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MEMORY RECONSOLIDATION OF AN INHIBITORY AVOIDANCE TASK IN MICE INVOLVES CYTOSOLIC ERK2 BIDIRECTIONAL MODULATION

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Abstract—Reconsolidation has been defined as the process of memory stabilization after retrieval involving, among others, gene expression regulation and post-translational modifications. Many of these mechanisms are shared with memory consolidation. Here, we studied hippocampal ERK participation on memory reconsolidation of an inhibitory avoidance task in CF-1 mice. We found a retrieval-induced cytosolic ERK2 activation in the hippocampus (HIP) 15 min after memory reactivation, and an inhibition at 45 min. PD098059, a MEK1/2 (MAPK/ERK kinase) inhibitor, administered in the HIP immediately after retrieval impaired memory in a dose-dependent fashion. However, infusions of the highest dose of PD098059 performed 40 min after retrieval enhanced memory in mice trained with a weaker footshock. These results suggest for the first time that ERK2 is involved in memory reconsolidation in a biphasic fashion. Furthermore, the inhibition of ERK could either impair or enhance mice performance depending on ERK state of activation.

Key words: ERK, inhibitory avoidance, memory reconsolidation, MAPK, mice, PD098059

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Abbreviations: AD, Alzheimer's disease; DG, dentate gyrus; dHIP, dorsal hippocampus; EDTA, ethylenediaminetetraacetic acid; ERK/MAPK, extracellular signal-regulated kinase/mitogen-activated protein kinase (also known as p42/p44MAPK); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HFS, high footshock-trained animals; HIP, hippocampus; IA, inhibitory avoidance; LFS, low footshock-trained animals; LTP, long-term potentiation; LTST, latencies to step through; mPFC, medial prefrontal cortex; N, naïve animals; NF-κB, Rel/nuclear factor-κB; NR, non-reactivated animals; PD, PD098059 (a MEK1/2 inhibitor); pERK, phosphorylated ERK; R, memory-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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INTRODUCTION

Memory consolidation refers to the underlying processes occurring after a learning situation where the information, initially labile, becomes progressively stabilized and strengthened (McGaugh, 1966, 2000). It was shown that memory consolidation is dependent on gene transcription (Alberini, 2009), protein synthesis (Flexner et al., 1963; Davis and Squire, 1984), post-translational modifications (Kandel and Schwartz, 1982), and several neurotransmitters, hormones and peptides are also able to modulate this process (McGaugh and Izquierdo, 2000).

In 1968, Misanin (Misanin et al., 1968) posited that reactivation renders memory susceptible to electroconvulsive shock as it is during memory consolidation. Controversially, a year later, Dawson and McGaugh were not able to replicate Misanin's results (Dawson and McGaugh, 1969), but the raised controversy was shortly after (''paradoxically'' in the memory field) forgotten. Many years later, Ledoux's group reported that intra-amygdala infusion of a protein synthesis inhibitor immediately after memory reactivation of a cued fear conditioning task results in a poor performance of experimental subjects on subsequent tests (Nader et al., 2000), suggesting, similarly to what Misanin had shown, that memory can become labile again when reactivated. This new period of sensitivity was named ''reconsolidation'' (Przybyslawski et al., 1999). In addition, other studies failed to report that post-retrieval treatment affects subsequent retention using a variety of training conditions and amnesic agents (Dawson and McGaugh, 1969; Squire et al., 1976; Lattal and Abel, 2001; Taubenfeld et al., 2001; Vianna et al., 2001; Power et al., 2006).

Reconsolidation hypothesis posits that retrieval-induced reactivation can render memory labile and susceptible to disruption again (Nader et al., 2000; Sara, 2000; Dudai and Eisenberg, 2004). Similar to consolidation, it involves gene expression regulation and post-translational modifications. However, a new debate has arisen regarding whether memory consolidation and reconsolidation share common mechanisms, brain areas or have, in terms of molecular pathways, similar activation kinetics profiles (Alberini, 2005; Blake et al., 2014). Nowadays the functional role, as well as the biological significance and the evolutionary relevance of memory reconsolidation are hot and debatable topics (Forcato et al., 2014).

There are also several boundary conditions regarding memory reconsolidation: strength of the US used during training (Boccia et al., 2004; Suzuki et al., 2004), the

age of the memory (Milekic and Alberini, 2002; Boccia et al., 2006), the structure of the reminder: duration of the CS (Pedreira and Maldonado, 2003), mismatch between what is expected and what actually happens (Pedreira et al., 2004) and prediction error (Exton-McGuinness et al., 2014).

Inhibitory avoidance (IA) task has proven to be effective in order to study memory consolidation and reconsolidation. One of its main advantages resides in the short time required for training (e.g., 5–15 s), avoiding overlapping between acquisition and the initial stages of memory consolidation (Gold, 1986). In this task, reconsolidation has shown to be dependent on nuclear factor-κB (NF-κB) and cholinergic system (Boccia et al., 2007, 2010; Blake et al., 2012), and constrained by phosphodiesterase type 5 (Baratti et al., 2009; Boccia et al., 2011). Protein synthesis is necessary for IA memory consolidation, and it also seems to be a request for IA memory reconsolidation, depending on the brain area considered. In this sense, intra-hippocampal infusion of anisomycin does not impair the reconsolidation of IA memories (Taubenfeld et al., 2001; Power et al., 2006). However, Milekic et al. (2007) demonstrated that intra-amygdala anisomycin infusion impairs IA memory reconsolidation. Recently, Kida and coworkers (Fukushima et al., 2014) demonstrated that calcineurin, a proteasoma-dependent protein degradation and CREB-dependent gene expression are needed for reconsolidation-mediated memory enhancement. In this work, a protein synthesis inhibitor infused in the amygdala after retrieval not only blocked memory enhancement of the IA task, but also impaired memory reconsolidation; whereas protein synthesis inhibition in the hippocampus (HIP) or medial prefrontal cortex (mPFC) abolished only the retrieval-induced enhancement of the IA memory.

Several intracellular signaling cascades have been involved in learning and memory processes. Among them, extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) has been exhaustively studied in the last two decades. Activation of the MAPK cascade during long term potentiation (LTP)-inducing stimuli delivery is required for the NMDA-dependent induction of LTP (English and Sweatt, 1997). Moreover, its nuclear activation can be induced by as few as three to seven dendritic spinés potentiation in rat CA1 pyramidal neurons (Boggio et al., 2007; Zhai et al., 2013), suggesting signaling integration to some extent. In rat cortical neurons, activation of ERK in synaptic and somatic compartments has been shown to occur simultaneously and very rapidly (Boggio et al., 2007). These findings support that ERK plays an important role in the local modulation of synaptic function, and propose that it can have early and late actions both centrally in the cell nucleus and peripherally at synaptic contacts. However, evidence is lacking regarding ERK compartmentalization in memory processes.

It is worth pointing out that this pathway constitutes an evolutionarily conserved memory mechanism. A role for MAPK in *Aplysia* neuronal plasticity was prompted by Kandel's group (Bailey et al., 1997; Martin et al., 1997), and also in another invertebrate memory model, the crab *Neohelice granulata* (Feld et al., 2005).

ERK is involved in several downstream cascades of different receptor systems (Sweatt, 2004). Among them, NMDA- and nAChR-induced ERK activation are proposed to play a relevant role in mechanisms of neuronal plasticity, learning and memory (Dineley et al., 2001; Wang et al., 2001; Krapivinsky et al., 2003; Ivanov et al., 2006). Furthermore, amyloid-β peptide (Aβ), a major component of extracellular neuritic plaques in Alzheimer's disease (AD) (Selkoe, 2001), has been shown to activate different neural plasticity and memory-associated signaling pathways, such as ERK/MAPK and NF-κB, among others. Recently, we published that ERK overactivation in a mouse model of AD (3 × Tg) might account for the memory impairment in the novel object recognition task. Moreover, ERK inhibition was able to reverse the memory deficit observed (Feld et al., 2014).

Different authors highlighted a role of ERK in memory reconsolidation, although it still remains elusive (Kelly et al., 2003; Duvarci et al., 2005; Miller and Marshall, 2005; Cestari et al., 2006; Martijena and Molina, 2012; Besnard et al., 2013).

The aim of the present work was to study ERK1/2 activation in subcellular fractions of the HIP after memory reactivation of an IA task in mice. Moreover, pharmacological ERK inhibition was performed in order to establish the requirement for its activation in memory reconsolidation.

EXPERIMENTAL PROCEDURES

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 Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication N° 80-23/96) and local regulations. All efforts were made to minimize animal suffering and to reduce the number of animals used.

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 midsagittal suture and DV: −2.2 mm from a flat skull

surface (Franklin and 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 intrahippocampal 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 and Paxinos, 1997). Coronal sections containing the deepest reach of the needle were Nissl stained to estimate the damage produced during the procedure (Fig. 4D). 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.

Drugs

Five milligrams of 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one (PD098059) (Sigma, St. Louis, MO, USA) were dissolved in 250 μl of dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, 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 HIP. Vehicle solution was 100% DMSO. No symptoms of toxicity were observed in mice infused either with vehicle or PD (Korzus et al., 2004; Feld et al., 2014).

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

IA task

IA behavior was studied in a one-trial learning, step-through type situation (Boccia et al., 2004; Blake et al., 2008), 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 a footshock (1.2 mA, 50 Hz, 1 s; except when stated) as it stepped into the dark compartment. The footshock employed yielded median retention scores at the ceiling and was used in order to reduce the influence of extinction on retention performance (Boccia et al., 2004, 2006).

At the times indicated for each experimental group, the 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 score of 300 s (ceiling score). In the retention test session the footshock was omitted.

Experiments were carried out in a blinded fashion with regard to drug treatments.

Nuclear and cytosolic protein extraction

Animals were sacrificed by cervical dislocation and HIP were dissected. Cytosolic and nuclear protein extracts were obtained as described previously (Freudenthal et al., 1998). For cytosolic protein extracts, tissue was resuspended in 250 μl of buffer A (10 mM Hepes pH 7.9; 1.5 mM $MgCl_2$; 10 mM KCl; 1 mM DTT; 1 μg/ml Pepstatin A; 10 μg/ml Leupeptin; 0.5 mM PMSF; and 10 μg/ml Aprotinin) and homogenized with eight strokes in a Dounce homogenizer, type B pestle. The homogenate was centrifuged for 15 min at 1000 × *g*, and the supernatant (cytosolic fraction) was aliquoted and kept at −20 °C. For nuclear protein extracts, the pellet was re-suspended in an equal volume of buffer B (20 mM Hepes, pH 7.9; 1.2 M KCl; 1.5 mM $MgCl_2$; 0.4 mM EDTA; 0.5 mM DTT; 50% glycerol; 1 μg/ml Pepstatin A; 10 μg/ml Leupeptin; 0.5 mM PMSF; and 10 μg/ml Aprotinin) and incubated for 25 min at 0 °C. Finally, a centrifugation was performed for 15 min at 10,000 × *g*. The supernatant was aliquoted and kept at −80 °C until used. All the extraction protocol was performed at 4 °C. Nuclear presence and integrity were checked with trypan blue in an optic microscope. Protein quantity in the samples was determined by the Bradford method in triplicates.

Western blots

For ERK activation determination, 15 μg of protein were electrophoresed in 12.5% sodium dodecyl (SDS)–polyacrylamide gel electrophoresis (PAGE) at 150 V for 1:30 h and then electroblotted to PVDF membranes (BIO-RAD, Hercules, California, USA) at 100 V for 1 h. Immunodetection was performed using primary antibodies detailed below, at the corresponding dilutions, followed by IgG-HRP secondary antibody at 1/5000 dilution. The immunoblots with anti-pERK antibody were subsequently stripped using a harsh stripping protocol (2% SDS; 0.8% β-mercaptoethanol; 62.5 mM Tris–HCl pH 6.8 for 45 min at 50 °C, and tap water washings for 1–2 h), and reblocked with 5% non fat milk. Then, the blots were reprobed with anti-tERK1/2 antibody. Detection was made with Luminol chemiluminescence kit (Amersham General Electric, Pittsburgh, PA, USA) as described by the manufacturer, exposing the membranes in a FUJI chemiluminescence detector. The relative optical density was estimated using NIH ImageJ 1.45s software. Replicates are indicated in figure legends.

Antibodies for immunoblot

The antibodies used for immunoblotting included the following: total extracellular signal-regulated kinase (tERK, 1:1000; Cell Signaling Technologies cat.#9102, Danvers, MA, USA); phospho-ERK (pERK, 1:2500; Cell

Signaling Technologies cat.#9101, Danvers, MA, USA). Anti-actin antibody (1:2000; Santa Cruz Biotechnology cat.#sc-1616, Dallas, Texas, USA) and anti-βlamin antibody (1:2000; Santa Cruz Biotechnology cat.#sc-56143, Dallas, Texas, USA) were used as protein loading controls for cytosolic and nuclear extracts, respectively. Secondary IgG-HRP antibodies were purchased from Santa Cruz Biotechnologies (anti-rabbit, cat.#sc-2030; anti-mouse, cat.#sc-2005).

Data analysis

Behavioral data are expressed as median latencies (s) to step-through 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.

For Western blot analysis, specific bands were quantified with ImageJ software. Anti-actin and anti-βlamin antibodies incubation signals were used to eliminate those cytosolic and nuclear extracts where the variation on the intensity of the signal was over 10%. Western blot data were analyzed by a one way ANOVA. Subsequent Newman–Keuls multiple comparisons test 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.

RESULTS

ERK1/2 activation on memory reconsolidation of an IA task

For the first experiment two groups of eight mice each were used. Mice trained in IA as described in Experimental procedures. One group of mice received the footshock (Sh group) immediately after entering the dark compartment and the remaining group did not receive the footshock (USh group). Forty eight hours later mice were tested for retention (T1), and 24 h afterward a new retention test was performed (T2).

Results are depicted in Fig. 1. Latencies to step through (LTST) during training did not statistically differ between groups ($U = 24$, $p = 0.16$). Significantly different LTSTs were observed when comparing T1 between both groups. This difference was also disclosed when T2 was compared ($U = 0$, $p = 0.0004$ for T1 USh vs T1 Sh groups; $U = 0$, $p = 0.0004$ for T2 USh vs T2 Sh groups). There were no significant differences when comparing T1 vs T2 either in the Sh or USh group of mice ($U = 28.5$, $p = 0.10$ for T1 vs T2 Ush group; $U = 31.5$, $p = 1.00$ for T1 vs T2 Sh group). These results indicate the USh group, unlike the Sh group, behaves significantly different regarding the TR conditions.

To evaluate ERK1/2 participation in memory reconsolidation, four groups of seven to eight mice each were trained in the IA task. Two of them were Sh and the remaining groups were USh. Forty eight hours later

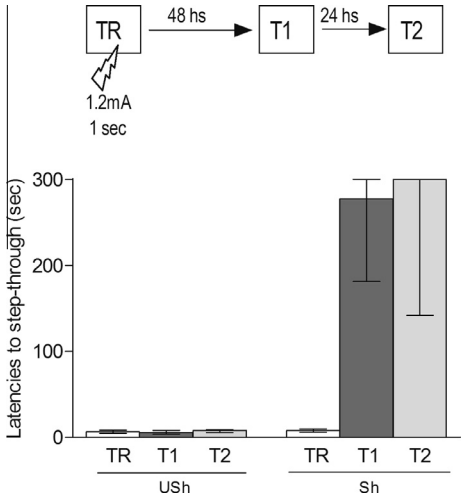

Fig. 1. Behavioral characterization of experimental groups in an inhibitory avoidance task. Animals were trained in the inhibitory avoidance task in the presence (Sh) or absence (USh) of a footshock (1.2 mA, 1 s). Representation of the behavioral protocol is depicted (top). Each bar represents the median and interquartile range ($n = 8$ mice/group). TR: training session, T1–2: retention tests, Sh: shocked group, Ush: unshocked group.

memory was reactivated (T1) and mice were sacrificed either 15 or 45 min later (pairs of Sh and USh groups). Two additional naïve groups (N) were included for each time point as baseline controls for ERK determinations. HIP from mice 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. 2).

Fifteen minutes after T1 (Fig. 2A.1), a significant difference in cytosolic ERK2 ($F = 7.93$; $p = 0.005$), but not ERK1 activation ($F = 0.005$; $p = 0.99$), was observed. Cytosolic ERK2 activation levels from both USh and Sh groups were significantly higher than the N group ($q = 3.14$ for USh and $q = 5.59$ Sh groups, $p < 0.05$ in both cases). No differences among groups were observed either in nuclear ERK1 or ERK2 activation ($F = 2.29$ and $F = 1.7$; $p = 0.12$ and $p = 0.2$ for ERK1/2, respectively) (Fig. 2A.2).

Forty-five minutes after T1 (Fig. 2B.1), a significant difference in cytosolic ERK2 activation ($F = 5.024$; $p = 0.023$) was also disclosed. In this case, a decrease was observed specifically in mice receiving the footshock during TR (Sh group) but not in those that did not receive the footshock (USh group) ($q = 3.975$ compared vs N, $p < 0.05$; and $q = 3.66$ compared vs USh, $p < 0.05$). Similarly to the previous experiment, neither cytosolic ERK1 ($F = 1.36$; $p = 0.28$, Fig. 2B.1) nor nuclear ERK1/2 activation showed significant differences among groups ($F = 2.61$ and $F = 0.24$; $p = 0.10$ and $p = 0.79$ for ERK1/2 respectively) (Fig. 2B.2).

Thus, ERK2 is activated 15 min after memory retrieval in Sh as well as USh groups and becomes inhibited 45 min after T1, particularly in the Sh group.

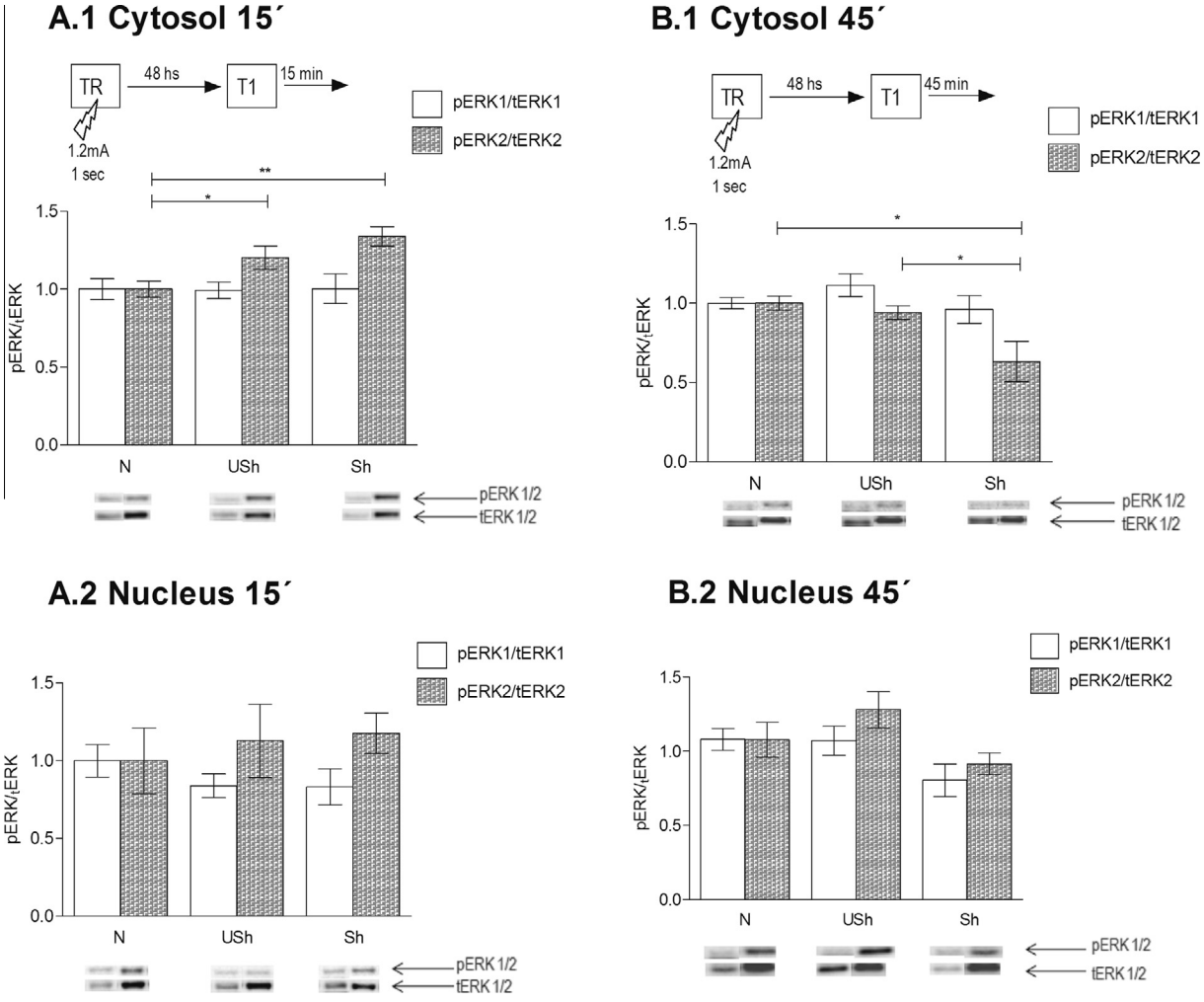

Fig. 2. Cytosolic and nuclear activation levels of ERK1/2 at different time points after the reactivation session. Cytosolic (A.1, B.1) and nuclear (A.2, B.2) activation levels of ERK1/2 from hippocampal protein samples obtained at 15 (A) and 45 min (B) after the reactivation session (T1). Mean relative optic density ± SEM of pERK/ERK1/2 bands obtained with specific antibody in Western blots. TR: training session, T1: retention test, N: naïve group, USh: unshocked group, Sh: shocked group. $^{*}p < 0.05$; $^{**}p < 0.01$ (Newman–Keuls Multiple Comparison Test). (A.1, A.2) Samples obtained 15 min post T1. N: $n = 8$; USh: $n = 7$; Sh: $n = 8$. (B.1, B.2) Samples obtained 45 min post T1. N: $n = 7$; USh: $n = 7$; Sh: $n = 8$.

Activation of ERK2 is specific to memory reconsolidation

Our next experiment was aimed to determine whether the cytosolic increase in pERK2/tERK2 is a specific event involved in the memory reconsolidation process, induced when memory reactivation (T1) took place. Thus, two groups of 10 mice each were trained in the IA task as described above, and 48 h later only one group was subjected to the reactivation test (T1). Seventy-two (T2) hours after TR both groups were tested (Fig. 3A). There were no differences in T2 LTSTs between reactivated (R) and non-reactivated (NR) mice ($U = 40$; $p = 0.17$), showing memory is not altered. In another independent experiment, cytosolic protein extracts from HIP obtained 15 min and 45 min after T1 (or equivalent time of the day for NR and N groups) were analyzed as previously described (Fig. 3B, C, respectively). Significant differences in cytosolic ERK2 activation (but not ERK1) were observed ($F = 4.98$; $p = 0.027$ for cytosolic ERK2; $F = 0.61$; $p = 0.56$, for cytosolic pERK1). In the group of mice where memory was reactivated, a significantly higher cytosolic ERK2 activation was observed ($q = 3.97$ and $q = 3.74$, compared with N and NR groups, respectively; $p < 0.05$ in both cases), suggesting that the ERK2 activation takes place specifically when memory is reactivated.

Post-reactivation administration of PD098059 impaired memory reconsolidation of an IA task

The following set of experiments was aimed at determining whether hippocampal ERK1/2 activation is necessary for memory reconsolidation. In the first experiment, four groups of 10 mice each were trained with a footshock in the IA task. The first retention test (T1) was performed 48 h after training and immediately after it, mice received a bilateral dHIP infusion of vehicle or PD098059 (0.1, 0.5 or 1 μg/HIP). Mice were tested again (T2) 24 h later. As shown in the Fig. 4A, PD098059, administered immediately after memory

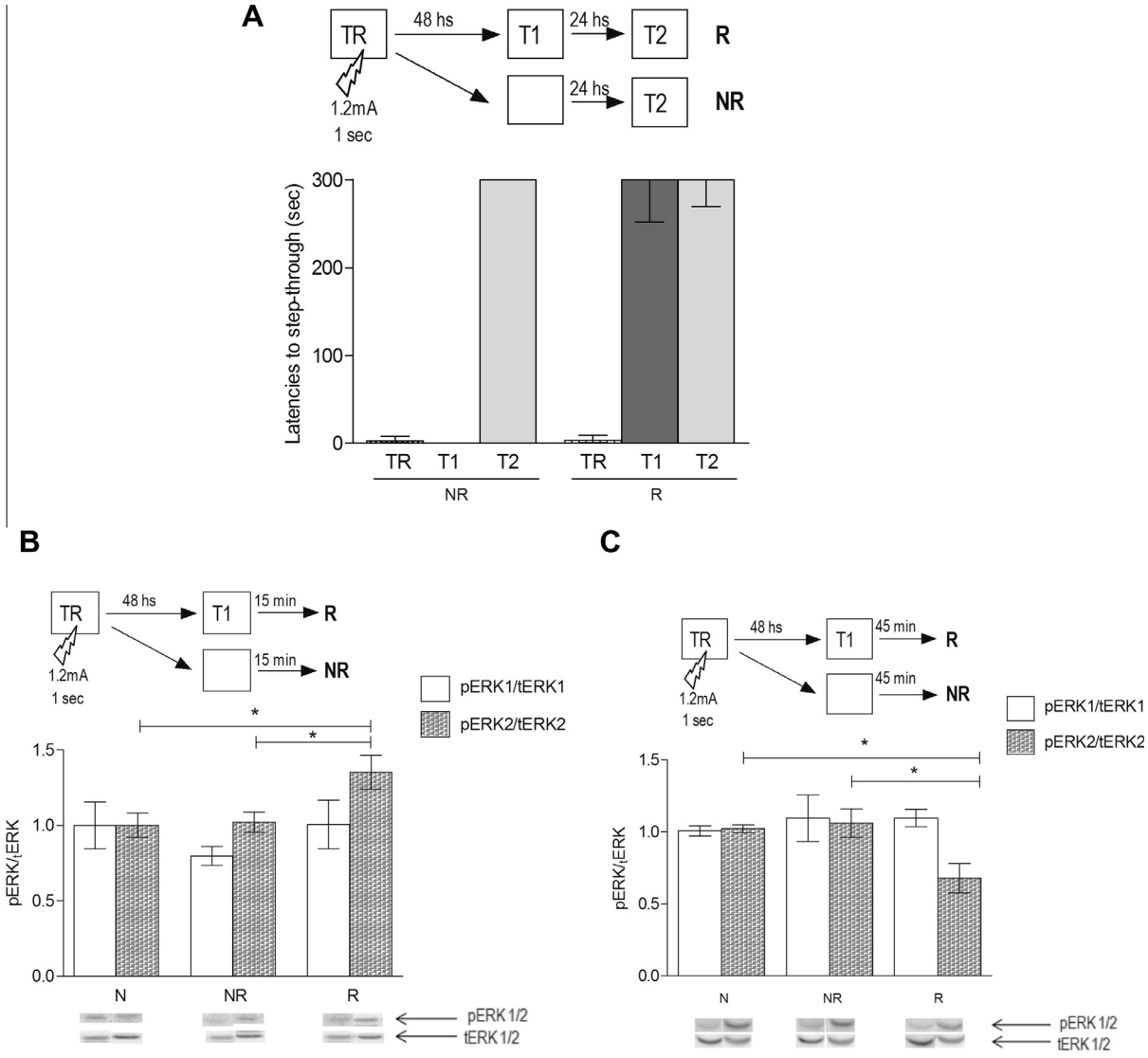

Fig. 3. Cytosolic activation of ERK2 only occurs after memory reactivation. (A) Two groups of animals were trained (TR) in the IA task. Forty-eight hours later only one of them (R group) was tested (memory reactivation, T1). Seventy-two hours after TR both groups were tested (T2). Each bar represents the median and interquartile range (n = 10 mice/group). TR: training session, T1–2: retention tests, R: reactivated group, NR: non-reactivated group. (B and C) Cytosolic activation levels of ERK1/2 fifteen (N, n = 7; NR, n = 6; R, n = 7) or forty-five (N, n = 8; NR, n = 6; R, n = 8) minutes 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: naïve group, NR: non-reactivated group, R: reactivated group. $^{*}p < 0.05$ (Newman–Keuls Multiple Comparison Test).

reactivation, significantly impaired retention latencies at T2 in a dose-dependent manner (H = 17.28; p = 0.006).

In order to answer if PD-induced memory impairment is specific to memory reactivation, two sets of experiments were carried out. In each of them, two Sh groups of mice were trained in the IA task. In the first one (Fig. 4B, left), forty eight hours later, mice received a bilateral dHIP infusion of either vehicle or PD (1 μg/HIP), but in this case T1 was omitted. The retention test (T2) was performed 72 h after TR. The administration of PD098059 48 h after TR without memory reactivation did not affect retention performance during T2, when compared to the Veh group (U = 19; p = 0.45).

In the other experiment (Fig. 4B, right), mice received a bilateral dHIP infusion of either vehicle or PD (1 μg/HIP) 180 min after T1, and T2 was performed 24 h later. PD098059 administered 3 h after T1, did not affect retention performance during T2 when compared to the Veh group (U = 31; p = 1).

Finally, to assess ERK activity after PD administration, two groups of mice were trained in the IA task (Sh groups). The first retention test (T1) was performed 48 h after it, and immediately after T1, mice received a bilateral dHIP infusion of vehicle (Sh Veh) or PD098059 (Sh PD, 1 μg/HIP). Fifteen minutes afterward, mice were sacrificed by cervical dislocation and HIP were dissected (Fig. 4C). Again, a naïve group (N) was included for basal ERK level determination. Cytosolic protein samples were analyzed by immunoblot as previously described.

There were differences in cytosolic ERK2 activation among groups (F = 13.65; p = 0.0006, Fig. 4C). As

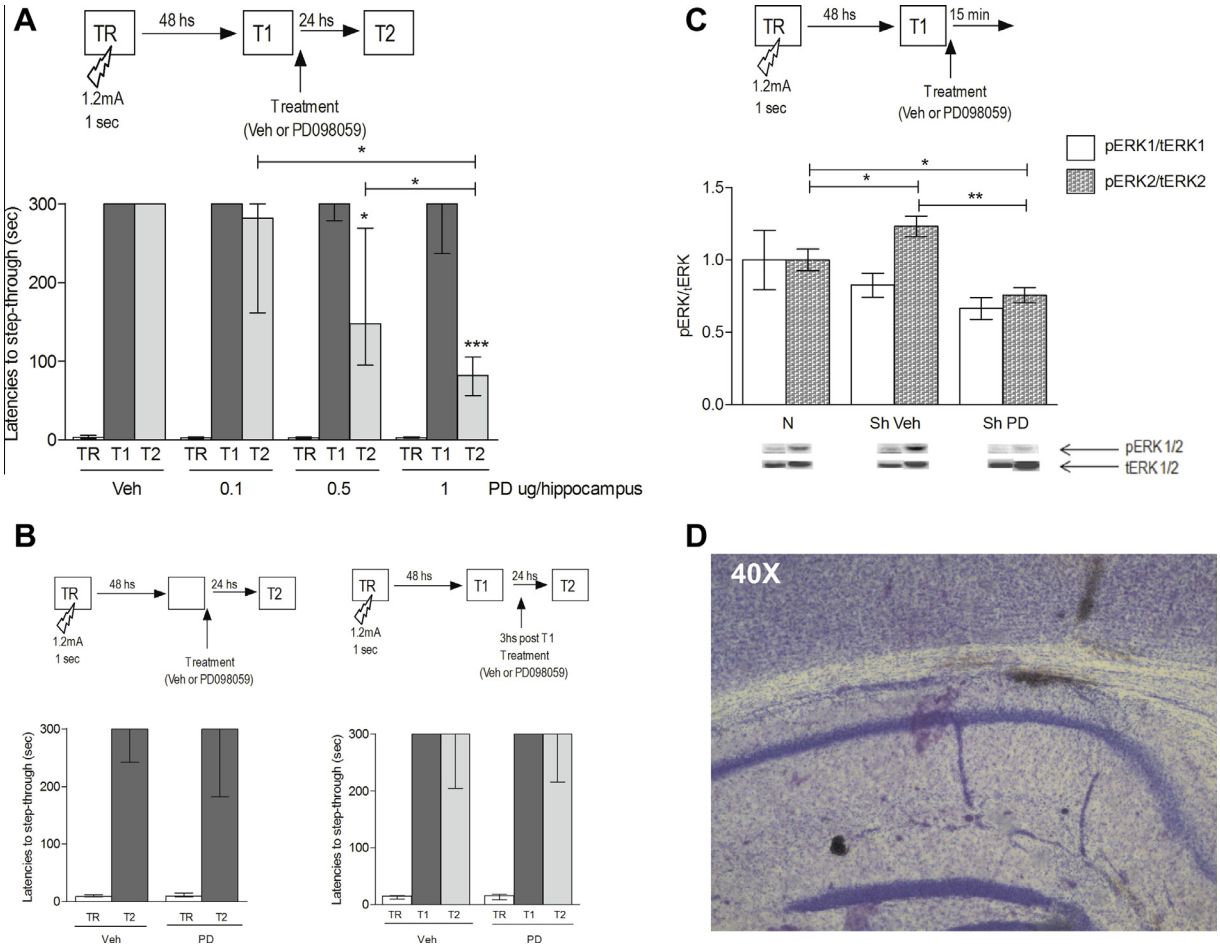

Fig. 4. Inhibition of ERK2 by PD098059 impairs memory reconsolidation. (A) Effects of PD098059 administered immediately after T1 on retention performance of mice trained in the IA task. Vehicle (Veh) or PD098059 (0.1, 0.5 or 1 μg/hippocampus) were given immediately after T1. The behavioral protocol is represented above the graph. Each bar represents the median and interquartile range (n = 10 mice/group). TR: training session, T1–2: retention tests. $^{*}p < 0.05$; $^{***}p < 0.001$ (T2 vs T2 Veh; Mann–Whitney U-test, two-tailed). (B) Effects of dHIP PD098059 infusion (1 μg/hippocampus) on retention performance when given 48 h after TR, without retrieval session (left) or 180 min after memory reactivation (right). The behavioral protocol is represented above each graph. Each bar represents the median and interquartile range (n = 8–10 mice/group). (C) Cytosolic activation levels of ERK1/2 in Naïve (N, n = 7) or trained mice (Sh Veh, Sh PD groups). Trained mice received a dHIP injection of vehicle (Veh; n = 7) or PD098059 (1 μg/hippocampus; n = 8) immediately after T1. Fifteen minutes after T1, animals were sacrificed and protein samples were obtained from hippocampi. Mean relative optic density ± SEM of pERK/ERK1/2 bands obtained with specific antibody in Western blots. $^{*}p < 0.05$; $^{**}p < 0.01$ (Newman–Keuls Multiple Comparisons Test). (D) Example of 200-μm coronal section showing the deepest reach of the needle (Cresyl Violet staining).

expected, ERK2 activation in the Sh Veh group was significantly higher than the N group (q = 3.45, $p < 0.05$). A 39% inhibition of cytosolic ERK2 relative to the Sh Veh group was disclosed in the Sh PD group (q = 3.78 vs N group, $p < 0.01$; Fig. 4C).

Although there was a tendency to a decrease in ERK1 activation levels compared to naïve mice, no significant differences were observed (F = 1.58; p = 0.24; Fig. 4C).

ERK2 inhibition is also specific to memory reconsolidation

We observed that memory retrieval induces a differential ERK2 activity profile. There is an activation at 15 min and an inhibition at 45 min. Blocking retrieval-induced ERK activation impairs memory, suggesting ERK2 activation is necessary for reconsolidation. If ERK2 inhibition at 45 min after T1 was also a specific and necessary event in memory reconsolidation, achieving a similar inhibition after reactivation of a weaker memory might induce a retention level similar to that of a stronger trace. That is, enhancing ERK inhibition at 45 min might allow memory improvement.

Our next experiment was aimed at testing if this kind of mechanism is taking place. Three groups of 10 mice each were trained. One of them was trained as in the previous experiments (here referred as high foot-shock, HFS group). The other two groups were trained with a lower footshock (LFS groups; 0.8 mA, 50 Hz, 1 s), which induces retention latencies around 120–180 in later tests (Fig. 5).

Two days after training, mice were subjected to T1. Forty minutes after the test, mice received a bilateral dHIP infusion of vehicle (Veh) or PD (1 μg/HIP). Mice were tested again one day after T1 (T2). Results are shown in Fig. 5, PD administered 45 min after T1

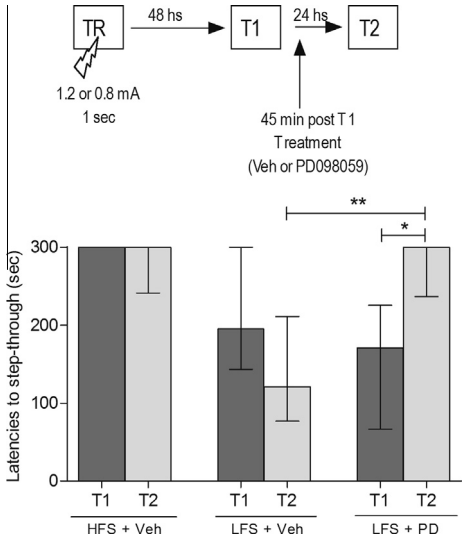

Fig. 5. Administration of PD098059 45 min after T1-enhanced memory reconsolidation. Animals were trained in the inhibitory avoidance task and received a footshock (0.8 or 1.2 mA, 1 s). Vehicle (Veh) or PD098059 (1 μg/hippocampus) were given 45 min after T1. Representation of the behavioral protocol is depicted (top). Each bar represents the median and interquartile range (n = 10 mice/group). HFS: mice trained with the higher footshock (1.2 mA), LFS: mice trained with the lower footshock (0.8 mA), T1–2: retention tests. $^{*}p < 0.05$; $^{**}p < 0.01$ (T2 vs T2 Veh; Mann–Whitney U-test, two-tailed).

significantly impaired retention performances during T2 (U = 55.5; p = 0.005, comparing LFS groups T2-Veh vs T2-PD). T1 LTSTs from LFS animals were similar between them but significantly lower than those from HFS animals (U = 144; p = 0.08, T1-Veh vs T1-PD and U = 42; p = 0.001, T1-HFS vs T1-LFS).

DISCUSSION

Several reports have demonstrated ERK participation on memory reconsolidation (Kelly et al., 2003; Duvarci et al., 2005; Miller and Marshall, 2005; Martijena and Molina, 2012; Besnard et al., 2013). Consistent with previous findings, the present study provides new evidence for a central role of ERK2 isoform and for a biphasic ERK modulation during memory reconsolidation. Not only cytosolic ERK2 activation increased compared to N levels (Fig. 2A), but 1 μg of PD098059 administered dHIP immediately after T1 (which significantly inhibited almost 40% of retrieval-induced ERK2 activation) also impaired memory retention (Fig. 4A). These results suggest ERK2 might be involved in memory trace re-stabilization. Moreover, 45 min after memory reactivation ERK2 is inhibited (Fig. 2B). In support that this inhibition is specifically involved in reconsolidation, we pharmacologically inhibited hippocampal ERK (PD 1.0 μg/HIP) 45 min after T1, and observed enhanced memory performance.

ERK2 inhibition at 45 min after T1 seems to correlate with subsequent retention latencies. In other words, the higher the inhibition of ERK the higher the retention latencies will be. How is ERK2 level of inhibition at 45 min after retrieval able to modulate behavioral expression? Further experiments are in progress in order to elucidate this question. If ERK2 inhibition at 45 min after T1 was a specific and necessary event in memory reconsolidation, achieving a similar inhibition after reactivation of a weaker memory might induce a retention level similar to that of a stronger trace. That is, enhancing ERK inhibition at 45 min might allow memory improvement. Altogether hippocampal ERK2 activity dynamics, that is, enhancement at 15 min and inhibition at 45 min, seems to be critical on memory reconsolidation of an IA task in mice.

With the aim of determining the effects of PD on memory reconsolidation, a dose–response curve was performed. For this purpose, different doses of PD were given dHIP immediately after memory reactivation. The effects of PD on memory reconsolidation were dose-dependent with a maximal effect using a dose of 1.0 μg/HIP.

We performed additional experiments in order to evaluate whether PD effects on behavior performance were specific to memory reactivation. For this purpose, PD was administered in mice that were not subjected to memory reactivation and they were tested for retention 24 h apart. The results from this experiment suggest that either the PD-induced impairment or enhancement on retention performance could not be attributed to a non-specific effect of the pharmacological treatment, since PD was unable to alter retention latencies in mice not undergoing memory reactivation (NR). At this point it is worth pointing out that mice that were not subjected to memory reactivation did not show either activation (15 min) or inhibition (45 min) of ERK2 (see Fig. 3B, C). These results suggest that ERK activation was also specific to memory reactivation.

With the aim of checking the specificity of ERK involvement in memory reconsolidation, we included an unshocked group of animals paired to the regular trained ones (Sh group). Although both groups showed ERK2 activation 15 min after T1, USh mice showed no difference with baseline levels (N group) at 45 min, indicating that ERK2 inhibition at this time point could account for the specificity of the association between context and stimulus accomplished (Sh group).

Most of the experiments regarding ERK1/2 participation on memory processes were performed using different pharmacological tools and led to the proposal that both ERK1 and ERK2 participate in memory processes. Discrimination between them was not possible since specific inhibitors are not available. However, Mazzucchelli and coworkers, using ERK1 knock-out mice, proposed a different model in which ERK1 would constrain ERK2 function (Mazzucchelli et al., 2002). Given that ERK2 (but not ERK1) knock-out mice are embryonically lethal, the idea that ERK1 plays an accessory function related to ERK2 seems plausible. In this sense, ERK2 conditional knock-out mice, in which ERK2 expression was abolished specifically in the CNS, were viable and fertile. However, they expressed altered social behavior and also long-term memory deficits (Samuels et al., 2008; Satoh et al., 2011). In these mice pharmacological inhibition of ERK1 did not further impair

memory. Finally, Molina and colleagues findings also suggested that the selective ERK2 activation in BLA following stress exposure is a determinant for the stress-induced enhancement effect on fear memory (Maldonado et al., 2014). Altogether, these results give additional support for a relevant role of ERK2 in memory-related processes and synaptic plasticity. Our pharmacological results reinforce this idea, by showing that ERK2 phosphorylation is specifically regulated during memory reconsolidation. However, activation is not the only necessary step in memory re-stabilization. Inhibition is also critical not only for memory re-stabilization, but also for memory strengthening. Previous work by our group has also shown biphasic kinetics for NF-κB, another memory relevant pathway (Boccia et al., 2007). However, more experiments are under way to support these findings.

To the best of our knowledge this is the first report showing a bidirectional regulation of ERK on memory reconsolidation of an IA task in mice. Ivanov and colleagues proposed that NMDARs are capable of exerting a dual role in the regulation of ERK based on their localization. That is, synaptic receptors activate ERK whereas the extrasynaptic ones control ERK inactivation (Ivanov et al., 2006). The authors proposed that ERK inhibition by extrasynaptic NMDARs might be one of the first signaling events detecting glutamate-induced pathology. However, taking into account our results, an alternative explanation might be that parallel processes are engaged in memory reconsolidation: synaptic strengthening and weakening. These processes might need differential ERK regulation at precise time points after retrieval.

Besnard et al. (2014) examined the relationship between the strength of a previously established contextual fear conditioning memory and neuronal activity throughout the HIP and amygdala. ERK1/2 phosphorylation was analyzed immediately after a retrieval session in mice trained with 0, 1 or 3 footshocks. In the dentate gyrus (DG), the number of pERK1/2 immunoreactive cells was significantly increased in all trained groups, as compared to naïve controls. However, the number of pERK1/2 immunoreactive cells was higher in the group of animals receiving no footshock during training compared to the animals receiving 1 or 3 footshocks. In our hands, unshocked animals also showed increased hippocampal ERK2 activation 15 min after retrieval test. A possible scenario would be that ERK activates after memory retrieval in response to contextual cues (named ''pseudotrained'' in Besnard et al. (2014)). If this is the case, contextual memory reactivation might be DG-dependent. Our results, as well as previous ones from our laboratory (Boccia et al., 2007), regarding activation of NF-κB in mice trained in an IA without footshock, are consistent with this idea. It has been proposed that the amygdala is required for memory reconsolidation and enhancement, whereas the HIP and mPFC are required only for retrieval-induced memory enhancement associated with additional learning that depend only on the amygdala (Fukushima et al., 2014). Moreover, recent work using transgenic mice designed to allow for the comparison of cells activated during the encoding versus cells activated during the expression of a memory, suggested that re-exposure to a fear-inducing context induced more freezing and had a greater percentage of reactivated cells in the DG and CA3 than mice exposed to a novel context, while the strength of the memory is related to reactivation in CA3 (Denny et al., 2014). Since our samples were performed using whole HIP, we were not able to discriminate which hippocampal subfield contributed to the different pattern of ERK activation.

We disclosed nuclear from cytosolic activation, and we observed only cytosolic ERK2 activation. It is well established that either cytosolic ERK function might precede nuclear activation or else cytosolic activation might have a role *per se* in this subcellular compartment. In this sense, *Aplysia* cell adhesion molecules (apCAM) internalization in the sensory neuron of this invertebrate is related to synaptic growth by 5-HT-induced long-term facilitation. The internalization was blocked by overexpression of transmembrane constructs with a single point mutation in the two MAPK phosphorylation consensus sites, as well as by injection of a specific MAPK inhibitor into sensory neurons. These data suggest MAPK phosphorylation at the membrane is an important event for apCAM internalization and, thus, may represent an early regulatory step in the growth of new synaptic connections that accompanies long-term facilitation (Bailey et al., 1997). In support of this, our group has already shown the need for cytosolic ERK activation in an invertebrate memory model (Feld et al., 2005). Cytosolic ERK2 activation has also been associated with long-term plasticity and memory-dependent protein synthesis regulation in mice (Kelleher et al., 2004); potassium-channel function regulation by direct phosphorylation of the pore-forming alpha subunit from Kv4.2 channels *in vitro* and *in vivo* (Adams et al., 2000; Yuan et al., 2002); alterations in dynamin–microtubule interactions in culture (Earnest et al., 1996); among others. Regarding cytosolic activation, it has also been proposed that scaffolding proteins and dimerization are key mediators for cytoplasmic ERK2 function *in vitro* (Casar et al., 2008), and that local translation is one of the relevant processes regulated by ERK signaling (Gong and Tang, 2006; Leal et al., 2014). These results, together with ours, support the idea that not only nuclear ERK activation may constitute an important event in long-term memory and plasticity, but cytosolic ERK function might also be relevant. We found here that this is an important step in memory reconsolidation of an IA task in mice.

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