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Reconsolidation-induced memory persistence: Participation of late phase hippocampal ERK activation

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ABSTRACT

Persistence is an attribute of long-term memories (LTM) that has recently caught researcher's attention in search for mechanisms triggered by experience that assure memory perdurability. Up-to-date, scarce evidence of relationship between reconsolidation and persistence has been described. Here, we characterized hippocampal ERK participation in LTM reconsolidation and persistence using an inhibitory avoidance task (IA) at different time points. Intra-dorsal-hippocampal (dHIP) administration of an ERK inhibitor (PD098059, PD, 1.0 μg/hippocampus) 3 h after retrieval did not affect reconsolidation of a strong IA, when tested 24 h apart. However, the same manipulation impaired performance when animals were tested at 7 d, regardless of the training's strength; and being specific to memory reactivation. To the best of our knowledge, this is the first report showing that persistence might be triggered after memory reactivation involving an ERK/MAPK-dependent process.

1. Introduction

Memory consolidation has been traditionally seen as a unidirectional process involving the stabilization of recently labile acquired information into a memory trace (McGaugh, 1966, 2000), while nowadays memory formation is considered less static (Bartlett, 1932; Nader, Schafe, & LeDoux, 2000).

After consolidation is completed, memory becomes "inactive" and is no longer sensitive to disruptors. However, retrieval, the process of accessing to a stored internal representation, returns memory to an "active" state enabling memory updating (adding details to or modifying items from the stored information). The transition from "inactive" to "active" is referred to as "memory reactivation" and it is usually achieved upon presentation of a reminder (Lewis, 1979; Nader, Hardt, & Wang, 2005). The conversion from an active and labile to an inactive and restabilized state after memory reactivation has been given the name of memory reconsolidation (Przybyslawski, Roullet, & Sara, 1999; Przybyslawski & Sara, 1997). However, the reactivation/reconsolidation process is not ubiquitous and the retrieval not always leads to reactivation or to an "active state". It depends on several boundary conditions, such as strength of training (Boccia, Acosta, Blake, & Baratti, 2004; Suzuki et al., 2004); the age of memory (Boccia, Blake, Acosta, & Baratti, 2006; Milekic & Alberini, 2002) the structure of the reminder: duration of the CS (Pedreira & Maldonado, 2003), mismatch between what is expected and what actually happens (Pedreira, Pérez-Cuesta, & Maldonado, 2004) and prediction error (Exton-McGuinness, Lee, & Reichelt, 2015).

What makes memory to endure for a lifetime? It has been shown that delayed infusion of protein synthesis inhibitors after training does not affect memory consolidation (Bekinschtein et al., 2008). Assuming that molecular substrate of memory does not endure for a lifetime; protein turnover might represent a constraint for persistent long-term memory (p-LTM). Consequently, it was suggested that different waves of protein

Abbreviations: dHIP, dorsal hippocampus; ERK/MAPK, extracellular-signal regulated kinase/mitogen-activated protein kinase (also known as p42/p44MAPK); HFS, high footshock-trained animals; HIP, hippocampus; IA, inhibitory avoidance task; LFS, low footshock-trained animals; LTM, long-term memory; N, näive animals; NF-κB, Rel/Nuclear factor-κB; NR, non-reactivated animals; PD, PD098059 (a MEK1/2 inhibitor); pERK, phosphorylated ERK; p-LTM, persistent long-term memory; R, reactivated animals; SDS-PAGE, Sodium dodecyl-polyacrylamide gel electrophoresis; Sh, shocked animals; T1, test 1 session; T2, test 2 session; TR, training session; USh, unshocked animals; VEH, vehicle-injected animals.

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synthesis might underlie memory persistence (Bekinschtein et al., 2008, 2010). As well, epigenetic mechanisms during memory consolidation have been found to be required for memory persistence (Federman et al., 2013). However, molecular mechanisms involved in memory persistence after consolidation and/or reconsolidation are not clearly understood.

Memory consolidation, reconsolidation and persistence are protein synthesis-dependent and share some molecular pathways such as BDNF, IGF2, Arc/Arg3.1, among others, and involve almost the same brain areas, notwithstanding, at different and critical time-points (Bekinschtein et al., 2008; Bevilaqua, Medina, Izquierdo, & Cammarota, 2008; Dudai, 2012; Izquierdo et al., 2008; Medina, Bekinschtein, Cammarota, & Izquierdo, 2008). Although mechanistically these processes may seem very similar it is important to underlie that they are not identical (Alberini, Milekic, & Tronel, 2006; Lee, Everitt, & Thomas, 2004; Taubenfeld, Milekic, Monti, & Alberini, 2001; Tronson & Taylor, 2007).

The role of extracellular signal-regulated kinases/mitogen-activated protein kinases (ERK/MAPK) concerning mechanisms of long-term memory (LTM) consolidation and reconsolidation has been extensively studied. It has been shown that ERK/MAPK activation is necessary for memory consolidation but, its participation on memory reconsolidation is discussed depending on the model (Besnard, Caboche, & Laroche, 2013; Cestari, Costanzi, Castellano, & Rossi-Arnaud, 2006; Duvarci, Nader, & LeDoux, 2005; Kelly, Laroche, & Davis, 2003; Martijena & Molina, 2012; Miller & Marshall, 2005).

MAPKs might have specific roles depending on its cellular localization (nucleus and/or cytoplasm). In this sense nuclear ERK1/2 activation has been linked to CREB-regulated transcription during memory consolidation, and cytosolic ERK1/2-dependent translation has also been involved in memory formation processes. However, further studies are needed in order to delineate memory-related ERK differential functions and/or kinetics, depending on its subcellular localization. Consistent with previous findings, Krawczyk et al. (2015) provided evidence for a central role of ERK2 isoform and for a biphasic cytosolic ERK modulation during memory reconsolidation of an IA task in mice. Furthermore, intrahippocampal administration of an ERK inhibitor (PD098059, PD) immediately after a high footshock-memory reactivation impaired retention when tested at 24 h. However, in these experiments, PD administration 45 min after mild footshock-memory reactivation enhanced memory retention. The fact that ERK inhibition or activation (respectively) subserves and is sufficient for expression of memories elicited upon different stimuli's strength suggested that ERK might be a critical step in reactivation-dependent memory strengthening.

Here we aimed to further characterize ERK involvement in long-term memory stabilization for longer periods. For this purpose, we studied ERK activity after a memory reactivation of mild/high footshock training memory at different time points. We also administered an ERK inhibitor at different time points after reactivation and showed its participation in memory persistence. To the best of our knowledge, the possibility that persistence might be triggered after memory reactivation involving an ERK/MAPK-dependent process has not been tested yet.

2. Materials and methods

2.1. Experimental subjects

CF-1 male mice from our own breeding stock were used (age: 60–70 d; weight: 25–30 g). They were caged in groups of 8–10 and remained housed throughout the experimental procedures. The mice were kept in a climatized animal room (21–23 °C) maintained on a 12-h light/12-h dark cycle (lights on at 6:00 AM), with *ad libitum* access to dry food and tap water. Experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23/96) and local regulations. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Intra-dorsal-hippocampal (dHIP) Injections

Mice were prepared (Boccia et al., 2004, 2006, 2007) for the intra-dorsal-hippocampal injections of vehicle or drug solutions 48 h before training, so that a minimum of time was necessary for injection, which was administered under light ether anesthesia in a stereotaxic instrument. The preliminary surgery was also performed under ether anesthesia and consisted of an incision of the scalp. Two holes were drilled in the skull without perforating the brain, at the following stereotaxic coordinates AP: −1.50 mm posterior to bregma, L/R + 1.50 mm from the midsagital suture and DV: −2.2 mm from a flat skull surface (Franklin & Paxinos, 1997), in order to bilaterally infuse the drugs after recovery. The skull was covered with bone wax and the mouse was returned to its home cage. Injections lasted 90 s and were driven by hand through a 30-gauge blunt stainless steel needle attached to a 5 µl Hamilton syringe with PE-10 tubing. The volume of each dHIP infusion was 0.5 µl. The accuracy of intra-dorsal-hippocampal injections was determined by histological determination of the needle position on an animal-by-animal basis. For this purpose, the brains of injected animals were dissected, fixed in 4% paraformaldehyde/buffer phosphate saline, and stored in 30% sucrose. They were then cut into 200 µm coronal sections with a vibratome. The deepest position of the needle was superimposed on serial coronal maps (Franklin & Paxinos, 1997). Coronal sections containing the deepest reach of the needle were Nissl stained to estimate the damage produced during the procedure. Animals were excluded from the statistical analysis if the infusions caused excessive damage to the targeted structure or if the needle tips extended outside the target structure.

2.3. Drugs

Five milligrams of 2-(2-amino-3-methoxyphenyl)-4H-1-benzo pyran-4-one (PD098059, PD) (Sigma, USA) were dissolved in 250 µl of dimethyl sulfoxide (DMSO) (Sigma, USA) (20 g/l) and stored at −20 °C. The stock solution was diluted immediately before use in DMSO at the final concentration and delivered bilaterally into the hippocampus. Vehicle solution was 100% DMSO. No symptoms of toxicity were observed in mice infused either with vehicle or PD (Korzus, Rosenfeld, & Mayford, 2004; Krawczyk et al., 2015).

All other reagents were of analytical grade and obtained from local commercial sources.

2.4. Inhibitory avoidance task

Inhibitory avoidance (IA) behavior was studied in a one-trial learning, step-through type situation (Blake, Boccia, & Baratti, 2008; Boccia et al., 2004), which utilizes the natural preference of mice for a dark environment. The apparatus consists of a dark compartment (20 × 20 × 15 cm) with a stainless-steel grid floor and a small (5 × 5 cm) illuminated, elevated platform attached to its front center. The mice were not exposed to the dark compartment before the learning trial. During training (TR), each mouse was placed on the platform and received either a high footshock (HFS: 1.2 mA, 50 Hz, 1 s) or a low footshock (LFS: 0.8 mA, 5 Hz, 1 s) as it stepped into the dark compartment (Boccia et al., 2004, 2006).

At the times indicated for each experimental group, re-exposure and retention tests were performed. Each mouse was placed on the platform again and the step-through latency was recorded. The retention test was finished either when the mouse stepped into the dark compartment or failed to cross within 300 s (ceiling score). In the latter case the mouse was immediately removed from the platform and assigned a ceiling score. In the retention test session the footshock was omitted. The footshocks employed yielded median latencies to step through either at the ceiling or at intermediate times, respectively (Boccia et al., 2004).

Experiments were carried out in a blinded fashion with regard to drug treatments and started 2 h after lights' onset.

2.5. Cytosolic and nuclear protein extraction

Animals were sacrificed by cervical dislocation and hippocampi (HIP) were dissected. Cytosolic and nuclear protein extracts were obtained as described previously (Feld et al., 2014; Freudenthal et al., 1998; Krawczyk et al., 2015). Protein quantity in the samples was determined by Bradford method in triplicates.

2.6. Western blots

For ERK activation determination, 15 μg of protein were electrophoresed in 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) coupled to immunodetection using the antibodies detailed below, as described elsewhere (Krawczyk et al., 2015). Detection was made with Luminol chemiluminiscence kit (GE Healthcare) as described by the manufacturer, exposing the membranes in a Syngene G-Box chemiluminiscence detector. The relative optical density was estimated using NIH ImageJ 1.45s software. Replicates are indicated in figure legends.

2.7. Antibodies for immunoblot

The antibodies used for immunoblotting were total ERK (tERK, 1:1000; Cell Signaling Technologies cat. #9102) and phospho-ERK (pERK, 1:2500; Cell Signaling Technologies cat. #9101). Secondary IgG-HRP antibodies were purchased from Santa Cruz Biotechnologies (anti-rabbit, cat. #sc-2030). To account for small differences in total ERK protein amount, the phospho-ERK signal was normalized to the total ERK signal in each lane. Data for treated animals were normalized to the average value of the näive controls.

2.8. Data analysis

Behavioral data are expressed as median latencies to step-through (s) and interquartile ranges during the retention tests and were analyzed, when appropriate, with the nonparametric analysis of variance of Kruskal-Wallis. The differences between groups were estimated by individual Mann-Whitney *U* tests (two-tailed) (Siegel, 1956). In all cases, $p < 0.05$ values were considered significant. Animals showing latencies during TR higher than 30 s were excluded from the analysis. No animals were excluded considering the testing score.

For western blot analysis, specific bands were quantified with ImageJ software. Western blot data were analyzed by one way ANOVA and subsequent Newman-Keuls multiple comparisons test between each group and the corresponding *näive* group was used when needed. Data were expressed as mean relative optic density values for each group (each pERK band relative to the corresponding tERK band) ± standard deviations. Samples showing more than three standard deviations from the mean were excluded from the analysis (less than 2 samples in all cases).

3. Results

3.1. ERK1/2 activation during memory reconsolidation of a LFS-training IA task

In a previous paper we reported that pharmacological ERK inhibition 45 min after memory reactivation in mice trained with a LFS (0.8 mA, 1 s) is able to enhance memory retention at levels similar to those expressed by mice trained with a HFS (1.2 mA, 1 s) (Krawczyk et al., 2015). To further investigate ERK1/2 participation on memory reconsolidation under such different training conditions (LFS vs HFS) we performed the following experiments.

Six groups of mice were trained in the IA task. Three of them were trained with a LFS, and the remaining groups did not receive the US during training (Unshocked groups, USh). Forty-eight hours later memory was reactivated (T1) and mice were sacrificed 15, 45 or 180 min afterwards (pairs of Sh and USh groups). Additional *näive* groups (N) were included, one for each time point, as baseline controls for ERK level determinations.

Mice hippocampi were dissected and cytosolic and nuclear protein extracts were obtained as described in Experimental Procedures. Samples were analyzed by immunoblotting with antibodies raised against phospho-ERK1/2 (pERK) and total ERK1/2 (tERK) proteins, band densitometry was performed and ratio pERK/tERK was calculated (Fig. 1). For clarity purposes data were plotted in relation to the training condition used (Fig. 1A: LFS, B: HFS, C: USh).

Significant differences in cytosolic pERK2, but not pERK1 levels, were observed 15 min (pERK1: $F = 0.19$; $p = 0.826$; pERK2: $F = 6.45$; $p = 0.008$), 45 min (pERK1: $F = 1.05$; $p = 0.37$; pERK2: $F = 4.31$; $p = 0.029$) and 180 min (pERK1: $F = 2.93$; $p = 0.0806$; pERK2: $F = 36.88$; $p < 0.0001$) after T1 among groups (Fig. 1A). No changes in nuclear pERK1/2 were disclosed ($F = 1.35$, $p = 0.289$; supplementary Fig. 1A).

pERK2 levels in Sh mice were significantly increased compared to NV group at all time points ($q = 4.573$; $q = 3.37$; $q = 10.3$ for 15, 45 and 180 min, respectively; $p < 0.05$ in all cases).

3.2. ERK1/2 activation 180 min after memory reactivation of a strong memory

Previously, we found that ERK shows biphasic activation after memory reactivation occurring 48 h after training of an IA in mice trained with a high footshock (HFS) (Krawczyk et al., 2015). We reported that there is an increase in hippocampal pERK2 15 min after memory reactivation in mice either trained with a HFS or unshocked (Fig. 1B and C). Interestingly, hippocampal ERK2 phosphorylation is inhibited 45 min after retrieval only in mice receiving the footshock during training. No changes in nuclear ERK1/2 activation were observed (supplementary Fig. 1B and C). To further elucidate ERK1/2 pattern of activation after memory reactivation, we decided to search whether pERK return to basal levels after longer post-reactivation intervals.

For this purpose, one group was trained in the IA task with a high footshock (HFS: 1.2 mA, 1 s) and a control unshocked group was included. Forty-eight hours later memory was reactivated (T1) and mice were sacrificed 180 min afterwards (pairs of Sh and USh groups). One additional näive groups (N) was also included as baseline controls for ERK determinations. Mice hippocampi were dissected and samples were obtained and processed as described above. For a better understanding of the results, each behavioral group was represented in a different graph (Sh: Fig. 1B; USh: Fig. 1C).

Three hours after T1 (Fig. 1B), a significant difference in cytosolic ERK2 ($F = 49.93$; $p < 0.0001$) and ERK1 activation

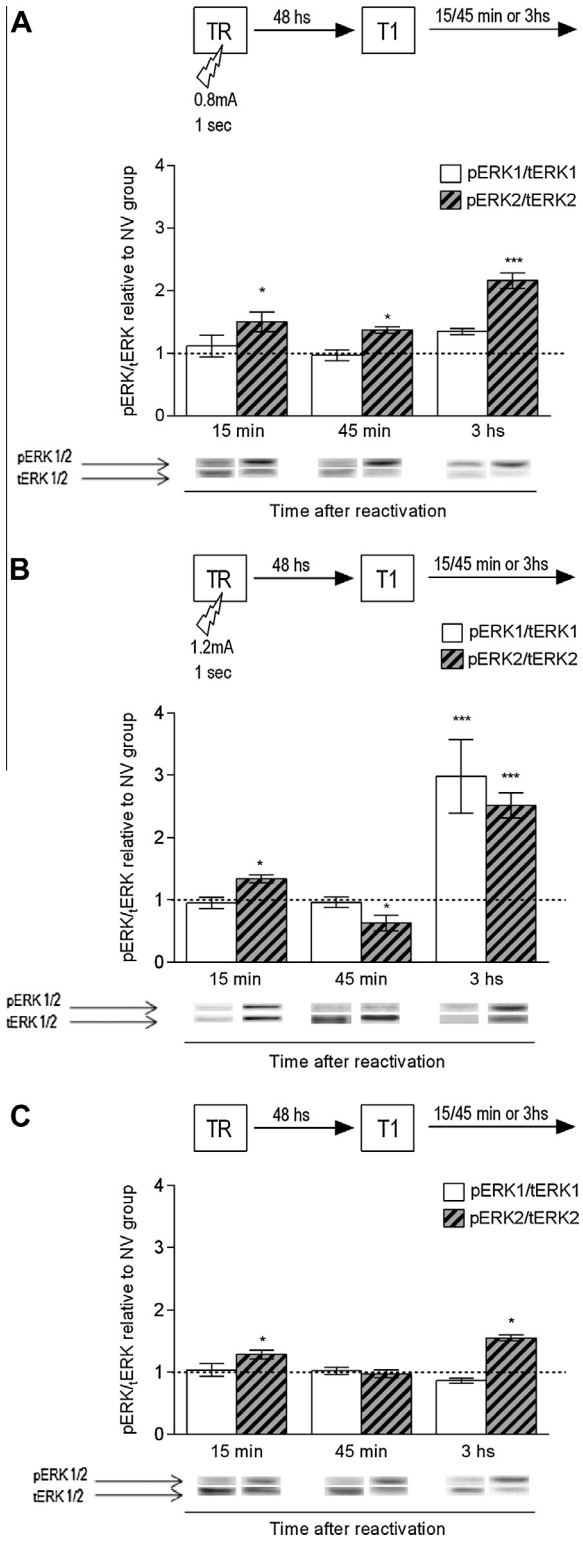

Fig. 1. Cytosolic ERK1/2 levels at different time points after the reactivation session in mice trained with a high, mild or no footshock. Cytosolic activity levels of hippocampal ERK1/2 at 15, 45 or 180 min after the reactivation session (T1). (A) Mice trained with a mild footshock, LFS (*Näive*: 15 min: n = 8; 45 min: n = 8; 180 min: n = 7). (B) Mice trained with a high footshock, HFS (*Näive*: 15 min: n = 7; 45 min: n = 7; 180 min: n = 8). (C) Mice trained without any footshock, USh (*Näive*: 15 min: n = 15; 45 min: n = 14; 180 min: n = 8). Mean relative optic density ± SEM of pERK/ERK1/2 bands obtained with specific antibody in western blots. TR: training session, T1: retention test. Dotted lines, näive levels; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Newman-Keuls Multiple Comparisons Test). Behavioral protocol is represented above each graph.

($F = 5.205$; $p = 0.0172$) was observed. Cytosolic ERK1 and ERK2 activation levels from Sh animals were significantly different from N group (ERK1: $q = 4.533$, $p < 0.05$; ERK2: $q = 13.98$; $p < 0.01$). No differences among groups were observed either in nuclear ERK1 or ERK2 activation ($F = 2.39$ and $F = 1.7$; $p = 0.12$ and $p = 0.07$ for ERK1/2, respectively; supplementary Fig. 1B).

3.3. ERK1/2 activation after memory reactivation in unshock-trained mice

As was stated, unshock-trained mice were included in both experiments described above. For the sake of simplicity, we pooled the data and plotted a single graph (Fig. 1C).

Significant differences in cytosolic pERK1 ($F = 11.05$; $p < 0.0001$) and pERK2 ($F = 12.39$; $p < 0.0001$) were observed among groups. ERK2 phosphorylation levels from USh mice were significantly higher than NV group 15 ($q = 5.34$, $p < 0.05$) and 180 min ($q = 3.07$, $p < 0.05$) after T1.

3.4. ERK2 activation 180 min post retrieval is specific to memory reactivation

We previously reported that pERK2/tERK2 in animals trained with a HFS 15 and 45 min is a specific event involved in the memory reconsolidation process, induced only when memory reactivation (T1) took place (Krawczyk et al., 2015). With that in mind, four groups of mice were trained in the IA task. Two of them were trained with a LFS and the other two with a HFS. Forty-eight hours later, only one group under each training conditions was subjected to T1 (R groups). The remaining groups were not tested for retention (NR groups). Three hours after retrieval, all groups (R and NR) were sacrificed. Two additional näive groups (N) were also included as baseline controls for ERK determinations. Mice hippocampi were dissected and samples were obtained and processed as described above (Fig. 2). Significant differences in cytosolic ERK1 and ERK2 activation were observed among groups under both training conditions ($F = 36.88$; $p < 0.0001$ for cytosolic ERK2 and $F = 5.948$; $p = 0.01$, for cytosolic pERK1 [LFS training]; $F = 35.08$; $p < 0.0001$ for cytosolic ERK2 and $F = 7.93$; $p = 0.0034$, for cytosolic pERK1 [HFS training]). Again, cytosolic ERK2 activation was significantly higher in R groups in both experiments ($q = 10.3$ and $q = 10.72$, compared with N and NR groups, respectively [LFS training]; $q = 11.48$ and $q = 8.63$, compared with N and NR groups, respectively [HFS training]; $p < 0.05$ in all cases), suggesting that ERK2 activation takes place specifically when memory is reactivated. ERK1 was only activated in the R in mice trained with the HFS ($q = 5.50$ and $q = 3.81$, compared with N and NR groups, respectively; $p < 0.05$ in all cases). At variance with R groups, ERK activation in NR groups did not differ statistically from N groups. No changes in ERK1/2 activation levels were found in the nuclear fraction (Suppl. Fig. 2).

3.5. Pharmacological ERK inhibition after retrieval alters memory persistence

We have already shown that intrahippocampal administration of an ERK phosphorylation inhibitor (PD098059, 1.0 μg/hippocampus) 3 h after memory reactivation in mice trained with a HFS did not affect performance on a subsequent test 24 h later (Krawczyk et al., 2015). This finding suggested that ERK participation on memory reconsolidation seems to be enclosed within a time-window shorter than 3 h.

To further analyze whether delayed (180 min post T1) infusions of PD098059 (PD) affect memory reconsolidation depending on training conditions, the following experiments were performed.

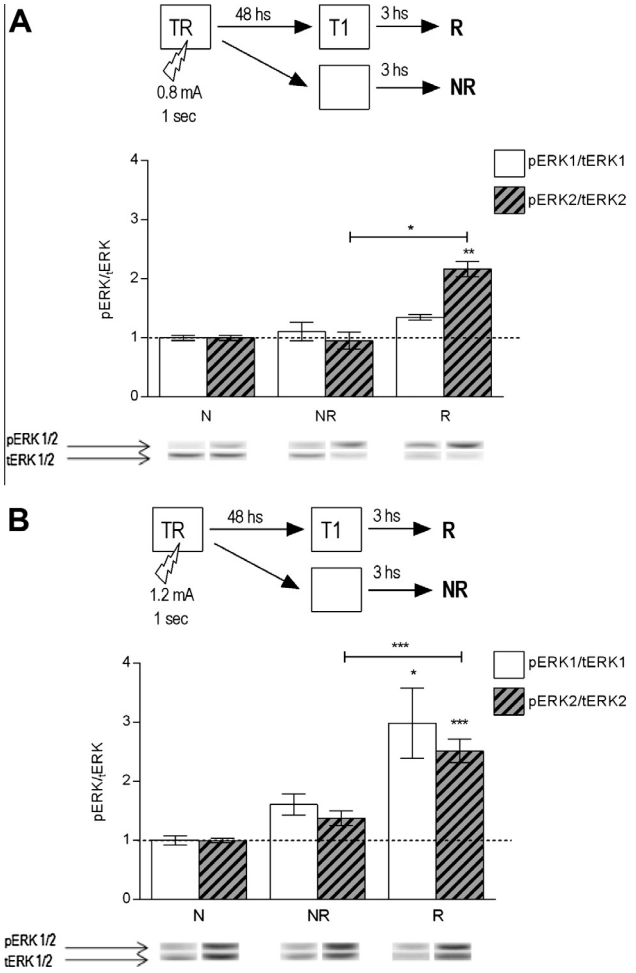

Fig. 2. ERK 1/2 activation only occurs after memory reactivation. Cytosolic activation levels of ERK1/2 180 min after memory reactivation in mice either trained with LFS (N, n = 7; NR, n = 6; R, n = 7) or HFS (N, n = 8; NR, n = 6; R, n = 8) in the presence (R) or absence (NR) of T1. Mean relative optic density ± SEM of pERK/ERK1/2 bands obtained with specific antibody in western blots. N: näive group, NR: non–reactivated group, R: reactivated group. * $p < 0.05$ (Newman-Keuls Multiple Comparisons Test).

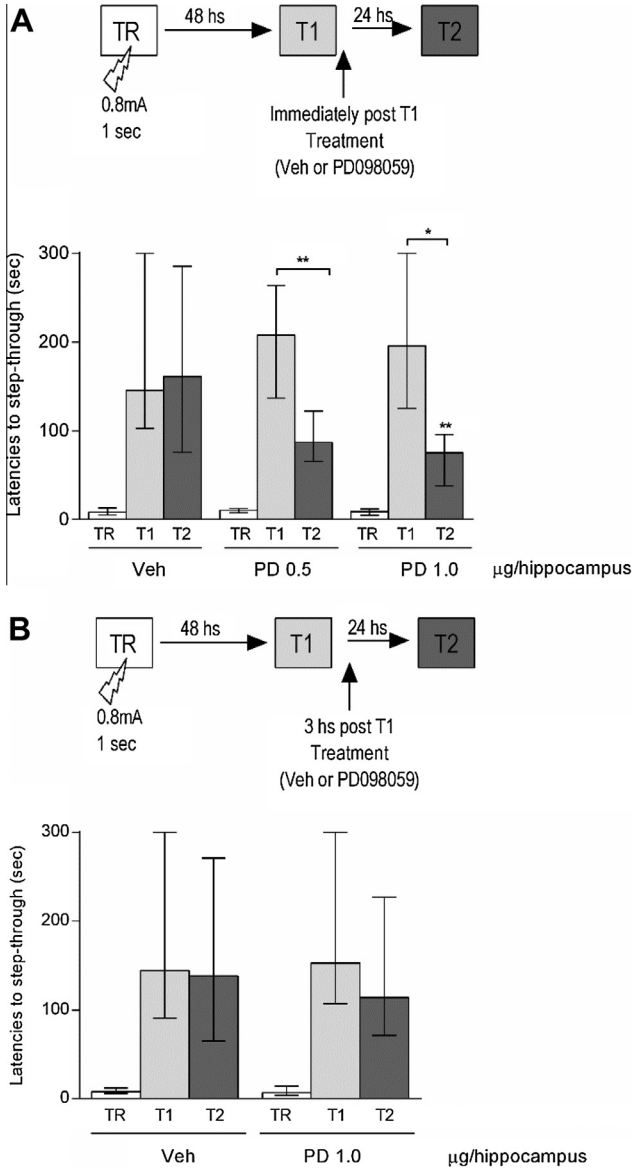

Fig. 3. PD098059 administered immediately after T1 impaired retention performance in mice trained with a mild footshock (LFS). (A) Effects of PD098059 administered immediately after T1 on retention performance of mice trained in the IA task. Vehicle (Veh) or PD098059 (PD 0.5 or 1.0 µg/hippocampus) were given immediately after T1. Each bar represents the median and interquartile range (n = 10 mice/group). TR: training session, T1–T2: retention tests. * $p < 0.05$; ** $p < 0.01$ (T2 vs T2 Veh; Mann–Whitney *U* test, two-tailed). (B) Effects of dHIP PD098059 infusion (1.0 µg/hippocampus) on retention performance when given 180 min after memory reactivation. Each bar represents the median and interquartile range (n = 8–10 mice/group). The behavioral protocol is represented above each graph.

First we evaluated whether PD has any effect on memory reconsolidation in mice trained with a mild footshock (LFS). For this purpose we trained three groups of mice receiving the LFS and 48 h later they were subject to a retention test. Immediately afterwards they received Veh or PD (0.5 or 1.0 µg/hippocampus). Twenty-four hours later mice were tested again, T2 (Fig. 3A) (T1 vs T2 comparison: Veh $p > 0.05$; PD 0.5 µg/hippocampus: $p < 0.05$; PD 1.0 µg/hippocampus: $p < 0.05$). However, when comparing T2 among all three groups, only the highest dose of PD shown retention impairment (T2 comparison: Veh vs PD 0.5 $p > 0.05$; Veh vs PD 1.0 $p < 0.05$).

In the next experiment, two groups of mice were trained with a LFS; 48 h later memory was reactivated (T1) and 180 min afterwards either Veh or PD (1.0 µg/hippocampus) were intrahippocampally infused. Twenty-four hours later mice were subjected to a second test, T2. In this case, PD did not affect performance in mice injected 180 min after the first reactivation session (T1) (Fig. 3B; T2: Veh vs PD; $p > 0.05$).

Since we observed higher cytosolic pERK2 levels in Sh groups 180 min after memory reactivation for both training conditions and PD administration was ineffective at this time point, we decided to test whether this activation observed in the Sh group was related to memory persistence. With this in mind, 4 groups of mice were trained as described above. Half of them were trained with a LFS and the remaining ones with a HFS. Forty-eight hours post training mice were tested (T1) and 180 min later they received either Veh of PD (1.0 µg/hippocampus). Seven days after T1 mice were subjected to a new retention test, T2 (Fig. 4A and B). The administration of PD 180 min after the first reactivation session (T1), impaired retention in both training conditions when tested 7 days apart from T1 (HFS T2: Veh vs PD, $p < 0.05$; LFS T2: Veh vs PD, $p < 0.01$).

In order to control whether long lasting memory effects of PD administered 180 min after T1 were specific to memory reactivation, we performed the following experiment: four groups of ten mice each were trained as already described. Two groups received a LFS while the remaining one received a HFS. Fifty-one hours after TR they received an intrahippocampal injection of either Veh or PD

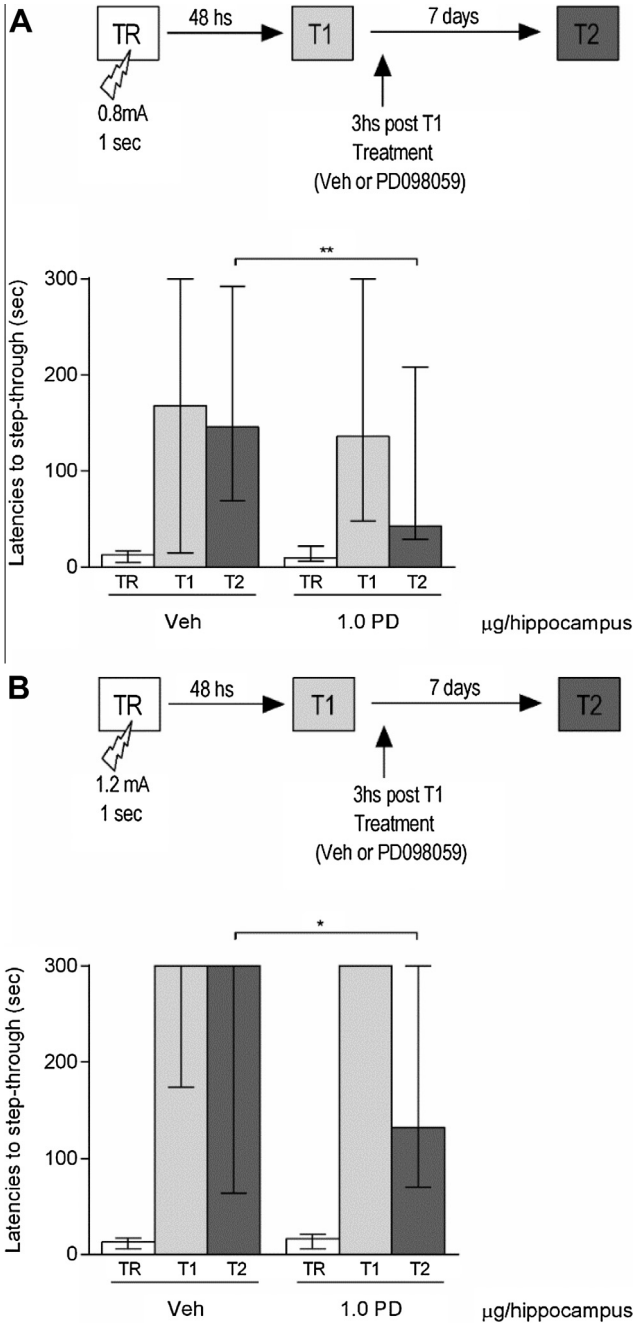

Fig. 4. Administration of PD098059 180 min post retrieval and evaluated 7 d afterwards impairs retention performance in both training conditions. Effects of PD098059 administered 180 min post T1 and tested 7 d later on retention performance in mice trained with a LFS (A) or a HFS (B) in an IA task. Vehicle (Veh) or PD098059 (1.0 µg/hippocampus) were given 180 min after T1. Each bar represents the median and interquartile range (n = 10 mice/group). The behavioral protocol is represented above the graph. TR: training session, T1–T2: retention tests. * $p < 0.05$; ** $p < 0.01$ (T2 vs T2 Veh; Mann–Whitney *U* test, two-tailed).

(1.0 µg/hippocampus) without being previously subjected to a retention test (that means, no T1 was experienced prior to drug administration). Seven days after the drug administration mice were tested for the first time (T1). The results are depicted in Fig. 5. PD infused 51 h after TR and in absence of memory reactivation, did not affect mice performance under both training conditions. These results suggest that memory reactivation is a necessary condition for the effects of retrieval-delayed infusions of PD on memory persistence and discard a non-specific effect of the drug.

4. Discussion

Here we report for the first time that persistence might be triggered after memory reactivation via ERK/MAPK-dependent process. We were also able to demonstrate a new time window of susceptibility to alter memory persistence induced by memory reactivation. To the best of our knowledge, reactivation-induced p-LTM susceptibility had not been described. Moreover, cytosolic ERK activation seems to be a critical molecular step involved in this process.

In the first set of experiments we described hippocampal cytosolic ERK kinetics after memory reactivation. We had previously shown a bidirectional cytosolic ERK regulation at 15 and 45 min after memory reactivation (activation and inhibition, respectively) in mice trained with a high footshock (Krawczyk et al., 2015). In the same report, using pharmacological tools, we demonstrated that PD infused 40 min after T1 in mice trained with LFS is able to enhance memory, achieving retention latencies similar to those expressed by mice trained with a HFS. In the present paper we observed that a "mild" training (e.g., performed with a LFS) induced ERK2 activation after T1 at all time points assayed. Thus, there is a critical difference in ERK2 activity 45 min after memory reactivation depending on the training condition (see Fig. 1A and B). ERK2 inhibition at 45 min after T1 seems to correlate with subsequent retention latencies. In other words, the lower the ERK2 activation the higher the retention latencies will be. How is ERK2 level of phosphorylation at 45 min after retrieval able to modulate behavioral expression? If ERK2 inhibition at 45 min after T1 was a specific and necessary event in memory reconsolidation of a strong memory, achieving a similar inhibition after reactivation of a weaker memory might induce a retention level similar to that of a stronger trace (Krawczyk et al., 2015). That is, enhancing ERK inhibition at 45 min might allow memory improvement. These results further support our previous work reinforcing our original hypothesis which stated that hippocampal ERK2 inhibition 45 min after retrieval (e.g. induced by HFS memory retrieval or pharmacologically imposed over a LFS memory retrieval) specifically subserve memory strengthening. Interestingly, ERK2 activation 15 min after T1 in mice not receiving a footshock during training was similar to those determined in mice trained either with HFS or LFS (higher than NV group), while at 45 min there was no difference from NV levels (see Fig. 1C).

We had previously demonstrated that PD administered immediately after memory reactivation was able to impair memory retention 24 h apart in mice trained with a HFS (Krawczyk et al., 2015). In the present paper we further explored whether ERK activation immediately after memory reactivation is necessary for memory reconsolidation in mice trained with LFS. PD administered immediately after T1 in mice trained with a LFS was able to impair memory retention 24 h after T1. Altogether, these results suggest that ERK activation after memory reactivation seems to be crucial for reconsolidation of an IA in mice under both training conditions.

We also widened ERK activation kinetics by studying its phosphorylation levels 3 h after T1. Surprisingly, we observed higher levels of cytosolic pERK2 in all training conditions (Fig. 1A–C). In particular, memory reactivation in mice trained with a LFS induced only ERK2 activation at 3 h, while in those trained with a HFS induced ERK1/2 activation. However, delayed intrahippocampal infusion of PD after T1 did not affect 24 h retention in mice trained either with a HFS (Krawczyk et al., 2015) or with a LFS (present results). Since in both training conditions delayed ERK inhibition was not able to affect memory reconsolidation tested 24 h apart, we proposed that ERK2 activation at this time point might support other memory processes.

It is worth pointing out that an increase in ERK2 activation was also disclosed at 15 min and 3 h in USh-trained animals. These

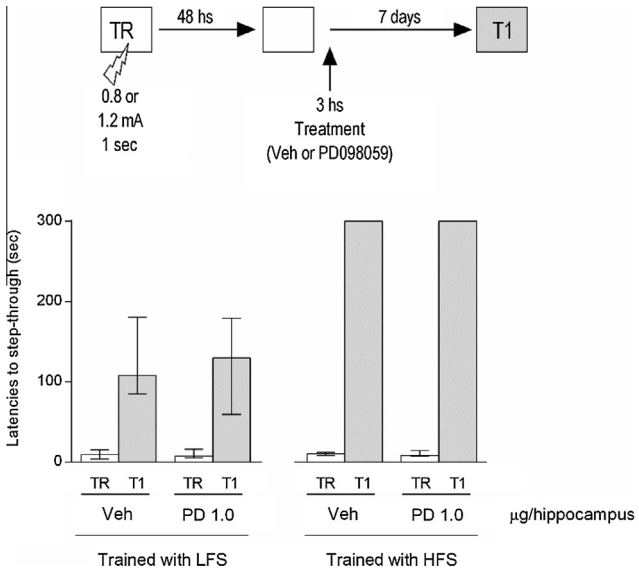

Fig. 5. Memory impairment by administration of PD098059 does not occur in the absence of memory reactivation. Four groups of animals were trained (TR) in the IA task, two of them with a mild footshock and the other two with a high footshock. Fifty-one hours later Vehicle (Veh) or PD098059 (1.0 µg/hippocampus) were given without retrieval session. Seventy-two hours after TR all groups were tested for the first time (T1). The behavioral protocol is represented above the graph. Each bar represents the median and interquartile range (n = 10 mice/group). TR: training session, T1: retention test.

findings suggest that these animals also formed a memory that can be reactivated by re-exposing them to the training context. This has already been observed when studying the role of NF-kappa B in consolidation (Freudenthal et al., 2005) and reconsolidation (Boccia et al., 2007), and deserves further clarifications.

It's important to highlight that we found ERK1/2 activation /inhibition specifically in the cytosol. The cytosolic ERK component has been proposed as an essential requirement for cellular proliferation to take place (Casar, Pinto, & Crespo, 2008). Moreover, β-arrestins facilitate GPCR-mediated ERK activation but inhibit ERK-dependent transcription by binding to phospho-ERK1/2, leading to its retention in the cytosol (Tohgo, Pierce, Choy, Lefkowitz, & Luttrell, 2002). Although a nuclear transcription-regulating role has traditionally been described for this pathway, in the last years a body of evidence has arisen that supports its extra-nuclear function at different levels. Several cytosolic ERK substrates have been identified (Mao & Wang, 2015), highlighting its physiological and morphological relevance in this subcellular localization (e.g. local synaptic function and morphology). A recent paper from our lab also described the synaptic dynamics of the NF-kappa B pathway in the hippocampus during memory consolidation (Salles et al., 2015), suggesting a cytosolic function for this transcription factor. In other reports, this technique has also allowed to describe nuclear ERK activation, especially involved in beta-amyloid-induced mnesic alterations (Feld, Galli, Piccini, & Romano, 2008; Feld et al., 2014). To our knowledge, an accurate description of the subcellular compartments where these pathways' activation and/or inhibition take place is missing in the field.

Originally, memories were thought as unidirectional processes (McGaugh, 2000). Whereas short-term memories (STM) were necessary for LTM, the latter would also be necessary for long lasting LTM (or persistent memories). However, there is evidence, although scarce, regarding the existence of independent mechanisms for LTM and STM (Emptage & Carew, 1993; Izquierdo, Medina, Vianna, Izquierdo, & Barros, 1999; Izquierdo et al., 2002). The first report supporting this proposal came from Carew's lab. They showed that short-term and long-term synaptic facilitation are induced by 5-HT in parallel in the sensory neurons from the mollusk *Aplysia* (Emptage & Carew, 1993). Moreover, several drugs affecting different molecular targets and infused in different brain regions were able to modulate differentially STM and LTM using an IA task in rats (Cammarota, Bevilaqua, Medina, & Izquierdo, 2007). Noteworthy, intrahippocampal PD impaired STM, leaving LTM intact, when infused immediately after training, while 180 min-delayed infusion impaired LTM without affecting STM (Walz et al., 1999). Thus, STM cannot be assumed as a necessary condition for LTM to occur, and independent and parallel molecular processes might be responsible for the behavioral output (Izquierdo et al., 2002).

Similarly, the same could be applied to memory persistence. There is no clear definition or distinction among memory consolidation and persistence. However it was proposed that two types of memory consolidation might exist, one fast and dependent on early molecular and cellular events (synaptic or cellular consolidation) and a slower one involving the interaction among medial temporal lobes and neocortical structures (systems consolidation, for a review see Squire, Genzel, Wixted, & Morris, 2015). The precise time window for each one has not been determined yet and there has not been a complete agreement either. Once a memory is consolidated it might persist for days, months or years (McGaugh, 2000). It is known that several factors are able to modulate memory strength (degree of training, arousal, massed vs spaced training, etc.) (Gold & Van Buskirk, 1975; Hermitte, Pedreira, Tomsic, & Maldonado, 1999). Less is known about the molecular and cellular events that mediates long lasting memories or their persistence. It has been found that epigenetic mechanisms, like histone acetylation, are involved in the determination of the strength and persistence of memory (Federman, Fustiñana, & Romano, 2009; Federman et al., 2013). In addition, it was reported that activation of dopaminergic cells in the ventral tegmental area (VTA) immediately and 12 h after training is critically involved in memory persistence (Rossato, Bevilaqua, Izquierdo, Medina, & Cammarota, 2009). Moreover, it was postulated that upon dopaminergic activation of VTA, downstream hippocampal BDNF production 12 h after training is a determinant molecular step for long lasting-LTM (Bekinschtein et al., 2008). Interestingly, BDNF-induced persistence is ERK-dependent, since ERK inhibitor administration 15 min before BDNF infusions (12 h after TR) prevented its effects on long lasting memories (Bekinschtein et al., 2008). It was also shown that, since ERK activity follows circadian oscillation, its pharmacological and physiological interference after memory consolidation impairs its persistence. This suggests that different rounds of ERK activation in the hippocampus might be able to explain, at least in part, how memory persists (Eckel-Mahan et al., 2008).

To the best of our knowledge, there is only one report suggesting that memory persistence might be induced after memory reactivation. In this work, authors showed that late Arc expression (at 12 h) is essential for the persistence of newly-acquired and reactivated memories (Nakayama, Hashikawa-Yamasaki, Ikegaya, Matsuki, & Nomura, 2016). Here we show that although ERK pharmacological inhibition 180 min after retrieval did not affect performance at 24 h (Krawczyk et al., 2015), it impaired memory when tested at 7 days (Fig. 4). This effect was independent of the training protocol applied (e.g., LFS or HFS), suggesting that this would be a general mechanism for IA memory persistence in mice. At this point one might ask whether this effect was due to lingering consolidation process triggered by a learning experience. In this sense the results depicted in Fig. 2 shows that delayed ERK activation occurred only when memory was reactivated. Moreover, we pharmacologically interfered with ERK pathway at the same time point after training (51 h), without memory reactivation (Fig. 5), and there was no

effect on mice performance. These experiments confirmed that PD effect is specific to processes triggered and initiated by reactivation that would determine memory persistence. In any case, 51 h after TR there would be no consolidation-induced persistence-related hippocampal ERK1/2 activation, and retrieval would be necessary to elicit memory persistence mechanisms sensitive to ERK inhibition.

Although several evidences suggest that brain areas different from hippocampus (cortices) are involved in systems consolidation (Izquierdo & Murray, 2004; Izquierdo et al., 2002), this structure has also been implicated in memory reconsolidation and consolidation-induced persistence (Bekinschtein et al., 2008; Federman et al., 2013; Katche, Cammarota, & Medina, 2013). Notwithstanding this, our results highlight a hippocampal role on reactivation-induced persistence, independent from consolidation.

Amygdalar PKA activation was involved in memory reconsolidation. On the one hand, PKA activation was able to enhance memory reconsolidation and, on the other hand, its inhibition impaired it (Tronson, Wiseman, Olausson, & Taylor, 2006). That paper and other by Frenkel et al., were the first to report memory reconsolidation enhancement (Frenkel, Maldonado, & Delorenzi, 2005). Moreover, the latter highlights the fact that memory strengthening might be triggered by a real-life episode during reconsolidation. We propose that persistence modulation might be induced by real-life memory reactivation events. Finally, it was also proposed in humans that memory reconsolidation is able either to impair or to enhance a declarative memory (Forcato, Fernandez, & Pedreira, 2013; Forcato et al., 2007).

The involvement of ERK in memory reconsolidation has been studied by several groups, although it still remains elusive (Besnard et al., 2013; Cestari et al., 2006; Duvarci et al., 2005; Kelly et al., 2003; Martijena & Molina, 2012; Miller & Marshall, 2005). Moreover, this pathway was shown to play a role in memory mechanisms from animal models evolutionarily distant from mammals. In the mollusk *Aplysia*, not only was MAPK-mediated neuronal plasticity demonstrated (Bailey et al., 1997; Martin et al., 1997), but it was also assigned a role establishing a molecular context permissive for LTM formation (Philips, Ye, Kopec, & Carew, 2013). Additionally, it was also involved in one-trial conditioning in the mollusk *Hermissenda* (another well-known model) (Crow, Xue-Bian, Siddiqi, Kang, & Neary, 1998). Finally, memory consolidation in the crab *Neohelice granulata* also depends on ERK activation (Feld, Dimant, Delorenzi, Coso, & Romano, 2005). In this crustacean model, beta-amyloid fibrils administration induced nuclear ERK activation 1 h after training (not observed after saline administration) and memory impairment at 24 h (Feld et al., 2008). Recently we reported a causal relationship between medial prefrontal cortex (mPFC) ERK1/2 pathway deregulation and memory deficits in 3xTg, a mouse model for Alzheimer's disease (Feld et al., 2014).

MAPK activation has different temporal phases triggered by learning experiences. In this sense, it was recently reported that different growth factors might be responsible, at least in part, for these phases (Kopec, Philips, & Carew, 2015). Inter-trial MAPK activation is required for more than one training trial LTM formation (Philips, Tzvetkova, & Carew, 2007; Philips et al., 2013). Nevertheless, it is not well understood what these phases stand for, or their functions.

In the present paper we provide the first evidence regarding reactivation/reconsolidation – induced memory persistence. Moreover, we demonstrate that ERK1/2 pathway is critically involved in these processes. However its functional significance and up-stream signaling responsible for its activation remains an open question. These results support the idea that hippocampal ERK activation may constitute an important event in long-term memory persistence.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.nlm.2016.06.013.

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