de Ciencias Exactas y Naturales, UBA, Ciudad Autónoma de Buenos Aires, Argentina

[7]Instituto Tecnológico de Buenos Aires, Ciudad Autónoma de Buenos Aires, Argentina

[8]Lead contact

*Correspondence: hviola@fmed.uba.ar

https://doi.org/10.1016/j.isci.2023.107278

Here, we used the inhibitory avoidance (IA) aversive task to study the classic "lag effect" defined as the tendency for longer intervals between learning sessions to produce better memory than shorter intervals (see Carpenter[32]). In this task, which is commonly used to study learning and memory processes in rodents,[33] animals typically receive a single aversive foot shock after stepping down from a little platform to the floor. Retention is tested later by measuring the rats' latency to step down to the floor when placed again on the platform. Our previous work showed that a weak IA training session results in IA-LTM if coupled with a contiguous open field (OF) session via the molecular mechanisms underlying the BT process. In this scenario, the weak IA training sets a learning tag, which is transient and lasts less than 2 h, and the OF exposure provides the PRPs captured by the tag to stabilize the memory.[6] It is, therefore, important to ask if the same mechanisms underlie LTM formation on retraining. Given that a single weak IA session does not establish LTM, we hypothesized that cooperation between two successive weak sessions via a BT mechanism is required to establish IA-LTM, which would explain the advantage of spaced training to induce LTM. In particular, we suggest that the retraining session would mainly retag the sites initially labeled by the prior training session. PRPs required for memory consolidation would be synthesized as a result of the sum or synergy of two consecutive weak training sessions when they are spaced by an appropriate inter-trial interval (ITI) and rendered available for capture at the tag from the second session. Using a pharmacological approach, we investigated the role of extracellular regulated kinases 1/2 (ERKs1/2), protein kinase A (PKA) and calcium/calmodulin-dependent protein family kinases (CaMKs) on tag setting and/or PRPs synthesis as these molecules are required for IA memory formation.[33] Blockade of these kinases during specific times of the training sessions allowed us to determine their participation and the necessity for the processes of protein synthesis and/or tag-setting.

We show that two weak IA training sessions spaced by a temporal window varying between 15 min and 6 h promote IA-LTM formation and that this phenomenon requires protein synthesis and the activation of ERKs1/2, CaMKs and PKA in the dorsal hippocampus. Our data indicate that CaMKs contribute to tag-setting, ERKs1/2 to PRPs synthesis and PKA to both processes. Finally, we show that the combination of two weak sessions of different hippocampal-dependent memory tasks does not promote LTM for either task, which suggests that overlap of the neuronal populations activated by each training session is required to promote the interaction between cellular resources at specific learning sites. Overall, our results suggest that the BT mechanism provides a valid account of IA-LTM formation via retraining and that the processes of tag setting and PRP synthesis may explain the molecular constraints of the classic effect of spaced learning on memory formation.

RESULTS

Two consecutive weak IA training sessions induce LTM when spaced by an interval between 15 min and 6 h

In the IA task used in our work, rats placed on an elevated platform learned to inhibit stepping down onto the floor of the conditioning apparatus as they received a foot shock on descent. In the one-trial protocol (Ctrl), a weak shock (0.3 mA, 2 s) does not induce LTM 24 h after training as the step-down latency in the test session is short and similar to that in the training session (Figure 1, $p > 0.05$ versus Training) (see also Moncada and Viola[6]).

To study the temporal limits of LTM formation induced by weak spaced training in this task, we performed two weak IA training sessions separated by different ITIs varying from 5 min to 24 h in eight independent groups of animals (5 min, 15 min, 1 h, 2 h, 4 h, 6 h, 9 h, and 24 h; Figure 1). A control group was trained with a single weak IA training session. Control and experimental groups were tested for LTM 24 h after training. Step-down latency results in seconds by group were presented as median [interquartile range] in Figure 1 (Training, 7.95 [6.45/22.15]; Ctrl, 17.00 [9.25/39.22]; 5min, 21.55 [14.55/42.52]; 15min, 118.40 [40.61/167.70]; 1h, 170.70 [23.34/300]; 2h, 166.40 [27.05/300]; 4h, 184.50 [59.45/300]; 6h, 91.47 [47.60/144.60]; 9h, 48.15 [18.46/89.66]; 24h, 13.18 [10.36/25.23]). IA-LTM was present in groups that experienced a second weak IA training session spaced from the first session by an ITI ranging from 15 min to 6 h (Figure 1, $p < 0.0001$ versus Training). These animals increased their latency to step down from the platform compared to the control group (Figure 1, $p < 0.05$–0.001 versus Ctrl). On the contrary, training sessions spaced by 5 min or by ITIs longer than 6 h (9 and 24 h) did not induce IA-LTM (Figure 1, $p > 0.05$ versus Ctrl and Training). Taken together, our results confirm, on the one hand, that a single weak training session is unable to promote 24-h LTM show, on the other hand, that two spaced weak training sessions can promote LTM if the ITI separating them is between 15 min and 6 h.

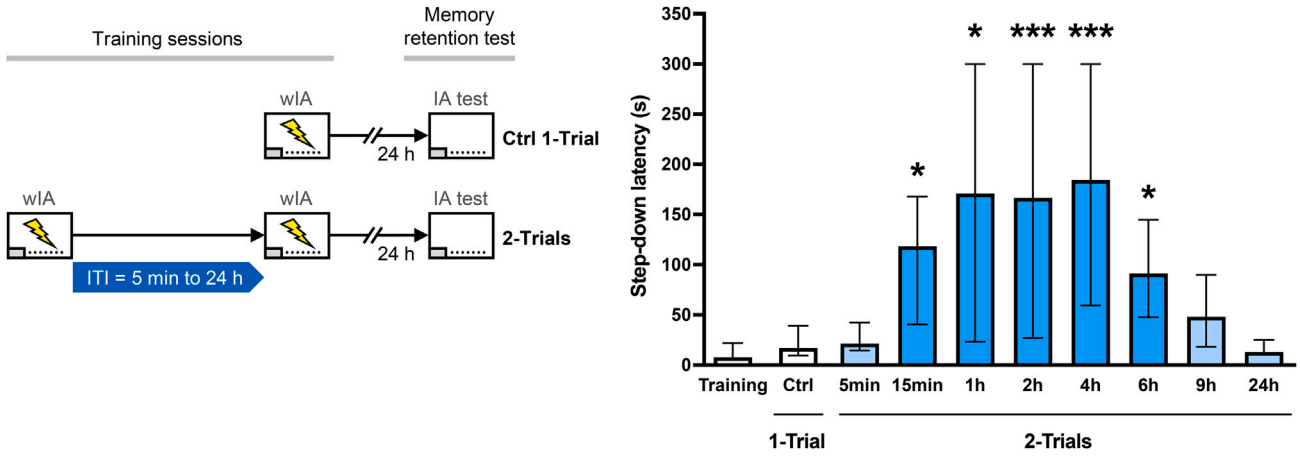


Figure 1. Two consecutive weak IA training sessions induce LTM when spaced by an interval between 15 min and 6 h

(Left) Diagram illustrating the experimental protocol. A total of 145 rats were randomly assigned to the control group or to an experimental group. The control group (Ctrl 1-Trial, n = 17) received a weak foot-shock in a single weak IA training. The groups trained with two weak IA sessions differed in the ITI, which varied from 5 min to 24 h (5 min, n = 17; 15 min, n = 18; 1 h, n = 18; 2 h, n = 19; 4 h, n = 17; 6 h, n = 17; 9 h, n = 14; 24 h, n = 8). The step-down latency was registered during a test session 24 h after the training. (Right) Latency results in seconds are expressed as median ± IQR. 'Training' indicates the latency recorded during a representative first training session (n = 18). Comparisons were done by means of a Kruskal-Wallis test with $p < 0.0001$ and Dunn's multiple comparisons between groups (***$p < 0.001$ and *$p < 0.05$ versus Ctrl 1-Trial; groups 15min to 6h are $p < 0.0001$ versus Training).

Combining a weak IA session with a weak SOR does not result in IA-LTM formation

To evaluate if LTM promotion by two consecutive training sessions is task specific, we used two weak, different hippocampal-dependent tasks spaced by an ITI of 4 h. In addition to train rats with a weak IA session, we also trained them with a weak Spatial Object Recognition (SOR) session. In this session, animals explored during 4 min two identical objects placed in adjacent corners of an arena. This exposure time does not lead to a 24-h LTM (SOR-LTM), because animals replaced in the arena in which one of the objects was displaced to a novel location do not allocate more time exploring the displaced object,[10,31] as they typically do when SOR-LTM is present.[30]

One group of rats received a weak IA training session followed by a weak SOR training session (IA + SOR) whereas another group experienced the reversed sequence of tasks (SOR+IA). Two further control groups were included, one trained with a single weak IA session (Ctrl) and another trained with two weak IA sessions also spaced by 4 h (IA + IA). All four groups were tested for IA-LTM 24 h after the end of training. Step-down latency results by group in seconds were presented as median [interquartile range] in Figure 2 (Training, 11.43 [8.14/16.56]; Ctrl, 23.80 [11.14/32.64]; IA + IA, 115.10 [65.65/174.10]; IA + SOR, 20.70 [13.06/36.25]; SOR+IA, 28.62 [11.71/52.55]).

Figure 2 shows that only the group trained with two consecutive weak IA sessions exhibited a significant 24-h IA-LTM reflected in their longer latency to step down from the platform (Figure 2, $p < 0.0001$ versus Training and $p < 0.05$ versus all other groups). IA-LTM was neither observed in the control group trained with a single weak IA session, nor in the groups having experienced combined weak IA and weak SOR sessions (Figure 2, $p > 0.05$ versus Training).

These results indicate that the process of LTM formation is task specific. Only two spaced sessions of the same task led to a 24-h IA-LTM, which suggests that common substrates and neuronal populations need to be activated by each training session to promote the interaction of cellular resources at the specific learning sites that lead to LTM.

Protein-synthesis inhibition in the dorsal hippocampus impairs IA-LTM formation after spaced training - A novel open field (OF) task prevents this effect

After showing that rats trained with two weak IA sessions spaced by 4 h form an IA-LTM observable 24 h after training, we assessed if this memory requires the synthesis of proteins. We thus performed hippocampal infusions of the protein-synthesis inhibitor emetine (EME) either before the first (Figure 3A) or the

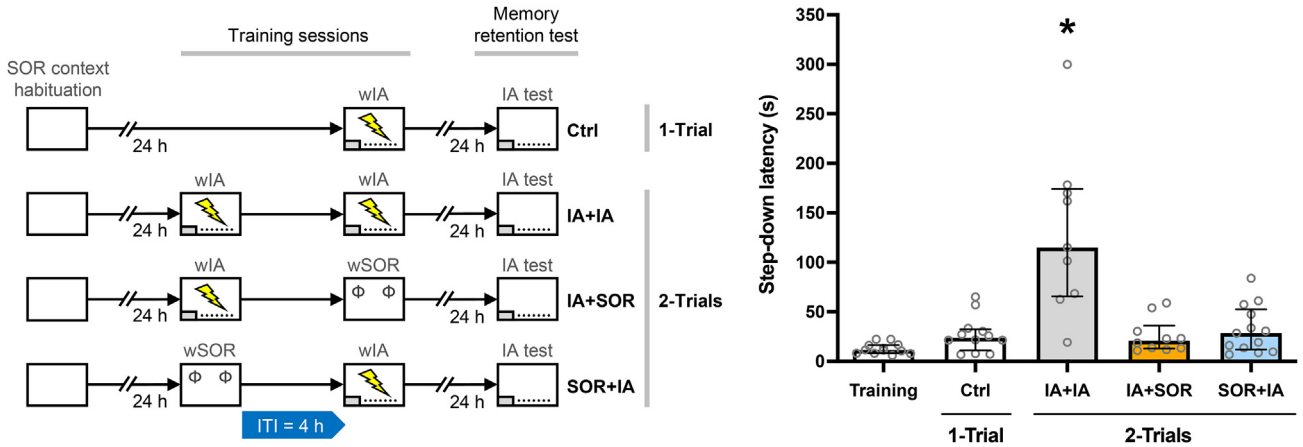


Figure 2. Combining wIA and wSOR does not result in IA-LTM formation

(Left) Diagram illustrating the experimental protocol. A total of 44 rats were randomly assigned to the control group or to an experimental group. Control group rats (Ctrl 1-Trial, n = 12) were trained with a single weak IA task, whereas the IA + IA group (n = 9) received 2 identical weak IA sessions (wIA) separated by 4 h. Other two groups were submitted to different tasks, a weak IA session 4 h before a weak SOR session (IA + SOR, n = 10) or a weak SOR session followed 4 h later by a weak IA session (SOR+IA, n = 13). The step-down latency was recorded during a test session 24 h after training. (Right) Latency results are represented as median ± IQR. 'Training' indicates the latency recorded during a representative first training session (n = 12). Comparisons were done by means of a Kruskal-Wallis test with $p < 0.001$ and Dunn's multiple comparisons between groups (*$p < 0.05$ versus Ctrl 1-Trial, IA + SOR and SOR+IA; $p < 0.0001$ IA + IA versus Training).

second weak IA training session (Figure 3B) to determine in a test performed 24 h after training if this inhibition affected IA-LTM formation. Control animals were injected with vehicle in parallel to experimental groups. An additional control group was trained with a single weak IA session and infused with vehicle before the session. Step-down latency results by group in seconds were presented as median [interquartile range] in Figure 3A (Ctrl, 21.01 [14.42/34.51]; Veh, 220.70 [144.30/293.70]; EME, 37.87 [26.57/47.42]; EME+OF, 225.00 [93.33/300]), and Figure 3B (Ctrl, 26.38 [19.87/35.84]; Veh, 205.60 [158.80/280.80]; EME, 55.99 [35.65/67.25]; EME+OF, 219.00 [156.00/300]). As expected, animals in the control group did not form IA-LTM 24 h after training (Figures 3A and 3B, Ctrl 1-Trial).

Rats trained with two weak IA sessions and infused with vehicle before the first session, expressed IA-LTM (Figure 3A, $p < 0.0001$ Veh versus Ctrl); on the contrary, animals infused with EME did not exhibit LTM (Figure 3A, $p > 0.05$ EME versus Ctrl), thus showing a dependency of IA-LTM on protein synthesis. Similar results were observed when rats received the infusions before the second weak IA session. In this case, animals infused with vehicle showed again a significant IA-LTM 24 h after training (Figure 3B, $p < 0.001$ Veh versus Ctrl) whereas animals infused with EME did not exhibit IA-LTM (Figure 3B, $p > 0.05$ EME versus Ctrl). These results indicate that the formation of IA-LTM induced by retraining requires protein synthesis in the dorsal hippocampus.

In a previous study, we showed that a weak IA training session results in IA-LTM if coupled to a contiguous OF session via the molecular mechanisms underlying the BT process, namely the setting of a learning tag by the weak IA training and the provision of PRPs by the OF exposure.[6] We thus reasoned that the amnesic effect of EME in IA retraining could be counteracted by OF exposure 1h before the second weak IA session. During this OF session, animals having experienced the first weak IA training session were allowed to explore the novel spatial context of a square arena during 5 min. Figures 3A and 3B show that in animals infused with EME either before the first or the second weak IA session, OF exploration restored IA-LTM (Figures 3A and 3B, $p < 0.05$ EME+OF versus EME). These results suggest that EME did not affect the setting of the weak IA learning tag and that the OF experience supplied the necessary resources to consolidate IA-LTM.

Inhibition of ERKs1/2 activation in the dorsal hippocampus impairs IA-LTM formation after spaced training - A novel open field (OF) task prevents this effect

Our previous work showed that a weak IA training session induces a transient learning-tag that lasts less than 2 h and that is independent of ERKs1/2 activation in the dorsal hippocampus.[6,34] We thus aimed at

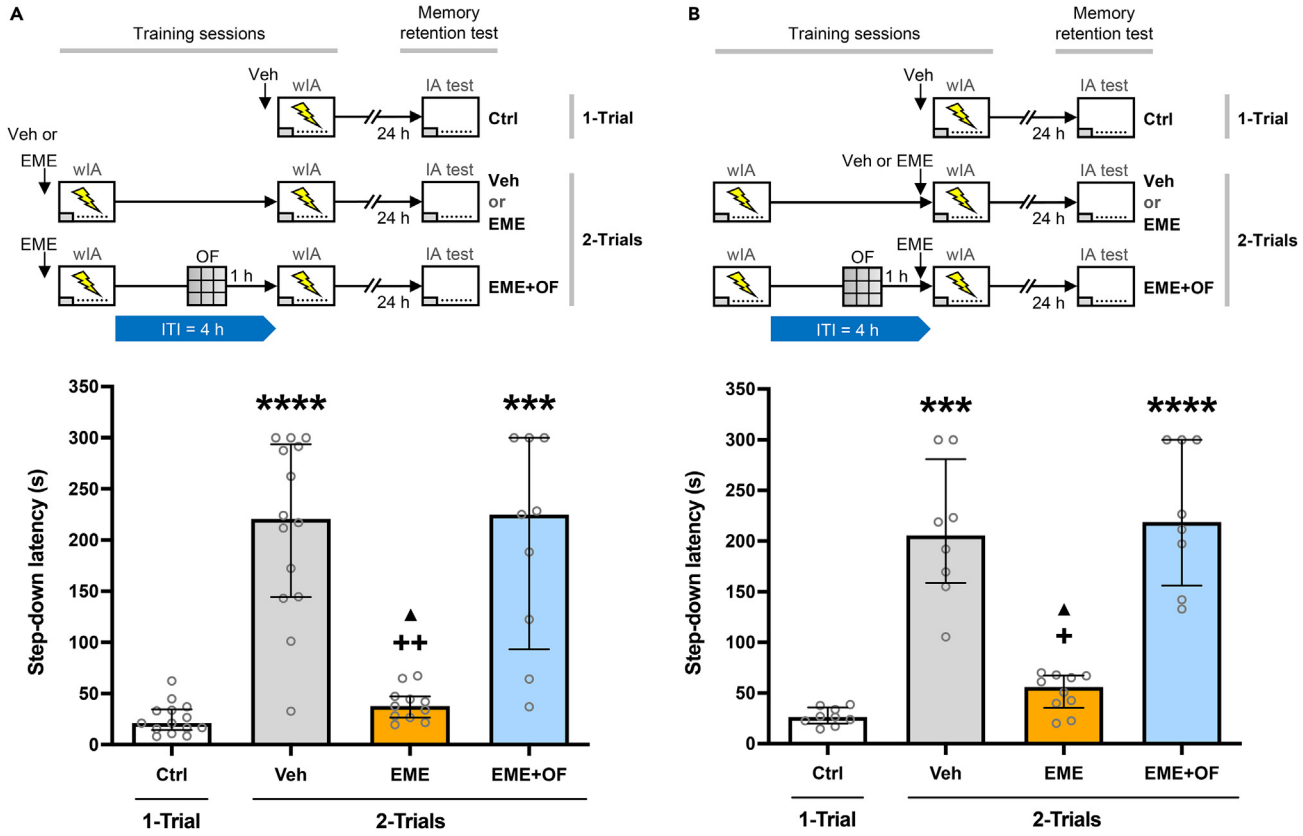


Figure 3. Protein-synthesis inhibition in the dorsal hippocampus impairs IA-LTM formation after spaced training - A novel Open Field (OF) task prevents this effect

(Top) Diagrams illustrating the experimental protocols. A total of 48 rats in (A) and 35 rats in (B) were randomly assigned to the control group or to an experimental group.

(A) Control-group rats (Ctrl 1-Trial, n = 14) received an intra-dorsal hippocampal infusion of vehicle (Veh) 15 min before a single weak IA training session. Experimental animals received intra-dorsal hippocampal infusions of vehicle (Veh 2-Trials, n = 14) or emetine (EME 2-Trials, n = 11) 15 min before the first weak IA session. Another group injected with emetine was also exposed to a novel OF session 1h before the second weak IA session, which was performed 4 h after the first one (EME+OF 2-Trials, n = 9). All groups were tested for IA-LTM 24 h later. Latency is represented as median ± IQR. Comparisons were done by means of a Kruskal-Wallis test with $p < 0.0001$ and Dunn's multiple comparisons between groups (****$p < 0.0001$ and ***$p < 0.001$ versus Ctrl 1-Trial; ++$p < 0.01$ versus Veh 2-Trials; ▲$p < 0.05$ versus EME+OF 2-Trials).

(B) Control-group rats (Ctrl 1-Trial, n = 9) received an intra-dorsal hippocampal infusion of vehicle (Veh) 15 min before a single weak IA training session. Experimental animals trained with a second weak IA session were injected with vehicle (Veh 2-Trials, n = 8) or emetine (EME 2-Trials, n = 10) 15 min before the second session, which was performed 4 h after the first one. Another group injected with emetine was also exposed to a novel OF session 1h before the second weak IA session (EME+OF 2-Trials, n = 8). All groups were tested for IA-LTM 24 h later. Latency is represented as median ± IQR. Comparisons were done by means of a Kruskal-Wallis test with $p < 0.0001$ and Dunn's multiple comparisons between groups (****$p < 0.0001$ and ***$p < 0.001$ versus Ctrl 1-Trial; +$p < 0.05$ versus Veh 2-Trials; ▲$p < 0.05$ versus EME+OF 2-Trials).

studying if ERKs1/2 activation is required during the formation of IA-LTM following two weak IA training sessions. We used an experimental design similar to that of the previous section, infusing animals either with vehicle or with the specific MEK inhibitor U0126 before the first (Figure 4A) or the second weak IA training session (Figure 4B). An additional control group not forming IA-LTM was trained with a single weak IA session and infused with vehicle before this session. Step-down latency results by group in seconds were presented as median [interquartile range] in Figure 4A (Ctrl, 14.94 [10.89/38.00]; Veh, 145.50 [60.56/240.10]; U0126, 29.45 [14.04/68.01]; U0126+OF, 190.10 [152.50/300]), and Figure 4B (Ctrl, 14.00 [5.90/21.40]; Veh, 92.75 [58.65/263.40]; U0126, 29.74 [18.94/33.39]; U0126+OF, 181.60 [80.41/283.00]).

Figure 4A shows that retrained experimental rats infused with vehicle before the first weak IA session exhibited significant IA-LTM ($p < 0.001$ Veh versus Ctrl) whereas rats infused with U0126 had no LTM ($p > 0.05$ U0126 versus Ctrl). A similar result was observed when rats were infused before the second weak IA session (Figure 4B): vehicle-infused animals exhibited significant IA-LTM ($p < 0.001$ Veh versus

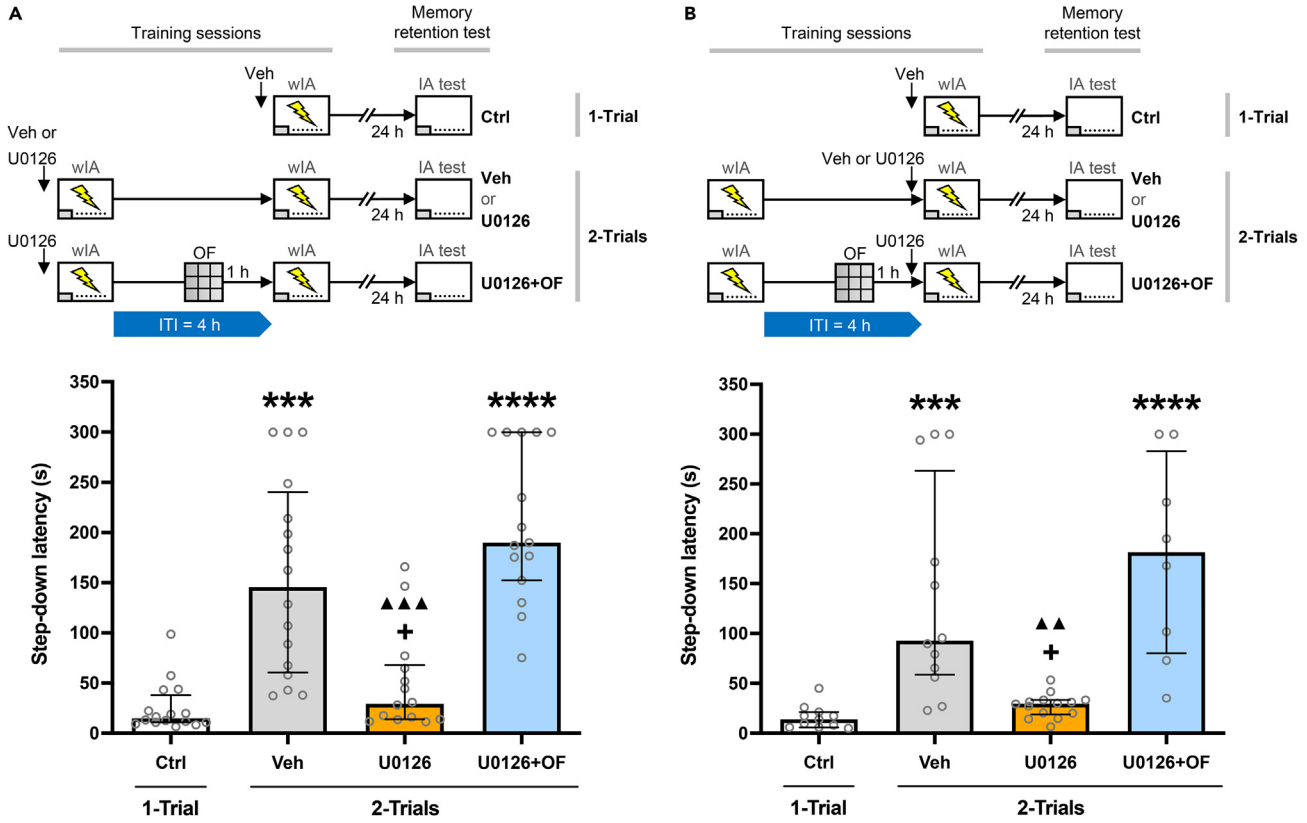


Figure 4. Inhibition of ERKs1/2 activation in the dorsal hippocampus impairs IA-LTM formation induced by retraining - A novel OF exposure prevents this effect

(Top) Diagrams illustrating the experimental protocols. A total of 61 rats in (A) and 45 rats in (B) were randomly assigned to the control group or to an experimental group.

(A) Control-group rats (Ctrl 1-Trial, n = 16) received an intra-dorsal hippocampal infusion of vehicle (Veh) 15 min before a single weak IA training session. Experimental, retrained animals received intra-dorsal hippocampal infusions of either vehicle (Veh 2-Trials, n = 16) or U0126 (U0126 2-Trials, n = 14) 15 min before the first weak IA session. Another group was injected with the MEK inhibitor U0126 and was exposed to a novel OF session 1h before the second weak IA session (U0126+OF 2-Trials, n = 15). All groups were tested for IA-LTM 24 h later. Latency is represented as median ± IQR. Comparisons were done by means of a Kruskal-Wallis test with $p < 0.0001$ and Dunn's multiple comparisons between groups (****$p < 0.0001$ and ***$p < 0.001$ versus Ctrl 1-Trial; +$p < 0.05$ versus Veh 2-Trials; ▲▲▲$p < 0.001$ versus U0126+OF 2-Trials).

(B) Similar to (A), control group rats (Ctrl 1-Trial, n = 11) received an intra-dorsal hippocampal infusion of vehicle (Veh) 15 min before a single weak IA training session. Experimental, retrained animals subjected to a second weak IA session were injected with either vehicle (Veh 2-Trials, n = 12) or U0126 (U0126 2-Trials, n = 14) 15 min before the second weak IA session, performed 4 h after the first one. Another retrained group injected with U0126 was also exposed to a novel OF session 1h before the second weak IA session (U0126+OF 2-Trials, n = 8). All groups were tested for IA-LTM 24 h later. Latency is represented as median ± IQR. Comparisons were done by means of a Kruskal-Wallis test with $p < 0.0001$ and Dunn's multiple comparisons between groups (****$p < 0.0001$ and ***$p < 0.001$ versus Ctrl 1-Trial; +$p < 0.05$ versus Veh 2-Trials; ▲▲$p < 0.01$ versus U0126+OF 2-Trials).

Ctrl) whereas U0126-infused animals did not exhibit IA-LTM ($p > 0.05$ U0126 versus Ctrl). Overall, these results indicate that the formation of IA-LTM induced by retraining requires the activation of ERKs1/2 in the dorsal hippocampus. However, they do not allow to determine if ERKs 1/2 had a selective role for tag setting and/or PRP synthesis.

To determine if ERKs1/2 participated in one of these processes, we included an additional group experiencing two weak IA training sessions spaced by an ITI of 4 h and an interspersed OF session occurring 1 h before the second weak IA session. Infusion of U0126 was performed either before the first (Figure 4A) or the second weak IA session (Figure 4B). If the infusion of U0126 impaired the synthesis of PRPs, OF exposure before the second weak IA session would contribute the PRPs restoring IA-LTM.

Our results show that irrespective of the timing of U0126 infusion, the OF session restored the IA-LTM that was suppressed by the MEK inhibitor (Figures 4A and 4B, $p < 0.01$–0.001 U0126+OF versus U0126). These

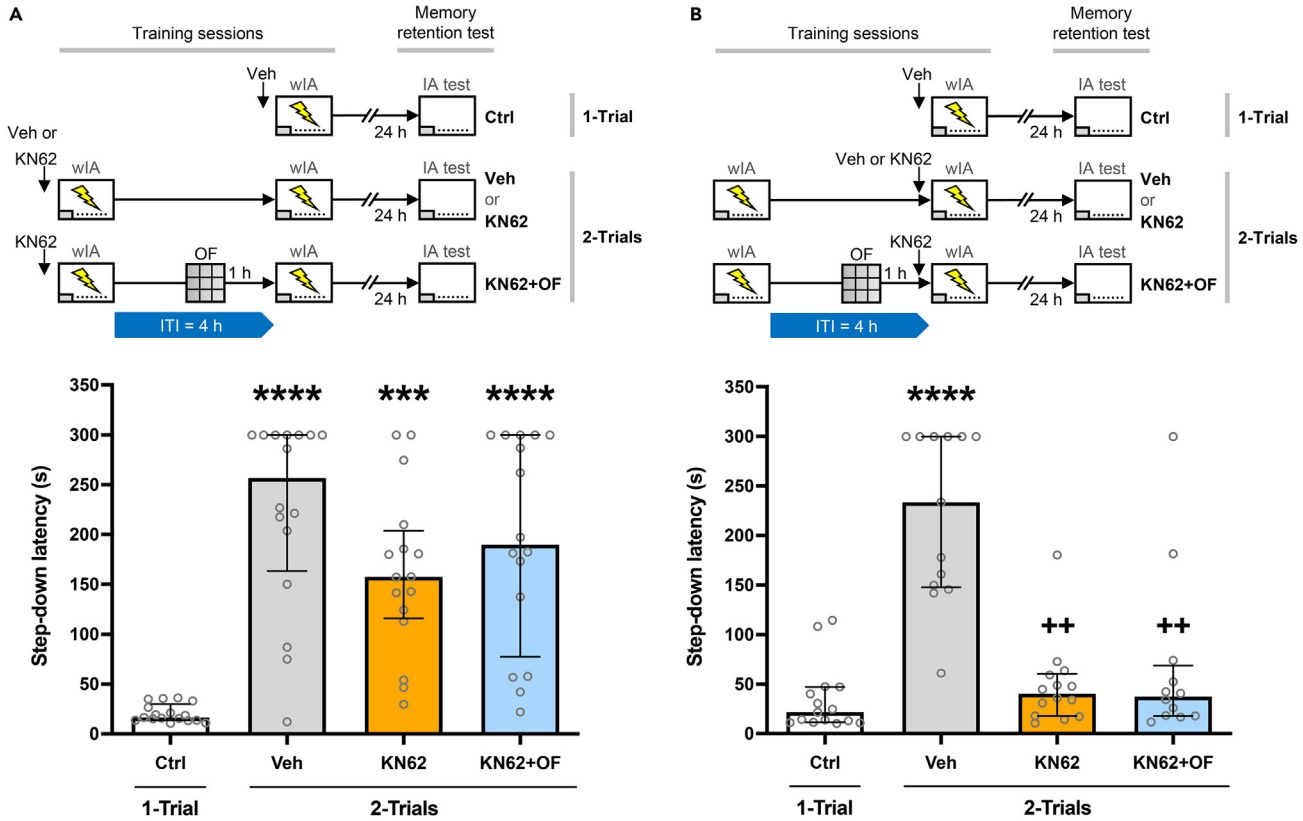


Figure 5. CaMKs inhibition in the dorsal hippocampus prior retraining impairs IA-LTM formation - A novel Open Field (OF) task does not prevent this effect

(Top) Diagrams illustrating the experimental protocols. A total of 65 rats in (A) and 54 rats in (B) were randomly assigned to the control group or to an experimental group.

(A) Control-group rats (Ctrl 1-Trial, n = 17) received an intra-dorsal hippocampal infusion of vehicle (Veh) 15 min before a single weak IA training session. Experimental retrained animals received intra-dorsal hippocampal infusions of either vehicle (Veh 2-Trials, n = 16) or KN62 (KN62 2-Trials, n = 16) 15 min before the first weak IA session. Another group injected with KN62 was also exposed to a novel OF session 1h before the second weak IA session (KN62+OF 2-Trials, n = 16). All groups were tested for IA-LTM 24 h later. Latency is represented as median ± IQR. Comparisons were done by means of a Kruskal-Wallis test with $p < 0.0001$ and Dunn's multiple comparisons between groups (****$p < 0.0001$ and ***$p < 0.001$ versus Ctrl 1-Trial).

(B) Similar to (A), control-group rats (Ctrl 1-Trial, n = 15) received an intra-dorsal hippocampal infusion of vehicle (Veh) 15 min before a single weak IA training session. Experimental, retrained animals subjected to a second weak IA session were injected with either vehicle (Veh 2-Trials, n = 13) or KN62 (KN62 2-Trials, n = 14) 15 min before the second weak IA session. Another group injected with KN62 was exposed in addition to a novel OF session 1h before the second weak IA session (KN62+OF 2-Trials, n = 12). All groups were tested for IA-LTM 24 h later. Latency is represented as median ± IQR. Comparisons were done by means of a Kruskal-Wallis test with $p < 0.0001$ and Dunn's multiple comparisons between groups (****$p < 0.0001$ versus Ctrl 1-Trial; ++$p < 0.01$ versus Veh 2-Trials).

results indicate that ERK1/2 activation is necessary during both training sessions to induce the synthesis of proteins. Moreover, the fact that OF exposure prevented amnesia induced by the infusion of U0126 before the second weak IA training session suggests that ERKs1/2 activation was not involved in the setting of the learning tag induced by the weak IA sessions.

CaMKs inhibition in the dorsal hippocampus prior retraining impairs IA-LTM formation - A novel open field (OF) task does not prevent this effect

To study the role of CaMKs in IA-LTM formation after spaced aversive training, we infused rats with either vehicle or the CaMKs inhibitor KN62 before the first (Figure 5A) or the second weak IA training session (Figure 5B). An additional control group not forming IA-LTM was trained with a single weak IA session and infused with vehicle before this session. Step-down latency results by group in seconds are shown as median [interquartile range] in Figure 5A (Ctrl, 16.38 [13.72/29.98]; Veh, 256.80 [163.50/300]; KN62, 157.50 [115.90/203.80]; KN62+OF, 189.90 [77.61/300]), and Figure 5B (Ctrl, 21.75 [11.48/47.40]; Veh, 233.80 [147.80/300]; KN62, 40.43 [17.88/60.43]; KN62+OF, 37.57 [18.06/68.77]).

Rats infused with either vehicle or KN62 before the first weak IA session exhibited a significant IA-LTM 24 h after training (Figure 5A, $p < 0.0001$ Veh versus Ctrl; $p < 0.001$ KN62 versus Ctrl). On the contrary, when KN62 was delivered before the second weak IA session, IA-LTM was impaired (Figure 5B, $p > 0.05$ KN62 versus Ctrl; $p < 0.01$ KN62 versus Veh). Infusion of vehicle before this session preserved IA-LTM as expected ($p < 0.0001$ Veh versus Ctrl).

To determine if activity of the CaMK-family kinases was involved in tag setting and/or PRP synthesis, we included additional groups that experienced two weak IA training sessions spaced by an ITI of 4 h and had an interspersed OF session occurring 1 h before the second weak IA session. KN62 was infused either before the first (Figure 5A) or the second weak IA session (Figure 5B). Of interest, OF exposure had a different effect on IA-LTM formation depending on the timing of KN62 infusion. When KN62 was infused before the first weak IA session, exploration of the OF did not change the results observed in the absence of OF experience (Figure 5A, $p > 0.05$ KN62-OF versus KN62), i.e., IA-LTM was observed in both cases (Figure 5A, $p < 0.001$ KN62 versus Ctrl; $p < 0.0001$ KN62-OF versus Ctrl). This result indicates that protein resources contributed by the OF event were redundant with available ones, i.e., that KN62 did not affect protein synthesis. Yet, when KN62 was infused before the second weak IA session, exploration of the OF did not restore IA-LTM (Figure 5B, $p > 0.05$ KN62-OF versus KN62). In other words, as the OF experience is known to induce PRP synthesis, what would be missing is the tag capturing them.

Besides acting on CaMK-family kinases, KN62 has been shown to antagonize the P2X7 purinergic receptor.[35,36] This effect is consistent for human P2X7 receptors but less significant for rat P2X7 receptors,[37] even if hippocampal administration of a P2X7 selective antagonist (A-740003) before fear conditioning slightly impaired memory formation. Despite this difference, we aimed nevertheless at verifying if the impairment of IA-LTM observed when KN62 was delivered before the second weak IA session (Figure 5B) was because of an off-target effect of KN62 on P2X7 receptors rather than on CaMK family kinases.

We thus administered locally A-740003 before the second weak IA session and determined the effects of this treatment on IA- LTM. Inhibiting the P2X7 receptors had no effect on IA-LTM (see Figure S1), thus ruling out the participation of P2X7 receptors in the amnesia induced by the administration of KN62 before IA training. Overall, these results support a critical role of CaMKs for tag setting -but probably not for PRP synthesis-in the dorsal hippocampus during the learning protocol used in our work.

PKA inhibition impairs IA-LTM formation induced by retraining - A novel Open Field (OF) prevents or not this effect depending on the timing of inhibition

Finally, we studied the role of PKA on IA-LTM formation after spaced training with two weak IA sessions. To this end, we infused rats with either vehicle or the PKA inhibitor Rp-cAMP before the first (Figure 6A) or the second weak IA training session (Figure 6B). As in the previous experiments, an additional control group not forming IA-LTM was trained with a single weak IA session and infused with vehicle before this session. Step-down latency results by group in seconds were presented as median [interquartile range] in Figure 6A (Ctrl, 30.24 [18.24/43.07]; Veh, 192.60 [111.30/300]; RP, 41.82 [17.10/63.47]; RP + OF, 199.60 [96.59/267.50]), and Figure 6B (Ctrl, 24.71 [21.84/31.55]; Veh, 76.45 [58.02/216.10]; RP, 19.90 [14.45/67.52]; RP + OF, 24.20 [15.39/37.56]).

Vehicle-infused retrained groups exhibited significant IA-LTM 24 h after training, irrespective of the timing of the vehicle infusion (Figure 6A, $p < 0.01$ Veh versus Ctrl; Figure 6B, $p < 0.05$ Veh versus Ctrl). On the contrary, IA-LTM was absent in Rp-cAMP-infused animals irrespective of the timing of infusion (Figures 6A and 6B, $p > 0.05$ RP versus Ctrl), thus showing the involvement of PKA activity in IA-LTM formation following spaced training with two weak IA sessions.

Including a session of OF exploration in additional groups infused with Rp-cAMP yielded results, which differed according to the infusion time. When OF exploration preceded Rp-cAMP infusion, i.e., when infusion occurred before the second weak IA session, LTM was not restored despite the potential contribution of PRPs by OF exploration (Figure 6B, $p > 0.05$, RP + OF versus RP). These results suggest that PKA activity participates in the setting of the learning tag by the second IA session as no LTM was formed even in the presence of PRP supply by the OF event. On the contrary, when OF exploration followed Rp-cAMP infusion, i.e., when infusion occurred before the first weak IA session, LTM was restored (Figure 6A, $p < 0.05$, RP + OF versus RP). This result indicates on the one hand that Rp-cAMP infusion before the first weak IA session did

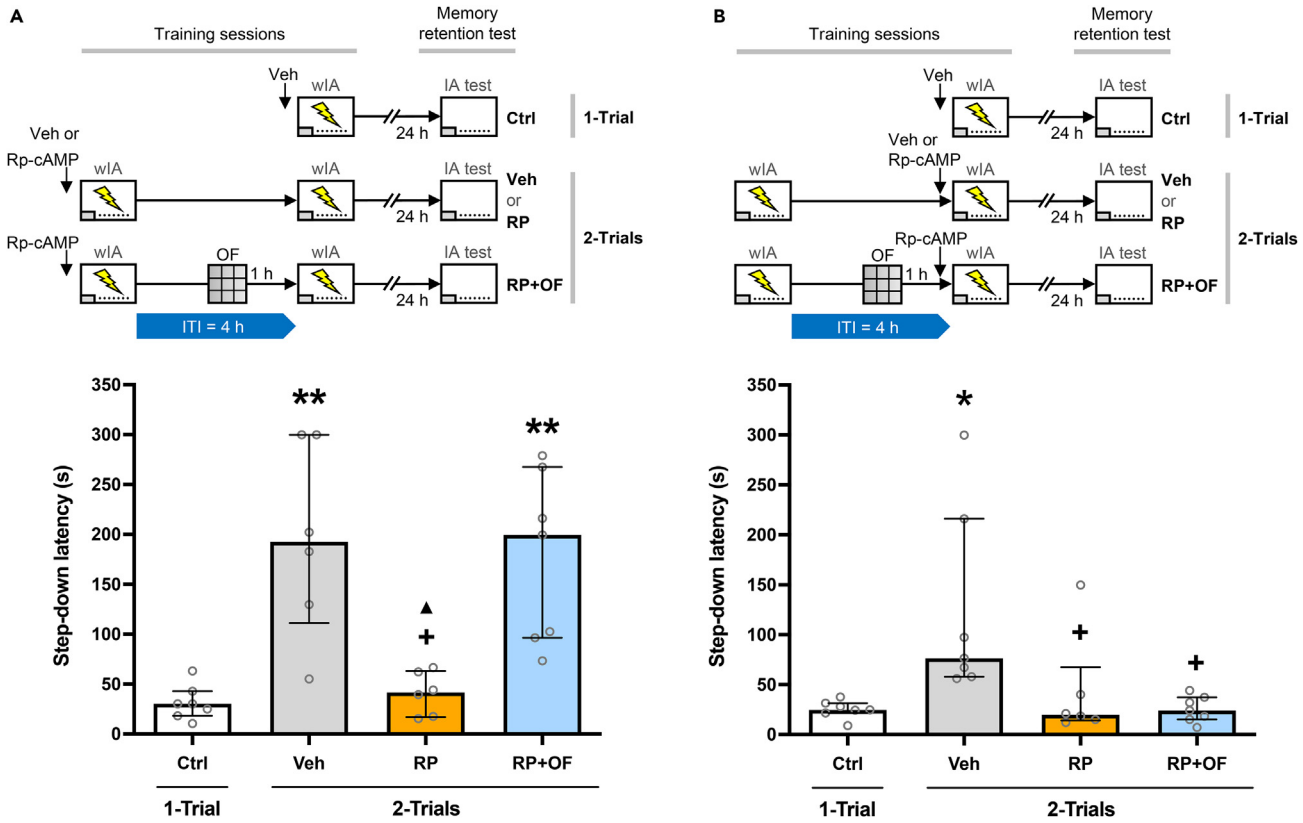


Figure 6. PKA inhibition impairs IA-LTM formation induced by retraining - A novel Open Field (OF) prevents or not this effect depending on the timing of inhibition

(Top) Diagrams illustrating the experimental protocols. A total of 26 rats in (A) and 27 rats in (B) were randomly assigned to the control group or to an experimental group.

(A) Control-group rats (Ctrl 1-Trial, n = 7) received an intra-dorsal hippocampal infusion of vehicle (Veh) 15 min before a single weak IA training session. Experimental groups received intra-dorsal hippocampal infusions of either vehicle (Veh 2-Trials, n = 6) or Rp-cAMP (RP 2-Trials, n = 6) 15 min before the first weak IA session. Another group also injected with Rp-cAMP before the first IA session was exposed in addition to a novel OF session 1h before the second weak IA session (RP + OF 2-Trials, n = 7). All groups were tested for IA-LTM 24 h later. Latency is represented as median ± IQR. Comparisons were done by means of a Kruskal-Wallis test with $p < 0.001$ and Dunn's multiple comparisons between groups (**$p < 0.01$ versus Ctrl 1-Trial; +$p < 0.05$ versus Veh 2-Trials; ▲ $p < 0.05$ versus RP + OF 2-Trials).

(B) Similar to (A), control-group rats (Ctrl 1-Trial, n = 7) received an intra-dorsal hippocampal infusion of vehicle (Veh) 15 min before a single weak IA training session. Experimental, retrained animals subjected to a second weak IA session were injected with either vehicle (Veh 2-Trials, n = 7) or Rp-cAMP (RP 2-Trials, n = 6) 15 min before the second weak IA session. An additional group similarly injected with Rp-cAMP was also exposed to a novel OF session 1h before the second weak IA session (RP + OF 2-Trials, n = 7). All groups were tested for IA-LTM 24 h later. Latency is represented as median ± IQR. Comparisons were done by means of a Kruskal-Wallis test with $p < 0.01$ and Dunn's multiple comparisons between groups (*$p < 0.05$ versus Ctrl 1-Trial; +$p < 0.05$ versus Veh 2-Trials).

not affect tag setting induced by the second IA session 4 h later as otherwise LTM would not be observable. It shows, on the other hand, that amnesia induced on injection of Rp-cAMP before the first IA session in the group that did not experience the OF event was because of an effect of the inhibitor on protein synthesis. Hence, when the OF experience was added to an equivalent group, LTM was restored. Overall, these results suggest a critical double role of PKA signaling in LTM formation following training with two spaced weak IA sessions: it is required for tag setting induced by weak IA sessions and for protein synthesis necessary to stabilize memory into an LTM form.

DISCUSSION

Our goal was to determine if the BT framework accounts for aversive LTM formation during spaced training trials in rats, and the possible role of different kinases in this process. We showed that two identical weak IA sessions spaced from 15 min to 6 h induce a 24-h LTM and that this process can be explained by a BT

mechanism, where ERKs are involved in PRP synthesis but not in tag setting, CaMKs is only needed for tag setting, and PKA is required for both processes. We thus extend the broad spectrum of learning and memory phenomena for which BT provides a valid mechanistic explanation to the case of aversive spaced learning.

Spaced training, neural assembly specificity and protein synthesis during memory consolidation

A recurrent feature of the process of memory formation in multiple species is that learning sessions spaced by long ITI (spaced training) lead to more robust memories than training sessions with little or no resting interval (massed training).[2] Our results show that while a single weak IA training session does not lead to IA-LTM, adding a retraining session within a time window in the range of 15 min to 6 h (Figure 1) induces LTM formation. In contrast, if a weak IA session is combined with a weak SOR session, no IA-LTM is formed (Figure 2) even if both learning forms depend on the dorsal hippocampus.[33,38] During the weak SOR session, animals briefly explored objects in a context *to which they had been previously familiarized* by two prior sessions of habituation. Thus, the SOR session did not meet the necessary condition of novelty required to promote PRP synthesis and IA LTM.[6] We thus suggest that the different nature of the tasks, and their weakness, may result in the activation of neural substrates with scarce overlap, leading to suboptimal sharing of cellular mechanisms and an insufficient induction of PRPs synthesis. In this sense, neuronal ensembles activated by initial learning are necessary for relearning and strengthening memories. For instance, when specific subset of neurons activated by initial fear conditioning are inhibited, relearning is impaired, thus suggesting that the activity of the initial ensemble is dedicated to the same learning and is not substitutable.[39]

We also observed that if the ITI between two weak IA session is shorter than 15 min or longer than 6 h, promotion of IA-LTM did not occur as the response observed in the 24-h test did not differ from that induced by training with a single weak IA session (Figure 1). Importantly, spaced training affects the specificity of the neural assemblies engaged in learning. In mice trained on an appetitive delayed matching-to-place task, which depends on the dorsomedial prefrontal cortex, calcium-imaging recordings of excitatory neurons in this brain region revealed that longer trial spacing increased the similarity of the population activity pattern on subsequent encoding trials and on retrieval.[40] Thus, spaced learning promotes reactivation of prefrontal neuronal ensembles and facilitates, therefore, sharing and cooperation between molecular processes at identical neural sites engaged in retraining.

Besides promoting neural specificity, spaced learning trials consolidate information acquired into LTM via the process of protein synthesis.[3,41] Therefore, it was important to verify that our conditioning protocol leading to a 24-h IA-LTM also induced protein synthesis. Our results showed that protein synthesis was indeed necessary for the formation of IA-LTM following two weak spaced training sessions as injection of the protein synthesis inhibitor EME in the dorsal hippocampus either before the initial training session or before the retraining session 4 h later, resulted in amnesia (Figures 3A and 3B). Thus, the lack of effectiveness of shorter ITIs (e.g., 5 min) to promote IA-LTM could be because of the inability of the second weak IA session to induce the synthesis of PRPs. Possibly, the interaction of intracellular signals triggered by each training session is required and needs to exceed a minimum ITI for this process to be operational at the time of the second session. In the case of ITIs longer that 6 h, which also failed to induce IA-LTM, molecular changes triggered by the first weak IA session would fade out before the second weak IA session, thus precluding any interaction or cooperation needed for LTM.

A BT account of LTM formation on aversive spaced training

The BT hypothesis postulates that learning induces tags in specific neural sites, which allow capturing PRPs to consolidate memory traces.[6,7] Both processes, tag setting and PRP synthesis, are necessary to consolidate LTM because the absence of any of them impairs memory formation.[34] For instance, training rats with a single weak IA session (the Ctrl group used in the present experiments) leads to tag setting.[6] Coupling this training with a relevant event such as a novel OF exploration, which supplies PRPs, induced a robust IA-LTM, which would not be observed in the absence of the OF experience.[6] The learning tag induced by the weak IA training session is short-lived and lasts less than 2 h. In consequence, OF exposure after this period fails to induce IA-LTM as the PRPs supplied cannot be longer captured by an absent tag.[6]

The time window between two weak IA training sessions required to promote IA-LTM (15 min–6 h) is wider than that separating a novel OF exposure as a provider of PRPs from the second IA session (1 h).[6] In the

framework of the BT hypothesis, we suggest that retraining will mainly retag the sites initially labeled by the prior training session and that PRPs required for memory consolidation will be synthesized as a result of the sum or synergy of the activation of intracellular signaling cascades induced by each weak training session. Our results suggest that these processes operate within a time window that extends up to 6 h between both weak IA sessions (Figure 1). Similar results have been recently reported for the SOR task.[31] Using two weak SOR sessions, each of which is unable to promote LTM on its own, SOR-LTM was promoted when the two sessions were spaced by an ITI ranging from 15 min to 7 h.[31] This ITI is 1 h longer than that found in the present work, thus suggesting that the molecular processes triggered by a first SOR session are more persistent in time.

ERKs1/2 activation is required to induce IA-LTM on spaced training: A role in protein synthesis

Studies focusing on aversive olfactory learning in flies, conditioning of the tail-elicited siphon withdrawal reflex in *Aplysia* or object location memory in mice found that changes in ERKs1/2 activities are required to promote or improve memories by retraining.[1,42–46] Previous works have also demonstrated the activation of ERKs1/2 in the dorsal hippocampus during IA-LTM formation.[47,48] Here, we studied the role of ERKs1/2 activity for the processes of tag setting and PRP synthesis during retraining (Figure 4). As retraining with the second weak IA session would mainly retag the sites labeled by the first training session, we used an ITI of 4 h to ensure that the transient learning-tag induced by the first weak IA session had already declined.[6] We found that the MEK inhibitor U0126, which blocks ERKs1/2 activation, impaired the formation of IA-LTM when injected both before the first weak IA session (Figure 4A) and before the second weak IA session (Figure 4B). However, exploration of a novel OF 1 h before retraining with the second IA session prevented this amnesia. As the OF event is known to provide PRPs,[10] LTM rescue would be achieved via this supply of PRPs. This result confirms previous findings indicating that ERKs1/2 activity was not required for the setting of the IA learning tag and indicates that the PRPs induced by the novel OF exposure are used in the process of IA-LTM formation.[34] Our data suggest that ERKs1/2 are key molecules involved in the process leading to PRP synthesis. Thus, ERKs1/2 blockade before the first or the second session would be responsible for the amnesia observed on injection with U0126. We propose that PRPs required for memory consolidation are synthesized as the consequence of summation or synergistic effect between the two consecutive, weak IA sessions experienced within an appropriate temporal window.

CaMKs activation is required to induce IA-LTM on spaced training: A role in tag setting

Kinases of the CaMK family are involved in the early phase of IA-LTM formation. In this task, training induces a rapid increase of αCaMKII activity in the dorsal hippocampus.[49] At the synaptic level, training results in the translocation of αCaMKII to the post-synaptic density, a dense lamina just beneath the postsynaptic membrane,[50] where it shapes activity-induced changes in the spine.[51] In addition, blocking CaMKs activity in the hippocampus after training induces amnesia for associative aversive and non-associative spatial tasks.[52–54] Consistently, complete loss of αCaMKII activity in a knock-in mouse model resulted in severe impairment of IA learning.[55]

Despite the well-studied role of CaMKII as an essential mediator of activity-dependent synaptic plasticity,[56] the participation of this kinase in learning protocols based on multiple trials has been less characterized. In infant mice trained to learn the nest location using olfactory cues during four learning sessions separated by minutes, genetical overexpression of this kinase in the hippocampus induces impaired olfactory-based spatial learning.[57] Conversely, an increase of CaMKII phosphorylation is observed in protein-phosphatase-1 genetically inhibited mice, which exhibit enhanced object recognition learning when trained with five trials separated by short ITIs.[58] Here, we inhibited CaMKs activity in the dorsal hippocampus before the first or the second weak IA session when these were separated by an ITI of 4 h. We found that CaMKs blockade via infusion of KN62 before the second weak IA session (but not before the first session) impairs the formation of IA-LTM, which, unlike the blockade of ERKs1/2, cannot be prevented by a novel OF exposure (Figure 5B). This result agrees with findings of Barros et al.[59] showing that the amnesic effect of KN62 injected in the hippocampus after IA training could not be reversed by the administration of drugs with known promnesic action. These data are compatible with the requirement of CaMKs activity for the establishment of the IA learning tag, as described by Moncada et al.[34] They are also in accordance with the role of CaMKII for synaptic tag setting induced by a tetanic stimulation capable of inducing LTP,[60] a model of synaptic plasticity associated with IA learning.[61] Using hippocampal slices tetanically stimulated, Redondo et al.[62] assigned a specific role to CaMKII for the setting of the synaptic tag and to the CaMKK/CaMKIV pathway

for the synthesis of PRPs. We showed that inhibiting the activity of CaMK-family kinases before the first weak IA session did not induce amnesia, suggesting that the participation of these kinases in protein synthesis, which depends on the contribution of both training sessions, is not crucial.

Importantly, in concluding this, we excluded a possible off-target effect of KN62 at the level of P2X7 receptors, which are also antagonized by KN62, besides the known action of this drug on CaMK-family kinases. Indeed, the delivery of the specific P2X7-receptor antagonist A-740003 before the second weak IA session did not affect memory, contrary to the impairment induced by KN62 delivery under similar conditions. The absence of effect observed on specific blockade of P2X7 receptors indicates that signaling through P2X7 receptors is not involved in the formation of IA-LTM and that the effect induced by KN62 was exclusive of the CaMK-family kinases. Moreover, the administration of P2X7 antagonists or the inhibition of the expression of this receptor in various rat studies led to an effect opposite to the one observed in our work on KN62 delivery, namely a prevention of memory deficits.[63–66] The results obtained on specific blockade of P2X7 receptors thus confirmed the validity of our conclusions on the participation of CaMKs activity in the establishment of the learning tag required for IA-LTM.

PKA activation is required for IA-LTM formation on spaced aversive learning: A double role in tag setting and protein supply

The activation of PKA constitutes an essential component of IA-LTM formation. The administration of specific PKA inhibitors to the hippocampus after IA training prevents the expression of both STM and LTM in rats.[67–70] Also, pharmacological inhibition of PKA impairs memory formation in day-old chicks trained on a single-trial passive avoidance task,[71] and several studies revealed that changes in the cAMP/PKA-signaling pathway influence rodent memory in the passive avoidance task.[72–74] Parsons and Davis[75] showed that PKA activity is also involved in the promnesic effects of spaced learning in a task in which animals learn to associate a light stimulus with a shock. In this case, a single learning trial is insufficient to induce LTM whilst two learning trials separated by an ITI ranging between 45 min and 7 days induced LTM. This phenomenon requires that both trials are equal and signaled by the same cue. The single training, which does not support formation of fear memory on its own, results in the phosphorylation of several PKA targets in the amygdala. Accordingly, blocking PKA activity in the amygdala before the first trial prevents memory formation when the second trial is delivered 24 h later.[75]

Our results indicate that PKA has a fundamental role in spaced training with two weak IA sessions. Inhibiting PKA activity in the dorsal hippocampus via infusion of Rp-cAMP before both the first and the second weak IA sessions impaired the formation of IA-LTM, yet possibly via two different pathways. In the first case (injection before the first weak IA session; Figure 6A), adding an OF session before the second weak IA session restored IA-LTM because Rp-cAMP did not affected tag setting by the distant second session and PRPs were delivered by the OF event. In the absence of OF, tag setting by the second IA session was still possible so that the observed amnesia may have been because of a blockade of PRP synthesis on Rp-cAMP injection.

In the second case (injection before the second weak IA session; Figure 6B), OF exposure was unable to prevent amnesia, which suggests that blockade of PKA activity affected also tag setting. This explains the ineffectiveness of the OF session to restore LTM. When Rp-cAMP was injected before the second session (Figure 6B), not only tag setting would be impaired but, in addition, the tag induced by the first weak IA session would have already declined. In consequence, PRPs supplied by the OF would lack a capture site, thus leading to amnesia.

Conclusions

Overall, our results are consistent with previous findings highlighting the importance of the activity of several kinases as molecular determinants of the spacing effect leading to LTM. One proposal is that they contribute to increase CREB activity and thus to gene transcription,[76] which is associated with the formation or enhancement of LTM after spaced training in fear conditioning learning.[77,78] Alternatively, these kinases could contribute to protein translation at synaptic terminals, affecting thereby memory consolidation.[79,80] However, the signaling routes induced by each of the two training sessions may differ. In *Aplysia*, the study of trial spacing on the induction of LTM for sensitization of the tail-elicited siphon withdrawal reflex showed that LTM could be induced with only two spaced training trials spaced by 45 min[42]; this effect depended on the delayed nuclear MAPK activity induced 45 min after the first trial (tail shock) plus the activation of PKA induced by the second stimulation. In our work, the activities of ERKs1/2 and PKA induced by

Table 1. Summary of the contribution of different kinases to the processes of learning-tag setting and/or protein synthesis proposed by the behavioral tagging model of LTM formation

	Time of procedure	Drug DH infusion pre 1st wIA session	Drug DH infusion pre 2nd wIA session	Drug DH infusion pre 1st or 2nd wIA sessions	Test 24 h after TR
Drug	Target of action (inhibition)	Impairment of learning tag induced by 1st wIA session[a]	Impairment of learning tag induced by 2nd wIA session	Impairment of PRPs synthesis resultant of cooperation between 1st and 2nd wIA sessions	IA-LTM
Veh	–	No	No	No	Yes
Emetine	Protein synthesis	No	No	Yes	No
U0126	ERKs 1/2	No	No	Yes	No
KN-62	CaMKs	Yes	Yes	No	No
Rp-cAMP	PKA	Yes	Yes	Yes	No

The table focuses on the main molecular actors targeted, specifies the effect of each drug on the first and second training sessions and on protein synthesis (PRPs), and indicates whether treatments led to IA-LTM. Drugs were infused into the dorsal hippocampus (DH) previous to the 1st or the 2nd weak IA training sessions (TR) spaced by 4h.

[a]Data was from Moncada et al.,[34] using a single wIA session.

both weak IA sessions are required to form IA-LTM. Thus, removing any of these components impaired LTM formation after spaced training.

The BT hypothesis postulates that at least two processes are required to form LTM, namely the establishment of a transient and specific learning tag and the synthesis of PRPs captured by the tag. Our results show that these processes are also required to promote the formation of IA-LTM after spaced training. Our results suggest that tagging and re-tagging induced by the first and the second weak IA sessions, respectively, depend on the activity of CaMKs and PKA. Moreover, our data suggest that the process of protein synthesis requires the activity of ERKs1/2 and PKA in both training sessions. Their activity would overlast the half-life of the tag and it would be summed or synergized to induce the synthesis of PRPs in the second training session. Table 1 summarizes the contribution of these kinases to the processes of tag setting and/or protein synthesis contemplated in the BT model for LTM formation.

The temporal dynamics of kinase activation would be fundamental to promote a cooperative and/or synergic phenomenon that cannot be achieved at very short or widely separated ITIs. Also, the local distribution of kinase activity, i.e., the specificity of the neural substrates engaged by each training session, would be a constraint for IA-LTM promotion after retraining. In other words, when two different, weak tasks are used consecutively, LTM is not observed probably because of the lower overlapping of neurons activated by each session. The learning tag and the PRPs necessary for memory consolidation must share both space and time (here an ITI ranging from 15 min to 6 h) to be effective. Overall, our findings suggest that the BT hypothesis provides a valid account for the well-known effect of spaced training on the promotion of LTM in IA learning in rodents. Further studies should show if this account is also valid for other forms of spaced learning in different animal species.

Limitations of the study

Our work presents some limitations inherent to the use of a behavioral pharmacological approach, such as the possible effect of drugs on other molecular targets. For instance, KN62 potently inhibits CaMKII but also antagonizes CaMKI and CaMKIV at similar concentrations.[81] We thus extended our conclusions on learning tag setting related to the use of KN62 to the CaMK family. Furthermore, as Epac (exchange protein activated by cAMP) is an alternative cAMP effector,[82] Rp-cAMP could also act via Epac inhibition in addition to its effect on PKA. Yet, there is little evidence for an effect of Epac on memory processes and existing findings do not support its participation in the effects observed in our paper.[83,84] Rp-cAMP could also act on cyclic nucleotide channels (HCN). Impairments in spatial learning and memory have been associated with an increase in HCN2 in cell membranes of hippocampal CA1 area.[85,86] However, septal infusion of a selective HCN channel blocker did not affect the acquisition or retention of a continuous multiple inhibitory avoidance task.[87] Other works obtained contradictory results concerning the involvement of the HCN in very short-term memories.[88,89] Overall, these findings do not support the involvement of HCN in the clear

strong amnestic effect on IA-LTM induced by the administration of Rp-cAMP before IA training sessions (Figures 6A and 6B).

Our behavioral approach has the limitation of not enabling a direct visualization of learning tag and protein synthesis. Using *in vivo* imaging of structural and functional changes at the level of individual spines during retraining would allow overcoming this limitation. Also, an essential aspect of our BT account in the case of retraining is that the same neural populations are engaged on each weak IA session. Yet, we did not dispose of the methodology to demonstrate this neural coincidence along retraining. Using a cellular compartment analysis of temporal activity by fluorescent in situ hybridization (catFISH) for specific IEGs, it would be, in principle, possible to determine the activity history of neurons during the consecutive training sessions and estimate possible coincidence in the numbers of active neurons.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
  - Experimental animals
- METHOD DETAILS
  - Surgery and drug infusion
  - Drugs
  - Behavioral procedures
  - Inhibitory avoidance task
  - Spatial Object Recognition task
  - Open field task
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.107278.

ACKNOWLEDGMENTS

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 Haydee Viola.

M.G. thanks the Institut Universitaire de France for generous support.

AUTHOR CONTRIBUTIONS

Conceptualization, H.V.; Methodology, H.V., P.B., and R.T.; Investigation, P.B., R.T., J.C., M.V., and P.M.; Resources, H.V.; Writing – Original Draft, H.V., M.G., and P.B.; Writing – Review and Editing, H.V., M.G., P.B., M.V., P.M., R.T., and J.C.; Supervision, H.V.; Project Administration, H.V.; Funding Acquisition, H.V.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: August 28, 2022
Revised: September 14, 2022
Accepted: June 30, 2023
Published: July 4, 2023

REFERENCES

1. Philips, G.T., Ye, X., Kopec, A.M., and Carew, T.J. (2013). Cellular/Molecular MAPK Establishes a Molecular Context That Defines Effective Training Patterns for Long-Term Memory Formation. J. Neurosci. *33*, 7565–7573. https://doi.org/10.1523/JNEUROSCI.5561-12.

2. Smolen, P., Zhang, Y., and Byrne, J.H. (2016). The right time to learn: Mechanisms and optimization of spaced learning. Nat. Rev. Neurosci. *17*, 77–88. https://doi.org/10.1038/nrn.2015.18.

3. McGaugh, J.L. (2000). Memory - A century of consolidation. Science *287*, 248–251. https://doi.org/10.1126/science.287.5451.248.

4. Martin, S.J., Grimwood, P.D., and Morris, R.G. (2000). Synaptic Plasticity and Memory: An Evaluation of the Hypothesis. Annu. Rev. Neurosci. *23*, 649–711. https://doi.org/10.1146/annurev.neuro.23.1.649.

5. Frey, U., and Morris, R.G. (1997). Synaptic tagging and long-term potentiation. Nature *385*, 533–536. https://doi.org/10.1038/385533a0.

6. Moncada, D., and Viola, H. (2007). Induction of long-term memory by exposure to novelty requires protein synthesis: Evidence for a behavioral tagging. J. Neurosci. *27*, 7476–7481. https://doi.org/10.1523/JNEUROSCI.1083-07.2007.

7. Moncada, D., Ballarini, F., and Viola, H. (2015). Behavioral Tagging: A Translation of the Synaptic Tagging and Capture Hypothesis. Neural Plast. *2015*, 650780. https://doi.org/10.1155/2015/650780.

8. Redondo, R.L., and Morris, R.G.M. (2011). Making memories last: The synaptic tagging and capture hypothesis. Nat. Rev. Neurosci. *12*, 17–30. https://doi.org/10.1038/nrn2963.

9. Viola, H., Ballarini, F., Martínez, M.C., and Moncada, D. (2014). The Tagging and Capture Hypothesis from Synapse to Memory. In Progress in Molecular Biology and Translational Science (Elsevier), pp. 391–423. https://doi.org/10.1016/B978-0-12-420170-5.00013-1.

10. Ballarini, F., Moncada, D., Martinez, M.C., Alen, N., and Viola, H. (2009). Behavioral tagging is a general mechanism of long-term memory formation. Proc. Natl. Acad. Sci. USA *106*, 14599–14604. https://doi.org/10.1073/pnas.0907078106.

11. Ballarini, F., Martínez, M.C., Díaz Perez, M., Moncada, D., and Viola, H. (2013). Memory in Elementary School Children Is Improved by an Unrelated Novel Experience. PLoS One *8*, e66875. https://doi.org/10.1371/journal.pone.0066875.

12. de Carvalho Myskiw, J., Furini, C.R.G., Benetti, F., and Izquierdo, I. (2014). Hippocampal molecular mechanisms involved in the enhancement of fear extinction caused by exposure to novelty. Proc. Natl. Acad. Sci. USA *111*, 4572–4577. https://doi.org/10.1073/pnas.1400423111.

13. Liu, J.F., Yang, C., Deng, J.H., Yan, W., Wang, H.M., Luo, Y.X., Shi, H.S., Meng, S.Q., Chai, B.S., Fang, Q., et al. (2015). Role of hippocampal ß-adrenergic and glucocorticoid receptors in the novelty-induced enhancement of fear extinction. J. Neurosci. *35*, 8308–8321. https://doi.org/10.1523/JNEUROSCI.0005-15.2015.

14. Moncada, D. (2017). Evidence of VTA and LC control of protein synthesis required for the behavioral tagging process. Neurobiol. Learn. Mem. *138*, 226–237. https://doi.org/10.1016/J.NLM.2016.06.003.

15. Bae, S.E., and Richardson, R. (2018). Behavioral tagging in infant rats. Learn. Mem. *25*, 580–586. https://doi.org/10.1101/lm.047605.118.

16. Gros, A., and Wang, S.H. (2018). Behavioral tagging and capture: long-term memory decline in middle-aged rats. Neurobiol. Aging *67*, 31–41. https://doi.org/10.1016/j.neurobiolaging.2018.02.023.

17. Naseem, M., Tabassum, H., and Parvez, S. (2019). PKM-ζ Expression Is Important in Consolidation of Memory in Prelimbic Cortex Formed by the Process of Behavioral Tagging. Neuroscience *410*, 305–315. https://doi.org/10.1016/j.neuroscience.2019.03.060.

18. Chen, N., Tsai, T.C., and Hsu, K.S. (2020). Exposure to Novelty Promotes Long-Term Contextual Fear Memory Formation in Juvenile Mice: Evidence for a Behavioral Tagging. Mol. Neurobiol. *57*, 3956–3968. https://doi.org/10.1007/S12035-020-02005-1.

19. Navakkode, S., Gaunt, J.R., Pavon, M.V., Bansal, V.A., Abraham, R.P., Chong, Y.S., Ch'ng, T.H., and Sajikumar, S. (2021). Sex-specific accelerated decay in time/activity-dependent plasticity and associative memory in an animal model of Alzheimer's disease. Aging Cell *20*, e13502. https://doi.org/10.1111/ACEL.13502.

20. Lu, Y., Ji, Y., Ganesan, S., Schloesser, R., Martinowich, K., Sun, M., Mei, F., Chao, M.V., and Lu, B. (2011). TrkB as a potential synaptic and behavioral tag. J. Neurosci. *31*, 11762–11771. https://doi.org/10.1523/JNEUROSCI.2707-11.2011.

21. Almaguer-Melian, W., Bergado-Rosado, J., Pavón-Fuentes, N., Alberti-Amador, E., Mercerón-Martínez, D., and Frey, J.U. (2012). Novelty exposure overcomes foot shock-induced spatial-memory impairment by processes of synaptic-tagging in rats. Proc. Natl. Acad. Sci. USA *109*, 953–958. https://doi.org/10.1073/pnas.1114198109.

22. Dong, Z., Gong, B., Li, H., Bai, Y., Wu, X., Huang, Y., He, W., Li, T., and Wang, Y.T. (2012). Mechanisms of hippocampal long-term depression are required for memory enhancement by novelty exploration. J. Neurosci. *32*, 11980–11990. https://doi.org/10.1523/JNEUROSCI.0984-12.2012.

23. Nomoto, M., Ohkawa, N., Nishizono, H., Yokose, J., Suzuki, A., Matsuo, M., Tsujimura, S., Takahashi, Y., Nagase, M., Watabe, A.M., et al. (2016). Cellular tagging as a neural network mechanism for behavioural tagging. Nat. Commun. *7*, 12319–12411. https://doi.org/10.1038/ncomms12319.

24. Vishnoi, S., Raisuddin, S., and Parvez, S. (2022). Behavioral Tagging: Role of Neurotransmitter Receptor Systems in Novel Object Recognition Long-Term Memory. ACS Omega *7*, 11587–11595. https://doi.org/10.1021/ACSOMEGA.1C05865.

25. Cassini, L.F., Sierra, R.O., Haubrich, J., Crestani, A.P., Santana, F., de Oliveira Alvares, L., and Quillfeldt, J.A. (2013). Memory reconsolidation allows the consolidation of a concomitant weak learning through a synaptic tagging and capture mechanism. Hippocampus *23*, 931–941. https://doi.org/10.1002/hipo.22149.

26. Rabinovich Orlandi, I., Fullio, C.L., Schroeder, M.N., Giurfa, M., Ballarini, F., and Moncada, D. (2020). Behavioral tagging underlies memory reconsolidation. Proc. Natl. Acad. Sci. USA *117*, 18029–18036. https://doi.org/10.1073/PNAS.2009517117.

27. Salvetti, B., Morris, R.G.M., and Wang, S.H. (2014). The role of rewarding and novel events in facilitating memory persistence in a separate spatial memory task. Learn. Mem. *21*, 61–72. https://doi.org/10.1101/lm.032177.113.

28. Lopes da Cunha, P., Villar, M.E., Ballarini, F., Tintorelli, R., and Ana María Viola, H. (2019). Spatial object recognition memory formation under acute stress. Hippocampus *29*, 491–499. https://doi.org/10.1002/hipo.23037.

29. Lopes da Cunha, P., Tintorelli, R., Correa, J., Budriesi, P., and Viola, H. (2022). Behavioral tagging as a mechanism for aversive-memory formation under acute stress. Eur. J. Neurosci. *55*, 2651–2665. https://doi.org/10.1111/EJN.15249.

30. Correa, J., Tintorelli, R., Budriesi, P., and Viola, H. (2022). Persistence of Spatial Memory Induced by Spaced Training Involves a Behavioral-Tagging Process. Neuroscience *497*, 215–227. https://doi.org/10.1016/J.NEUROSCIENCE.2022.02.032.

31. Tintorelli, R., Budriesi, P., Villar, M.E., Marchal, P., Lopes da Cunha, P., Correa, J., Giurfa, M., and Viola, H. (2020). Spatial-Memory Formation After Spaced Learning Involves ERKs1/2 Activation Through a Behavioral-Tagging Process. Sci. Rep. *10*, 98. https://doi.org/10.1038/s41598-019-57007-4.

32. Carpenter, S.K. (2017). Spacing Effects on Learning and Memory. In Learning and Memory: A Comprehensive Reference

(Elsevier), pp. 465–485. https://doi.org/10.1016/B978-0-12-809324-5.21054-7.

33. Izquierdo, I., and Medina, J.H. (1997). Memory formation: The sequence of biochemical events in the hippocampus and its connection to activity in other brain structures. Neurobiol. Learn. Mem. *68*, 285–316. https://doi.org/10.1006/nlme.1997.3799.

34. Moncada, D., Ballarini, F., Martinez, M.C., Frey, J.U., and Viola, H. (2011). Identification of transmitter systems and learning tag molecules involved in behavioral tagging during memory formation. Proc. Natl. Acad. Sci. USA *108*, 12931–12936. https://doi.org/10.1073/pnas.1104495108.

35. Baraldi, P.G., del Carmen Nuñez, M., Morelli, A., Falzoni, S., Di Virgilio, F., and Romagnoli, R. (2003). Synthesis and biological activity of N-arylpiperazine-modified analogues of KN-62, a potent antagonist of the purinergic P2X7 receptor. J. Med. Chem. *46*, 1318–1329. https://doi.org/10.1021/jm021049d.

36. Donnelly-Roberts, D.L., Namovic, M.T., Han, P., and Jarvis, M.F. (2009). Mammalian P2X7 receptor pharmacology: Comparison of recombinant mouse, rat and human P2X7 receptors. Br. J. Pharmacol. *157*, 1203–1214. https://doi.org/10.1111/j.1476-5381.2009.00233.x.

37. Humphreys, B.D., Virginio, C., Surprenant, A., Rice, J., and Dubyak, G.R. (1998). Isoquinolines as Antagonists of the P2X7 Nucleotide Receptor: High Selectivity for the Human versus Rat Receptor Homologues. Mol. Pharmacol. *54*, 22–32. https://doi.org/10.1124/MOL.54.1.22.

38. Mumby, D.G., Gaskin, S., Glenn, M.J., Schramek, T.E., and Lehmann, H. (2002). Hippocampal damage and exploratory preferences in rats: Memory for objects, places, and contexts. Learn. Mem. *9*, 49–57. https://doi.org/10.1101/lm.41302.

39. Matsuo, N. (2015). Irreplaceability of neuronal ensembles after memory allocation. Cell Rep. *11*, 351–357. https://doi.org/10.1016/j.celrep.2015.03.042.

40. Glas, A., Hübener, M., Bonhoeffer, T., and Goltstein, P.M. (2021). Spaced training enhances memory and prefrontal ensemble stability in mice. Curr. Biol. *31*, 4052–4061.e6. https://doi.org/10.1016/J.CUB.2021.06.085.

41. Costa-Mattioli, M., and Sonenberg, N. (2008). Chapter 5 Translational control of gene expression: A molecular switch for memory storage. In Progress in Brain Research (Elsevier), pp. 81–95. https://doi.org/10.1016/S0079-6123(07)00005-2.

42. Philips, G.T., Tzvetkova, E.I., and Carew, T.J. (2007). Transient mitogen-activated protein kinase activation is confined to a narrow temporal window required for the induction of two-trial long-term memory in Aplysia. J. Neurosci. *27*, 13701–13705. https://doi.org/10.1523/JNEUROSCI.4262-07.2007.

43. Pagani, M.R., Oishi, K., Gelb, B.D., and Zhong, Y. (2009). The Phosphatase SHP2 Regulates the Spacing Effect for Long-Term Memory Induction. Cell *139*, 186–198. https://doi.org/10.1016/j.cell.2009.08.033.

44. Seese, R.R., Wang, K., Yao, Y.Q., Lynch, G., and Gall, C.M. (2014). Spaced training rescues memory and ERK1/2 signaling in fragile X syndrome model mice. Proc. Natl. Acad. Sci. USA *111*, 16907–16912. https://doi.org/10.1073/pnas.1413335111.

45. Li, Q., Zhang, X., Hu, W., Liang, X., Zhang, F., Wang, L., Liu, Z.J., and Zhong, Y. (2016). Importin-7 mediates memory consolidation through regulation of nuclear translocation of training activated MAPK in Drosophila. Proc. Natl. Acad. Sci. USA *113*, 3072–3077. https://doi.org/10.1073/pnas.1520401113.

46. Miyashita, T., Kikuchi, E., Horiuchi, J., and Saitoe, M. (2018). Long-Term Memory Engram Cells Are Established by c-Fos/CREB Transcriptional Cycling. Cell Rep. *25*, 2716–2728.e3. https://doi.org/10.1016/j.celrep.2018.11.022.

47. Walz, R., Rockenbach, I.C., Amaral, O.B., Quevedo, J., and Roesler, R. (1999). MAPK and memory. Trends Neurosci. *22*, 495. https://doi.org/10.1016/S0166-2236(99)01473-3.

48. Cammarota, M., Bevilaqua, L.R., Ardenghi, P., Paratcha, G., Levi De Stein, M., Izquierdo, I., and Medina, J.H. (2000). Learning-associated activation of nuclear MAPK, CREB and Elk-1, along with Fos production, in the rat hippocampus after a one-trial avoidance learning: Abolition by NMDA receptor blockade. Brain Res. Mol. Brain Res. *76*, 36–46. https://doi.org/10.1016/S0169-328X(99)00329-0.

49. Cammarota, M., Bernabeu, R., Levi De Stein, M., Izquierdo, I., and Medina, J.H. (1998). Learning-specific, time-dependent increases in hippocampal Ca2+/calmodulin-dependent protein kinase II activity and AMPA GluR1 subunit immunoreactivity. Eur. J. Neurosci. *10*, 2669–2676. https://doi.org/10.1046/j.1460-9568.1998.00254.x.

50. Cammarota, M., Bevilaqua, L.R.M., Bonini, J.S., Rossatto, J.I., Medina, J.H., and Izquierdo, N. (2004). Hippocampal glutamate receptors in fear memory consolidation. Neurotox. Res. *6*, 205–212. https://doi.org/10.1007/BF03033222.

51. Dosemeci, A., Weinberg, R.J., Reese, T.S., and Tao-Cheng, J.H. (2016). The Postsynaptic Density: There Is More than Meets the Eye. Front. Synaptic Neurosci. *8*, 23. https://doi.org/10.3389/FNSYN.2016.00023.

52. Wolfman, C., Fin, C., Dias, M., Bianchin, M., Da Silva, R.C., Schmitz, P.K., Medina, J.H., and Izquierdo, I. (1994). Intrahippocampal or intraamygdala infusion of KN62, a specific inhibitor of calcium/calmodulin-dependent protein kinase II, causes retrograde amnesia in the rat. Behav. Neural. Biol. *61*, 203–205. https://doi.org/10.1016/S0163-1047(05)80001-9.

53. Vianna, M.R., Alonso, M., Viola, H., Quevedo, J., De Paris, F., Furman, M., De Stein, M.L., Medina, J.H., and Izquierdo, I. (2000). Role of hippocampal signaling pathways in long-term memory formation of a nonassociative learning task in the rat. Learn. Mem. *7*, 333–340. https://doi.org/10.1101/lm.34600.

54. Rossetti, T., Banerjee, S., Kim, C., Leubner, M., Lamar, C., Gupta, P., Lee, B., Neve, R., and Lisman, J. (2017). Memory Erasure Experiments Indicate a Critical Role of CaMKII in Memory Storage. Neuron *96*, 207–216.e2. https://doi.org/10.1016/j.neuron.2017.09.010.

55. Yamagata, Y., Kobayashi, S., Umeda, T., Inoue, A., Sakagami, H., Fukaya, M., Watanabe, M., Hatanaka, N., Totsuka, M., Yagi, T., et al. (2009). Kinase-dead knock-in mouse reveals an essential role of kinase activity of Ca2+/calmodulin-dependent protein kinase IIα in dendritic spine enlargement, long-term potentiation, and learning. J. Neurosci. *29*, 7607–7618. https://doi.org/10.1523/JNEUROSCI.0707-09.2009.

56. Sanhueza, M., and Lisman, J. (2013). The CaMKII/NMDAR complex as a molecular memory. Mol. Brain *6*, 10. https://doi.org/10.1186/1756-6606-6-10.

57. Wiedenmayer, C.P., Myers, M.M., Mayford, M., and Barr, G.A. (2000). Olfactory based spatial learning in neonatal mice and its dependence on CaMKII. Neuroreport *11*, 1051–1055. https://doi.org/10.1097/00001756-200004070-00030.

58. Genoux, D., Haditsch, U., Knobloch, M., Michalon, A., Storm, D., and Mansuy, I.M. (2002). Protein phosphatase 1 is a molecular constraint on learning and memory. Nature *418*, 970–975. https://doi.org/10.1038/nature00928.

59. Barros, D.M., Izquierdo, L.A., Sant'Anna, M.K., Quevedo, J., Medina, J.H., McGaugh, J.L., and Izquierdo, I. (1999). Stimulators of the cAMP cascade reverse amnesia induced by intra- amygdala but not intrahippocampal KN-62 administration. Neurobiol. Learn. Mem. *71*, 94–103. https://doi.org/10.1006/nlme.1998.3830.

60. Sajikumar, S., Navakkode, S., and Frey, J.U. (2007). Identification of compartment- and process-specific molecules required for "synaptic tagging" during long-term potentiation and long-term depression in hippocampal CA1. J. Neurosci. *27*, 5068–5080. https://doi.org/10.1523/JNEUROSCI.4940-06.2007.

61. Whitlock, J.R., Heynen, A.J., Shuler, M.G., and Bear, M.F. (2006). Learning induces long-term potentiation in the hippocampus. Science *313*, 1093–1097. https://doi.org/10.1126/science.1128134.

62. Redondo, R.L., Okuno, H., Spooner, P.A., Frenguelli, B.G., Bito, H., and Morris, R.G.M. (2010). Synaptic tagging and capture: Differential role of distinct calcium/calmodulin

kinases in protein synthesis-dependent long-term potentiation. J. Neurosci. *30*, 4981–4989. https://doi.org/10.1523/JNEUROSCI.3140-09.2010.

63. Chen, X., Hu, J., Jiang, L., Xu, S., Zheng, B., Wang, C., Zhang, J., Wei, X., Chang, L., and Wang, Q. (2014). Brilliant Blue G improves cognition in an animal model of Alzheimer's disease and inhibits amyloid-β-induced loss of filopodia and dendrite spines in hippocampal neurons. Neuroscience *279*, 94–101. https://doi.org/10.1016/j.neuroscience.2014.08.036.

64. Qin, S., Chen, Q., Wu, H., Liu, C., Hu, J., Zhang, D., and Xu, C. (2016). Effects of naringin on learning and memory dysfunction induced by gp120 in rats. Brain Res. Bull. *124*, 164–171. https://doi.org/10.1016/j.brainresbull.2016.04.020.

65. Li, X.-J., He, R.-F., Li, S., Li, X.-J., and Li, D.-L. (2012). Effects of progesterone on learning and memory and P2X7 receptor expression in the hippocampus after global cerebral ischemia/reperfusion injury in rats. Zhongguo Ying Yong Sheng Li Xue Za Zhi *28*, 472–475.

66. Das, S., Mishra, K.P., Ganju, L., and Singh, S.B. (2017). Andrographolide - A promising therapeutic agent, negatively regulates glial cell derived neurodegeneration of prefrontal cortex, hippocampus and working memory impairment. J. Neuroimmunol. *313*, 161–175. https://doi.org/10.1016/j.jneuroim.2017.11.003.

67. Vianna, M.R., Izquierdo, L.A., Barros, D.M., Medina, J.H., and Izquierdo, I. (1999). Intrahippocampal infusion of an inhibitor of protein kinase A separates short- from long-term memory. Behav. Pharmacol. *10*, 223–227. https://doi.org/10.1097/00008877-199903000-00011.

68. Izquierdo, L.A., Vianna, M., Barros, D.M., Mello E Souza, T., Ardenghi, P., Sant' Anna, M.K., Rodrigues, C., Medinam, J.H., and Izquierdo, I. (2000). Short- and long-term memory are differentially affected by metabolic inhibitors given into hippocampus and entorhinal cortex. Neurobiol. Learn. Mem. *73*, 141–149. https://doi.org/10.1006/nlme.1999.3925.

69. Quevedo, J., Vianna, M.R.M., Martins, M.R., Barichello, T., Medina, J.H., Roesler, R., and Izquierdo, I. (2004). Protein synthesis, PKA, and MAP kinase are differentially involved in short- and long-term memory in rats. Behav. Brain Res. *154*, 339–343. https://doi.org/10.1016/j.bbr.2004.03.001.

70. Furini, C.R.G., Myskiw, J.C., Schmidt, B.E., Marcondes, L.A., and Izquierdo, I. (2014). D1 and D5 dopamine receptors participate on the consolidation of two different memories. Behav. Brain Res. *271*, 212–217. https://doi.org/10.1016/j.bbr.2014.06.027.

71. Zhao, W.Q., Polya, G.M., Wang, B.H., Gibbs, M.E., Sedman, G.L., and Ng, K.T. (1995). Inhibitors of cAMP-Dependent Protein Kinase Impair Long-Term Memory Formation in Day-Old Chicks. Neurobiol. Learn. Mem. *64*, 106–118. https://doi.org/10.1006/nlme.1995.1049.

72. Bernabeu, R., Cammarota, M., Izquierdo, I., and Medina, J.H. (1997). Involvement of hippocampal AMPA glutamate receptor changes and the cAMP/protein kinase A/CREB-P signalling pathway in memory consolidation of an avoidance task in rats. Braz. J. Med. Biol. Res. *30*, 961–965. https://doi.org/10.1590/S0100-879X1997000800008.

73. Bernabeu, R., Bevilaqua, L., Ardenghi, P., Bromberg, E., Schmitz, P., Bianchin, M., Izquierdo, I., and Medina, J.H. (1997). Involvement of hippocampal cAMP/cAMP-dependent protein kinase signaling pathways in a late memory consolidation phase of aversively motivated learning in rats. Proc. Natl. Acad. Sci. USA *94*, 7041–7046. https://doi.org/10.1073/pnas.94.13.7041.

74. Sato, T., Tanaka, K.I., Ohnishi, Y., Teramoto, T., Irifune, M., and Nishikawa, T. (2004). Inhibitory effects of group II mGluR-related drugs on memory performance in mice. Physiol. Behav. *80*, 747–758. https://doi.org/10.1016/j.physbeh.2003.12.010.

75. Parsons, R.G., and Davis, M. (2012). A metaplasticity-like mechanism supports the selection of fear memories: Role of protein kinase A in the amygdala. J. Neurosci. *32*, 7843–7851. https://doi.org/10.1523/JNEUROSCI.0939-12.2012.

76. Naqib, F., Sossin, W.S., and Farah, C.A. (2012). Molecular determinants of the spacing effect. Neural Plast. *2012*, 581291. https://doi.org/10.1155/2012/581291.

77. Josselyn, S.A., Shi, C., Carlezon, W.A., Neve, R.L., Nestler, E.J., and Davis, M. (2001). Long-term memory is facilitated by cAMP response element-binding protein overexpression in the amygdala. J. Neurosci. *21*, 2404–2412. https://doi.org/10.1523/jneurosci.21-07-02404.2001.

78. Scharf, M.T., Woo, N.H., Lattal, K.M., Young, J.Z., Nguyen, P.V., and Abel, T. (2002). Protein synthesis is required for the enhancement of long-term potentiation and long-term memory by spaced training. J. Neurophysiol. *87*, 2770–2777. https://doi.org/10.1152/jn.2002.87.6.2770.

79. Banko, J.L., and Klann, E. (2008). Chapter 4 Cap-dependent translation initiation and memory. In Progress in Brain Research (Elsevier), pp. 59–80. https://doi.org/10.1016/S0079-6123(07)00004-0.

80. Gal-Ben-Ari, S., Kenney, J.W., Ounalla-Saad, H., Taha, E., David, O., Levitan, D., Gildish, I., Panja, D., Pai, B., Wibrand, K., et al. (2012). Consolidation and translation regulation. Learn. Mem. *19*, 410–422. https://doi.org/10.1101/lm.026849.112.

81. Davies, S.P., Reddy, H., Caivano, M., and Cohen, P. (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem. J. *351*, 95–105. https://doi.org/10.1042/0264-6021:3510095.

82. Lee, K. (2021). Epac: new emerging cAMP-binding protein. BMB Rep. *54*, 149–156. https://doi.org/10.5483/BMBRep.2021.54.3.233.

83. Wan, X., Torregrossa, M.M., Sanchez, H., Nairn, A.C., and Taylor, J.R. (2014). Activation of exchange protein activated by cAMP in the rat basolateral amygdala impairs reconsolidation of a memory associated with self-administered cocaine. PLoS One *9*, e107359. https://doi.org/10.1371/journal.pone.0107359.

84. Oliveira, S.L.B., Oliveira, M.G.M., and Hipolide, D.C. (2019). A1 adenosine receptors in the striatum play a role in the memory impairment caused by sleep deprivation through downregulation of the PKA pathway. Neurobiol. Learn. Mem. *160*, 91–97. https://doi.org/10.1016/j.nlm.2018.03.025.

85. Zhou, M., Luo, P., Lu, Y., Li, C.j., Wang, D.s., Lu, Q., Xu, X.l., He, Z., and Guo, L.j. (2015). Imbalance of HCN1 and HCN2 expression in hippocampal CA1 area impairs spatial learning and memory in rats with chronic morphine exposure. Prog. Neuro-Psychopharmacol. Biol. Psychiatry *56*, 207–214. https://doi.org/10.1016/j.pnpbp.2014.09.010.

86. Luo, P., Lu, Y., Li, C., Zhou, M., Chen, C., Lu, Q., Xu, X., He, Z., and Guo, L. (2015). Long-lasting spatial learning and memory impairments caused by chronic cerebral hypoperfusion associate with a dynamic change of HCN1/HCN2 expression in hippocampal CA1 region. Neurobiol. Learn. Mem. *123*, 72–83. https://doi.org/10.1016/j.nlm.2015.05.005.

87. Cissé, R.S., Krebs-Kraft, D.L., and Parent, M.B. (2008). Septal Infusions of the Hyperpolarization-Activated Cyclic Nucleotide-Gated Channel (HCN-Channel) Blocker ZD7288 Impair Spontaneous Alternation But Not Inhibitory Avoidance. Behav. Neurosci. *122*, 549–556. https://doi.org/10.1037/0735-7044.122.3.549.

88. Luo, P., Chen, C., Lu, Y., Fu, T., Lu, Q., Xu, X., Li, C., He, Z., and Guo, L. (2016). Baclofen ameliorates spatial working memory impairments induced by chronic cerebral hypoperfusion via up-regulation of HCN2 expression in the PFC in rats. Behav. Brain Res. *308*, 6–13. https://doi.org/10.1016/j.bbr.2016.04.020.

89. Wang, M., Ramos, B.P., Paspalas, C.D., Shu, Y., Simen, A., Duque, A., Vijayraghavan, S., Brennan, A., Dudley, A., Nou, E., et al. (2007). α2A-Adrenoceptors Strengthen Working Memory Networks by Inhibiting cAMP-HCN Channel Signaling in Prefrontal Cortex. Cell *129*, 397–410. https://doi.org/10.1016/j.cell.2007.03.015.

90. Paxinos, G., and Watson, C. (2007). The Rat Brain in Stereotaxic Coordinates, 6th edition (Academic Press).

91. Villar, M.E., Martinez, M.C., Lopes da Cunha, P., Ballarini, F., and Viola, H. (2017). Memory consolidation and expression of object recognition are susceptible to retroactive interference. Neurobiol. Learn. Mem. *138*, 198–205. https://doi.org/10.1016/J.NLM.2016.04.010.

92. Campos, R.C., Parfitt, G.M., Polese, C.E., Coutinho-Silva, R., Morrone, F.B., and Barros, D.M. (2014). Pharmacological blockage and P2X7 deletion hinder aversive memories: Reversion in an enriched environment. Neuroscience *280*, 220–230. https://doi.org/10.1016/J.NEUROSCIENCE.2014.09.017.

93. Bekinschtein, P., Cammarota, M., Katche, C., Slipczuk, L., Rossato, J.I., Goldin, A., Izquierdo, I., and Medina, J.H. (2008). BDNF is essential to promote persistence of long-term memory storage. Proc. Natl. Acad. Sci. USA *105*, 2711–2716.

94. Chai, N., Liu, J.F., Xue, Y.X., Yang, C., Yan, W., Wang, H.M., Luo, Y.X., Shi, H.S., Wang, J.S., Bao, Y.P., et al. (2014). Delayed noradrenergic activation in the dorsal hippocampus promotes the long-term persistence of extinguished fear. Neuropsychopharmacology *39*, 1933–1945. https://doi.org/10.1038/npp.2014.42.

95. Menezes, J., Alves, N., Borges, S., Roehrs, R., De Carvalho Myskiw, J., Furini, C.R.G., Izquierdo, I., and Mello-Carpes, P.B. (2015). Facilitation of fear extinction by novelty depends on dopamine acting on D1-subtype dopamine receptors in hippocampus. Proc. Natl. Acad. Sci. USA *112*, E1652–E1658. https://doi.org/10.1073/pnas.1502295112.

96. Vaynman, S., Ying, Z., and Gomez-Pinilla, F. (2007). The select action of hippocampal calcium calmodulin protein kinase II in mediating exercise-enhanced cognitive function. Neuroscience *144*, 825–833. https://doi.org/10.1016/j.neuroscience.2006.10.005.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Emetine dihydrochloride (EME)	Sigma	Cat# E2375
U0126 monoethanolate (U0126)	Sigma	Cat# U120
KN-62 (KN62)	Sigma	Cat# I2142
Rp-cAMPS triethylammonium salt (Rp-cAMP)	Sigma	Cat# A165
A-740003	MedChemExpress	Cat# HY-50697
Meloxicam 15 mg/1.5 ml	Roemmers	Cat# 436221-1
Gentamicin 40 mg/1 ml	Laboratorios Fabra	Cat# Gentamicina Fabra
Ketamine 50 mg/ml	Holliday	Cat# KETAMINA 50
Xylazine 100 mg/ml	König	Cat# sedomin
Deposited data		
Repository data	This paper	Mendeley Data: https://doi.org/10.17632/yrh5fs68r2.1
Software and algorithms		
GraphPadPrism 9.0.1	GraphPad Software	RRID: SCR_002798; www.graphpad.com

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Haydee Viola (hviola@fmed.uba.ar).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data have been deposited at Mendeley Data: https://doi.org/10.17632/yrh5fs68r2.1 and are publicly available as of the date of publication. DOIs are listed in the key resources table.
- This paper does not report original codes.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Experimental animals

550 Male adult Wistar rats between 2 and 3 months of age (weight, 250–400 g) obtained from the Faculty of Exact and Natural Sciences and the Faculty of Medicine of the University of Buenos Aires (Buenos Aires, Argentina) were used in this study. Animals were housed in groups of three *per* cage, with water and food *ad libitum* under a 12 h light/dark cycle with lights-on at 07:00 A.M. and a controlled temperature of 21°–23°C. Behavioral procedures took place during the light phase of the cycle. To avoid emotional stress, all animals were handled during 2 min for two consecutive days before each experiment. During behavioral procedures, the rats were individually moved from their housing cages to the arena and returned immediately after each trial session. All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, USA) and were approved by the Animal Care and Use Committee of the University of Buenos Aires (CICUAL), Buenos Aires, Argentina.

METHOD DETAILS

Surgery and drug infusion

For hippocampal administration of drugs or vehicle during the experimental procedures, a cranial cannulae implantation was performed. For this surgery procedure, the rats were deeply anesthetized (70 mg/kg ketamine and 7 mg/kg xylazine) and received a subdermal application of analgesic (meloxicam, 0.2 mg/kg) and antibiotic (gentamicin, 3 mg/kg). Then, bilateral 22-G cannulae were stereotaxically aimed at the CA1 region of the dorsal hippocampus at coordinates from Bregma A: −3.9 mm, L: ±3.0 mm, and D: −3.0 mm (Paxinos and Watson[90]) and cemented to the skull with dental acrylic. Finally, a short removable needle was attached at the open end of implanted cannulae to prevent clogging. Then, the animals were returned to the housing cages to recover from surgery procedures for at least four days. During the experiments, drugs or vehicle, were infused using a 30-G needle with 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. Infusion needles were left in place for one additional minute after administration to minimize backflow. Histological examination of cannulae placement was performed after the end of the behavioral procedures by the infusion of 0.8 μl of 4% methylene blue in saline solution. Animals were decapitated 15 min after the hippocampal infusion of methylene blue and their brains were sliced to verify the infusion area.[91] Only data from rats with correct cannulae implants (95%) were included in statistical analyses.

Drugs

Emetine (EME, 50 μg in 1 μl saline solution per side) was used as protein synthesis inhibitor. 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 ERKs1/2. KN62 (3.4 μg in 0.8 μl 20% DMSO-saline/side) was used as inhibitor of kinases of the CaMK family. Rp-cAMP (0.5 μg in 0.8 μl saline per side) was infused to inhibit PKA. A-740003 10 μM (4.7 ng in 1 μl 10% DMSO-saline/side) was used to block selectively P2X7 purinergic receptors. We used this dose because it was the only one which had some incidence on fear conditioning-memory when administered prior to training.[92] The doses chosen for the other different antagonists were based on our previously published studies[31,34] and are similar to those used by other research groups that performed learning and memory experiments with local administration of drugs.[68,93–96] Drugs were purchased from Sigma (St. Louis, MO, USA) and MedChemTronica (MedChemExpress Europe, Sweden).

Behavioral procedures

The experiments were carried out following the sequence exposed in the results section. Experimental designs, groups and treatments are presented as schematic diagrams in the figures. The sample size calculation was based on previously published work using IA, SOR and novel OF tasks.[10,34] In all experiments, rats were randomly assigned to the control group or to an experimental group and a randomized order of examination was performed. All test sessions were performed in a blind manner with respect to the experimental groups, including the number of training sessions (one or two), task type (weak IA, weak SOR, novel OF) or task combination, different inter-trial intervals or pharmacological treatments (drug or vehicle).

Inhibitory avoidance task

To evaluate the memory performance of animals, we use the inhibitory avoidance (IA) task in all experimental groups. The IA protocol is based on aversive learning as animals learn to not step-down from a little platform to the apparatus floor (thus inhibiting their natural tendency to explore) as it is associated with the delivery of a mild electric shock during training sessions.[33] The IA apparatus consists of a 60 × 25 × 30 cm box with a 10 × 25 × 5 cm platform on the left end of a series of conductive metal bars, which constitute the floor of the box. In the training session, a rat was placed on the box platform, and once it descended to the floor and put its four paws on the bars, it received a foot shock with an intensity of 0.3 mA for 2 s. This mild shock is characteristic of weak IA training sessions and is unable to induce LTM *per se* if experienced once. Depending on the experiment, animals were exposed to a single or double weak IA training sessions spaced by different inter-trial intervals (ITIs). After each training session, animals returned to their home cage and, depending on the experimental design to which they were assigned, they were submitted or not to another identical session. They were tested 24 h after training for LTM. During the memory test, each animal was placed again on the platform and was free to explore the IA apparatus; no foot shock

was delivered when the animal stepped-down from platform. A test latency time longer than that recorded during the training indicates the presence of memory.[6] In all cases, the latency to step-down from the platform was measured considering a ceiling time of 5 min.

Spatial Object Recognition task

To evaluate the effects of different, consecutive learning tasks on memory formation, a weak training session of the Spatial Object Recognition task (SOR) was included before or after a weak IA training session. Importantly, a single weak SOR session does not induce SOR-LTM on its own.[10,31] The SOR arena was a 60 cm wide 40 cm long 50 cm high acrylic box displaying different visual clues on its lateral white walls. The floor was white, the front wall was transparent, and the back wall was hatched. For habituation to the SOR context, all subjects were allowed to explore daily the arena without objects during 20 min for two consecutive days before training. In the weak SOR training session, two identical plastic or glass objects were placed in two adjacent corners of the arena and the animals were free to explore them during 4 min. The exploration time for each object, defined as sniffing or contacts with the nose or forepaws, was measured using a hand stopwatch. Rats were excluded from the analysis when they explored one of the two objects for more than 65% of the total object-exploration time in a training session.

Open field task

To study the mechanisms underlying retraining with weak IA spaced sessions, a novel Open Field session (OF) was included 1 h before retraining session in the experiments in which kinases or protein synthesis were pharmacologically inhibited. The exposure to the OF was used as a novel event able to induce the synthesis of PRPs.[6] In the OF session, the animal was allowed to explore the novel spatial context of the arena during 5 min. 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 rearings and floor line crossings was recorded in 1-min blocks during 5 min under attenuated room lighting conditions.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis of behavioral data was performed using GraphPad Prism software (GraphPad Software, Inc.). Differences between groups were assessed using non-parametric Kruskal–Wallis tests followed by post-hoc Dunn's multiple comparisons given that the step-down latency was measured considering a ceiling time of 5 min (see above, 'inhibitory avoidance task'). Effects were considered statistically significant when $p < 0.05$. Step-down latency results are expressed as median $\pm$ IQR (interquartile range).