Original Article

Role of ERK2 dimerization in synaptic plasticity and memory

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ABSTRACT

Extensive research has focused on extracellular-signal regulated kinase (ERK) 1/2 phosphorylation in different memory and plasticity models. However, the precise mechanism by which ERK activity leads to memory stabilization and restabilization remains largely elusive, and there are no reports on the role of ERK1/2 dimerization in those processes. ERK dimerization is critical for the binding and activation of cytoplasmic targets, many of which have been strongly associated with these processes. In this study, several key experimental interventions were performed. First, it was found that reactivation of a weak inhibitory avoidance (wIA) memory led to a significant decrease in hippocampal ERK2 dimerization. Additionally, intrahippocampal infusion of DEL-22379 (DEL), an ERK dimerization inhibitor, after memory reactivation had a bidirectional effect: it blocked the reconsolidation of a strong inhibitory avoidance (sIA) memory, whereas, in the case of the wIA memory, it enhanced reconsolidation. Moreover, DEL administration blocked hippocampal ERK2 dimerization in vivo and impaired high-frequency stimulation-induced long-term potentiation (LTP) in hippocampal slices. Taken together, these findings reveal for the first time that ERK2 dimerization occurs in the normal environment of the rodent nervous system and plays a critical role in plasticity and memory processes. Although more research is needed, these initial findings suggest a relevant role of ERK dimerization in plasticity and memory.

Keywords

Mice, DEL-22379, Memory reconsolidation, hippocampus, LTP, EGF

INTRODUCTION

Molecular mechanisms involved in learning and memory have been extensively assessed in the last decades using a wide variety of experimental paradigms and techniques. Long-term plasticity is considered the cellular correlate of memory formation[1,2]. In line with this proposal, certain signaling pathways have been associated with both plasticity and memory processes. Particularly, extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation has been shown to subserve both of them[3–8]. Pharmacological interventions demonstrated that ERK1/2 inhibition impairs memory formation as well as neuronal plasticity[reviewed in 9]. Mitogen-activated protein kinase (MAPK) family, including the ERK1/2 cascade, is a large group of Ser/Thr protein kinases that were initially associated with development, mitogenesis, and stress response[10]. ERK activation is achieved through dual phosphorylation of residues Thr and Tyr in a common TEY motif (pERK hereafter) by a MAPK/ERK kinase (MEK), which then translates into conformational changes of the enzyme leading to substrate binding and phosphorylation. Although initial results suggested that activation of the protein by phosphorylation led to dimerization and subsequent nuclear translocation, it was later disclosed that in fact dimerization guides extra-nuclear function[11], and contributes to binding and activation of cytosolic targets aided by scaffolding proteins[12]. Consistently, the inhibition of ERK1/2 dimerization selectively disrupted cytoplasmic signaling pathways[13]. Moreover, the multiplicity of ERK1/2 cytosolic targets previously described[14–19] supports the idea that extra-nuclear localization of the kinase is relevant for a wide variety of processes. Herrero and colleagues[13] described a novel compound that inhibits ERK dimerization (DEL-22379, DEL hereafter) and blocked the growth of hyperactive Ras-ERK1/2 signaling tumor cells, without decreasing its effectiveness by resistance mechanisms previously described for phosphorylation inhibitors. Thus, preventing ERK dimerization attenuated cellular proliferation, transformation and tumor development.

The role of ERK activation in synaptic plasticity and memory processes has been extensively studied although there is limited information on specific functions in different subcellular compartments. While oncological research has significantly advanced our understanding of ERK's role in cell function, neuronal activation of this pathway is likely to exhibit distinct characteristics based on subcellular localization and kinetics, particularly in processes such as synaptic plasticity, learning, and memory.

ERK phosphorylation increased at both pre- and post-synaptic sites of pyramidal neurons of the visual cortex after in vivo visual stimulation in dark-reared rats, and its temporal profile was similar to ERK activation in neuronal cell bodies[20]. High-frequency stimulation selectively induced ERK activation, regulating translation at specific subcellular compartments at active synapses in the medial perforant pathway[21]. Moreover, NR2A-induced ERK activation has been shown to play a role in the regulation of dendritic spine density in key brain areas involved in cognition[22]. Additionally, it is associated with neurodegenerative pathologies, depending on the stage of development of the organism, the type of cellular element in which the pathway is activated, and the anatomical neuronal structure[23].

Stability of consolidated memories can be challenged by reactivation, opening a new period of sensitivity named ‘‘reconsolidation” and triggering molecular mechanisms involved in re-stabilization[24–26]. Previous work from our group revealed that cytosolic ERK activation is important for the consolidation and reconsolidation of memories in invertebrates[27] as well as

vertebrates[28,29]. ERK cytosolic kinetics following inhibitory avoidance memory reactivation in mice regulated the reconsolidation, strength and persistence of the memory[28–30]. Moreover, ERK overactivation in the nuclear compartment was associated with memory impairment at 6 months in a mouse model of Alzheimer's disease, as partial inhibition reverted this retention deficit[31]. These results evidence a disregarded phenomenon which deserves further attention.

While ERK1/2 phosphorylation has been extensively studied regarding memory and plasticity, the role of ERK1/2 dimerization in these processes remains unexplored. We hypothesized that ERK dimerization constitutes a regulatory milestone for long-term memory (LTM) consolidation and reconsolidation. Here we demonstrate for the first time that ERK2 dimerization can be pharmacologically inhibited in mice hippocampi without affecting its phosphorylation. DEL administration blocked CA1 hippocampal LTP and affected memory reconsolidation depending on the strength of the inhibitory avoidance (IA) task. This represents the first report on the role of ERK dimerization *in vivo* within the nervous system and its involvement in memory processes. These findings emphasize the significant role of ERK dimerization in synaptic plasticity and memory processes.

MATERIALS AND METHODS

Animals

Different cohorts of CF-1 male mice aged either 5 weeks (weight range: 21 - 31 g kept at the Facultad de Ciencias Exactas y Naturales central mouse house, housed in groups of 3-5 individuals in 18.41 x 29.21 x 12.7 cm amber polysulfone cages) or 8-10 weeks old (weight range: 25 - 35 g, housed in groups of 18 - 22 individuals in stainless-steel 50 x 30 x 15 cm cages kept at the Laboratorio de Neurofarmacología de Procesos de Memoria) were used either for electrophysiological experiments or behavioral testing and biochemical analyses, respectively. The mice had *ad libitum* access to dry food and tap water. They were maintained under a 12h light/dark cycle (lights on at 6:00 AM) at a controlled temperature of 21-23ºC. Experiments were conducted during the light cycle. All procedures related to care and treatments of mice were approved by the local institutional animal care and use committees (CICUAL - CUDAP # 0044975-2016, Facultad de Farmacia y Bioquímica; CICUAL # 154b, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina), and in compliance with the standard European Union and United States National Institutes of Health ethical guidelines for Care and Use of Laboratory Animals. All efforts were made to minimize any possible distress or suffering experienced by mice, as well as to reduce the number of animals required to generate reliable scientific data.

Drugs

DEL-22379 (DEL), an ERK dimerization inhibitor[13], was purchased from MedChemExpress (Cat. No.: HY-18932) and dissolved in dimethyl sulfoxide (DMSO) as a vehicle (VEH). For behavioral experiments and hippocampal ERK2 dimerization assays, 0.5 µl of drug or VEH solution were injected per hemi-hippocampus (DEL final concentration: 500 nM). Epidermal growth-factor (EGF) was purchased from Thermo Fisher (Cat. No.: PHG0314) and diluted in sterile PBS to a final concentration of 100 µM (stock solution).

Hippocampal slices and extracellular field potential recordings

Electrophysiological recordings in slices were performed as described elsewhere[32]. Briefly, mice were deeply anesthetized with a mix of ketamine (160 mg/kg) and xylazine (8 mg/kg) before decapitation. Coronal brain slices including dorsal hippocampus (300-400 µm thick)

were obtained by gluing the brain onto a vibratome stage (PELCO, EasiSlicer, Ted Pella Inc., CA, USA), submerged in a chamber containing chilled low-sodium/high-sucrose solution (250 mM sucrose, 2.5 mM KCl, 3 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 0.4 mM ascorbic acid, 3 mM myo-inositol, 2 mM pyruvic acid, 25 mM D-glucose, and 25 mM $NaHCO_3$). Sequential slices were obtained in which Schaffer collaterals were transected near CA3 before being transferred to an incubation chamber and kept at 35 °C for 30 min. Recovery chamber was containing low Ca^{2+} / high Mg^{2+} normal artificial cerebrospinal fluid (ACSF; 125 mM NaCl, 2.5 mM KCl, 3 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 0.4 mM ascorbic acid, 3 mM myo-inositol, 2 mM pyruvic acid, 25 mM d-glucose, and 25 mM $NaHCO_3$ and aerated with 95% O_2 / 5 % CO_2, pH 7.4). Slices were then transferred to the recording chamber and perfused continuously with ACSF including bicuculline (20 µM; a GABA-A antagonist).
Recording electrodes were made with borosilicate glass (2–3 MΩ) filled with ACSF + bicuculline solution and placed in the *stratum radiatum* area of the CA1 region of the hippocampus. The field excitatory postsynaptic potentials (fEPSP) were recorded as measure of local excitatory synaptic transmission in the CA1 region. The stimulating electrode was placed at the Schaffer Collateral fibers and fEPSPs were evoked by test stimuli (0.033 Hz, 0.05-0.2 ms duration) every 30 sec. LTP was induced by applying high frequency stimulation (HFS; 5 trains, 100 Hz, 10 pulses) after a recorded baseline period of 10 min. Recordings were maintained for 30 min after LTP induction. The magnitude of LTP was calculated as a percentage of the fEPSP slope value after HFS compared to baseline fEPSP mean slopes. Changes in LTP were compared in hippocampal slices incubated with either DEL (30 min., 1,33 uM) or VEH. Signals were recorded using a MultiClamp 700 amplifier commanded by pCLAMP 10.0 software (Molecular Devices, CA, USA). Data was filtered at 5 kHz, digitized, and stored for off-line analysis.

Intra-hippocampal injections

Hippocampal injections were performed under isoflurane anesthesia (induction: 3.5 %, maintenance: 1 %) using a stereotaxic apparatus (Stoelting) at the indicated time points. Surgery lasted for no more than 5 min. A small incision was made in the scalp and two holes were drilled in the skull at the following coordinates: AP: -1.90 mm from bregma, DV: -2.2 mm from the skull surface and +/-1.2 mm left and right from the midsagittal suture[33] to bilaterally infuse the drugs. Injections were driven by an automatic pump (MD-1020, Bioanalytical Systems, Inc.) at a rate of 0.52 µL/min through a 30 gauge stainless steel needle attached to a 5 µl Hamilton syringe with PE-10 tubing. n. The drug infusion lasted 60 sec and the needle was left in place for another 30 sec to avoid reflux. Total volume administration was 0.5 µl (either drug or VEH solution) per hemi-hippocampus. At the end of the procedure, the incision was sutured and thoroughly disinfected. After the last testing session, animals were euthanized by cervical dislocation and brains were dissected, fixed by immersion in 4 % PFA overnight, and sectioned to verify the correct injection site placement. In all cases administration placement was confirmed in the target area.

Inhibitory Avoidance task

IA behavior was studied using a one-trial step-through protocol, which takes advantage of mice's natural preference for dark and confined spaces[34,35]. The apparatus consists of a dark closed compartment (20 x 20 x 15 cm) with a stainless-steel grid floor and a small illuminated open platform (5 x 5 cm) attached to its entrance. During the training phase (TR), each mouse was placed on the illuminated platform and received a foot shock upon entry into the dark compartment. Two different shock intensities were used: a 0.3 mA - 3 sec shock for weak inhibitory avoidance induction (wIA) and a 0.4 mA - 3 sec shock for strong inhibitory avoidance (sIA) memory formation[36]. Mice underwent two retention tests after TR. The first retention test,

176 48 h after TR, followed by a second retention test (TS) 24 h after T1. No shock was delivered
177 during either test session. Previous work showed that T1 also induces memory reactivation
178 (RE), and this process was controlled by means of a non-reactivated (NR) group[28,37–39]. The
179 retention tests were finished either when the mouse stepped into the dark compartment or
180 failed to enter within 300 sec after the beginning of the test. After the retention test, mice were
181 returned to their homecage. Step through latencies were manually registered by the
182 experimenter using a manual chronometer.

183 Sample preparation and ERK analysis

184 *Neuronal cultures.* Primary neuronal cultures were obtained as described before[40] according
185 to Kaech and Banker´s method[41]. Briefly, Wistar embryos were obtained on day 17 (E17), both
186 hippocampi were dissected from pups and digested with trypsin (Sigma, Sigma–Aldrich Co.,
187 St. Louis, MO, United States). Cells were plated onto poly-L-lysine (Sigma)–coated wells and
188 incubated in neurobasal media (NB; Invitrogen, Life Technologies Corporation, Carlsbad, CA,
189 United States) supplemented with B27 (Invitrogen) and Glutamax (Invitrogen). Cultures were
190 maintained in complete NB (NB + B27 + Glutamax) at 37°C and with 5 % CO_2 and one third
191 of the culture media was replaced every 2 days.
192 For the induction of ERK activation by EGF, 1 µl of the stock solution was diluted in 1 mL of
193 culture medium and incubated in the culture for 5 min. Chemical LTP (cLTP) induction was
194 achieved by a glycine stimulation protocol previously described[42] with some modifications.
195 Neuronal cultures were incubated with conditioned media containing 200 mM glycine for 3
196 min. Glycine solution was then removed and neurons were maintained in their media for 2 min
197 of incubation. Control groups were incubated with conditioned media for 3 min without Glycine
198 stimulation.
199 After treatment, neuronal cultures were cooled and homogenized by scraping cells in ice-cold
200 lysis buffer (20 mM HEPES, pH 7.5, 10 mM ethylene glycol tetra-acetic acid, 40 mM glycerol
201 2-phosphate, 1 % (w/v) IGEPAL, 2.5 mM $MgCl_2$, 2 mM sodium orthovanadate, 1 mM
202 dithiothreitol, 0.5 mM PMSF, 50 mM Sodium Fluoride, 1 µg/ml Pepstatin A, 10 µg/mL
203 Leupeptin, 10 µg/mL Aprotinin) as described previously[43]. Samples were centrifuged at 13000
204 x g for 10 min at 4 ºC, and the supernatants were collected and conserved at -20 ºC.
205
206 *Hippocampal* ERK activation and *tissue* protein extraction

207 For the induction of hippocampal ERK activation by EGF, two consecutive injections were
208 performed in each hemi-hippocampus: first either 0.5 µl of phosphate buffer saline (VEH) or
209 100 µM EGF followed by 0.5 µl of either DMSO (VEH) or 400 nM DEL. At the specified time
210 points, mice were quickly euthanized by cervical dislocation, decapitated and the hippocampi
211 were dissected. Each hemi-hippocampi was then homogenized using a Dounce tight
212 homogenizer and 125 µL of ice-cold lysis buffer (20 mM HEPES, pH 7.5, 10 mM ethylene
213 glycol tetraacetic acid (EGTA), 40 mM glycerol 2-phosphate, 1 % (w/v) IGEPAL, 2.5 mM
214 $MgCl_2$, 2 mM orthovanadate, 1 mM dithiothreitol, 0.5 mM PMSF, 50 mM Sodium Fluoride, 1
215 µg/ml Pepstatin A, 10 µg/mL Leupeptin, 10 µg/mL Aprotinin) by performing 8 strokes with a
216 tight glass pestle. Samples were centrifuged at 13000 x g for 10 min at 4 ºC, and the cytosolic-
217 enriched supernatants were collected and conserved at -20 ºC.

218 *Native gel electrophoresis*

219 Native polyacrylamide gel electrophoresis (PAGE) for ERK dimers detection was carried out
220 as described previously[43]. Briefly, protein levels in lysates were quantified by BCA Assay (Bio-
221 Rad, USA) and 60 µg of protein were loaded into an 8 % bis-acrylamide gel and run for 150
222 min at 70 V. Then samples were transferred to a nitrocellulose membrane (General Electric,
223 USA) overnight at 125 mA and 4 ºC.

224 *SDS-PAGE*

225 ERK phosphorylation levels were assessed by means of denaturing gel electrophoresis as
226 described before[28]. Briefly, 20 µg of protein were loaded in a 12.5 % bis-acrylamide gel and
227 run for 90 min at 100 V. The samples were then transferred to a PVDF membrane (Thermo
228 Scientific, USA) overnight at 300 mA and 4 ºC and stored in TBS until probed with antibodies.

229 *Antibodies used and detection*

230 Initially, membranes were blocked in 4% non-fat dry milk in TBS - 0.05 % Tween-20. A full list
231 of antibodies used, and dilutions can be found in Table 1. Primary antibodies were incubated
232 overnight at 4 ºC and secondary antibodies were incubated for 1 h in TTBS at room
233 temperature. ERK dimers were detected on nitrocellulose membranes by luminol
234 chemiluminescence kit (Bio Rad ECL, USA) using X-Ray film (Agfa ORTHO CP-GU, cat.
235 PTNAGCPGUM2, Voxel, Argentina). SDS-PAGE blots were developed using the same
236 chemiluminescence kit in a GE Amersham Imager.

237 Table 1.

Target	Supplier	Carrier solution	Dilution
ERK2	Santa Cruz (SC-1647)	4 % milk in TBS - 0.05 % (v/v) Tween	1:1000
pERK1/2	Cell Signaling (9101)	4 % milk in TBS - 1 % (v/v) Tween	1:2000
ERK1/2	Cell Signaling (9102)		1:2000
Rabbit IgG	Santa Cruz (SC-2357)		1:10000
Mouse IgG	Cell Signaling (7076)		1:15000

238

239 Data analysis

240 Data analyses were conducted using R (version 4.2.2,83)[44]. Generalized linear models
241 (GLMs) were employed using the glmmTMB package (version 1.1.6)[45]. For behavioral data
242 (latency to step-through), drug (VEH or DEL) and condition (RE or NR) were used as fixed
243 factors. The model was fitted with a Gamma distribution and log link function. For western blot
244 data, the model was fitted using a Gaussian distribution and identity link function. In the LTP
245 experiment, Time was added as a fixed factor in order to account for repeated measures. The
246 goodness-of-fit for each model was analyzed using the DHARMa package (version 0.4.6)[46].
247 Visual inspection of residual plots did not reveal any concerning deviations from normality or
248 homoscedasticity. Anova of the models is reported and, when applicable, post-hoc
249 comparisons were performed using the emmeans package (version 1.8.5)[47]. All reported p-
250 values were adjusted for multiple comparisons with the Šidak correction method. Two-tailed
251 Pearson coefficients were calculated to determine the correlation between phosphorylation
252 and dimerization at each time point.

253

254 **RESULTS**

255 *ERK dimerization in rodent nervous system and plasticity*

256 The latency to enter the dark compartment in the inhibitory avoidance (IA) task depends in
257 part on the intensity of the aversive stimulus delivered to the animal during training[30]. Previous
258 results from our group using the IA task in CF-1 mice showed that reactivation of a
259 consolidated memory by memory retrieval induced different levels of cytosolic phosphorylated
260 ERK2 (pERK2) 45 min later in the hippocampus, depending on stimulus strength. Weak IA
261 (wIA) memory reactivation induced significant pERK2 increase compared to naïve animals,
262 while strong IA (sIA) memory reactivation significantly decreased it. On the other hand,
263 contextual exposure alone did not alter pERK2 levels at this time point[29]. Consistently,
264 intrahippocampal administration of PD098059, a MEK1/2 inhibitor, 45 min after wIA retrieval
265 strengthened memory when tested 24 h later[28].

266 Considering that memory reconsolidation induces significant changes in cytoplasmic ERK
267 activity, and that dimerization regulates its subcellular localization[11], we aimed to investigate
268 whether this molecular mechanism also influences memory stabilization following reactivation
269 in mice, specifically using the inhibitory avoidance (IA) task. The small molecule DEL-22379
270 (DEL hereafter) was shown to inhibit ERK dimerization process, impairing the growth of tumor
271 cells[13] depending on the specific driver mutation for thyroid cancer onset and progression, and
272 with no apparent toxicity[48]. However, ERK dimerization has yet to be reported in nervous
273 tissue. Consequently, our first experiment aimed to characterize ERK2 dimerization in the
274 rodent brain (fig. 1). To induce ERK2 dimerization, two protocols were used: 1) chemical LTP
275 (cLTP) induced in rat primary neuronal cultures by glycine stimulation[42] and 2)
276 intrahippocampal stimulation with EGF. Then, protein-enriched samples from both protocols
277 were resolved in native polyacrylamide gels. In the first case, cultures were pre-incubated
278 either with vehicle (VEH) or DEL, and then cLTP was induced with a previously characterized
279 protocol[42]. As positive control, other cultures were stimulated with EGF, an ERK1/2 pathway
280 activator that also induces its dimerization[11]. Initial assessment of data showed a tendency to
281 ERK2 dimerization increase after EGF stimulation (fig. 1A). Furthermore, cLTP also
282 upregulated ERK2 dimerization and DEL pre-treatment for 30 min completely abolished it (fig.
283 1A). These results qualitatively validate that ERK dimerization occurs in primary neuronal
284 cultures.

285 However, primary neuronal cultures are devoid of the tissue context existing in the mouse
286 hippocampus; so, we decided to evaluate dimerization *in vivo*. For this purpose, in the second
287 experiment, we assessed the efficacy of DEL to inhibit ERK2 dimerization in CF-1 mice
288 hippocampus. We acutely administered either PBS (VEH) or EGF plus either VEH or DEL
289 solution in each hemi-hippocampus of CF-1 anesthetized mice. Afterwards, mice were
290 euthanized, and we performed SDS and native gel electrophoresis of protein-enriched
291 hippocampal samples and western blot. A schematic experimental design is shown on top of
292 figure 1B. This administration scheme was used in order to perform experimental and control
293 treatments in the same animal. Consistent with previous reports[49–52], EGF administration
294 altered hippocampal pERK2 levels 5 (fig. 1B left; $F_{2,13} = 5.381$, $p = 0.048$) and 15 min later (fig.
295 1B right; $F_{2,13} = 8.582$, $p = 0{,}0100$). EGF administration induced a rapid and transient significant
296 increase in hippocampal pERK2 at 5 min ($p = 0.0492$) which decreased under VEH baseline
297 levels at 15 min ($p = 0.0100$). It is important to point out that DEL treatment did not affect EGF-
298 induced pERK2 profile at the time points assessed ($p = 0.989$ at 5 min; $p = 0.555$ at 15 min in
299 EGF-VEH vs EGF-DEL contrasts), which were also statistically different from control ($p =$
300 $0{,}036$ at 5 min; $p = 0{,}009$ at 15 min in VEH-VEH vs DEL-EGF contrasts). However, ERK2
301 dimerization (fig. 1C) showed different kinetics after EGF administration: it did not change at

302 5 min (fig. 1C left; $F_{2,13} = 0.2703$; $p = 0.7694$), but it was significantly altered at 15 min (fig. 1C
303 right; $F_{2,13} = 11.623$; $p = 0.0044$). Consistent with previous results[13], EGF alone induced a
304 significant rise in ERK2 dimerization ($p = 0.014$). This effect was prevented by DEL pre-
305 treatment ($p = 0.030$) which induced dimer/monomer (dim/mon) levels comparable to VEH-
306 treated hippocampi ($p = 0.915$). Therefore, phosphorylation and dimerization of ERK2 showed
307 a strong negative correlation 15 min after stimulation (fig. 1D, $p = 0.049$). Thus, based on
308 these findings, it appears that under our experimental conditions, DEL prevents ERK2
309 dimerization induced by EGF in CF-1 mice hippocampi without affecting its phosphorylation.

310 Next, we wondered whether the inhibition of ERK2 dimerization has an impact on hippocampal
311 plasticity. In order to answer this question, we performed acute LTP experiments on
312 hippocampal slices. Slices were pre-incubated for 30 min in the presence of VEH or DEL, a
313 10 min baseline fEPSP was recorded to then extracellularly deliver a HFS protocol on Schaffer
314 Collaterals (5 trains, 100 Hz, 10 pulses). Neither fEPSP baseline recordings nor immediate
315 post-HFS fEPSPs were altered by DEL treatment (fig. 1E). However, around 15 min after HFS
316 application, DEL-treated slices begin to diverge from VEH-treated ones, reaching field
317 potential levels similar to baseline. In this last part of the experiment, fEPSP recordings were
318 significantly different between treatments ($p = 1.44 \times 10^{-10}$).

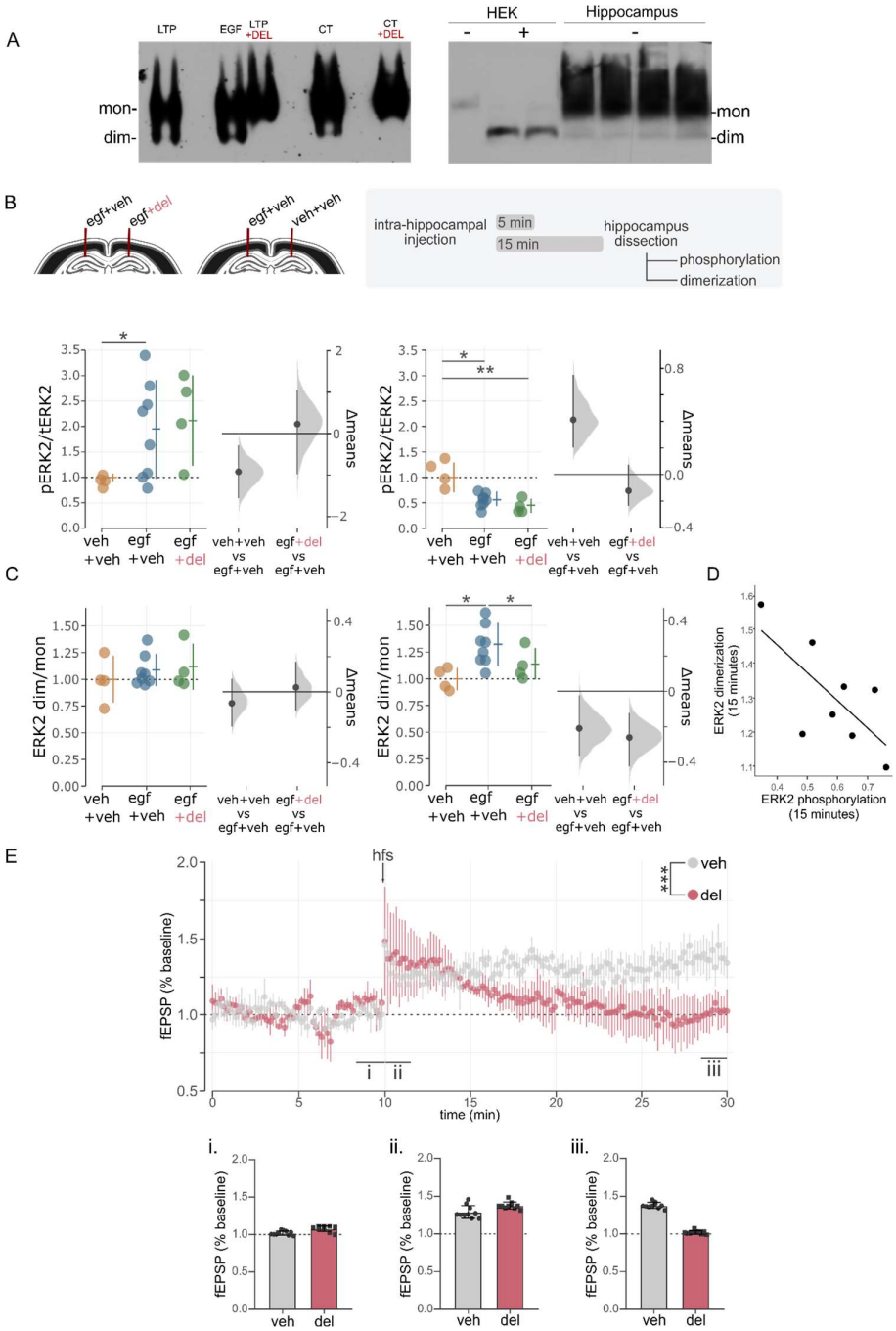


Figure 1. In vivo ERK2 dimerization in the rodent nervous system. **A)** Native PAGE western blot showing ERK2 dimers (dim) and monomers (mon) in samples from rat neuronal primary cultures subjected to either cLTP or EGF stimulation and-or DEL treatment (left). Control and EGF-stimulated HEK-293T culture cells and CF-1 mice hippocampal protein homogenates (right). **B-C)** ERK2 phosphorylation precedes dimerization in EGF-stimulated CF-1 mice hippocampal tissue. Schematic experimental design is shown on **top** of fig. B). Hippocampal ERK2 phosphorylation **(B)** and dimerization **(C)** were assessed 5 (left) or 15 (right) min after *in vivo* local EGF plus VEH or DEL administration. Dimer / monomer (dim/mon) ratios were normalized against "veh+veh" mean value in each case. Circles represent individual mice measurements, single lines represent mean ± s.d., and estimation plots shown on the right of each graph represent the difference between means (black dot) and the bootstrapped distribution of means (grey area) with 95% confidence intervals. *****, $p < 0.05$; ******, $p < 0.01$ in Tukey comparisons. n = 4 mice / group. **D)** Pearson correlation between ERK2 phosphorylation and dimerization assessed at 15 min in figures B and C. $R^2 = 0.71$; $p < 0.05$. **E)** DEL pre-treatment impairs LTP in CA1 hippocampal acute slices. Circles

represent the mean of 4 replicates and single lines represent s.d. Three points of interest are indicated throughout the recording: immediately before stimulation (i), immediately after stimulation (ii), and approx. 18-20 min after stimulation (iii). Bars in i), ii) and iii) are the mean ± s.d. of 100 sec recordings. ***, $p < 0.05$ (repeated measures comparison between VEH- and DEL-treated groups). n = 4 mice / group.

Memory reactivation after Weak IA decreases hippocampal ERK dimerization

Considering that ERK dimerization can modulate hippocampal plasticity, and that IA memory reconsolidation is fine-tuned by cytoplasmic ERK signaling at 45 min[28], we aimed to investigate whether its dimerization is regulated by memory reactivation in the hippocampus at this time point. Therefore, we trained animals in either strong or weak versions of the IA task and subjected them to memory reactivation (RE) 48 h later. As a control, identically trained animals were not exposed to reactivation (NR). Animals were euthanized 45 min after reactivation or the corresponding time for NR groups and their hippocampi were dissected in order to perform native polyacrylamide gel electrophoresis and ERK dimerization assessment (fig. 2, scheme).

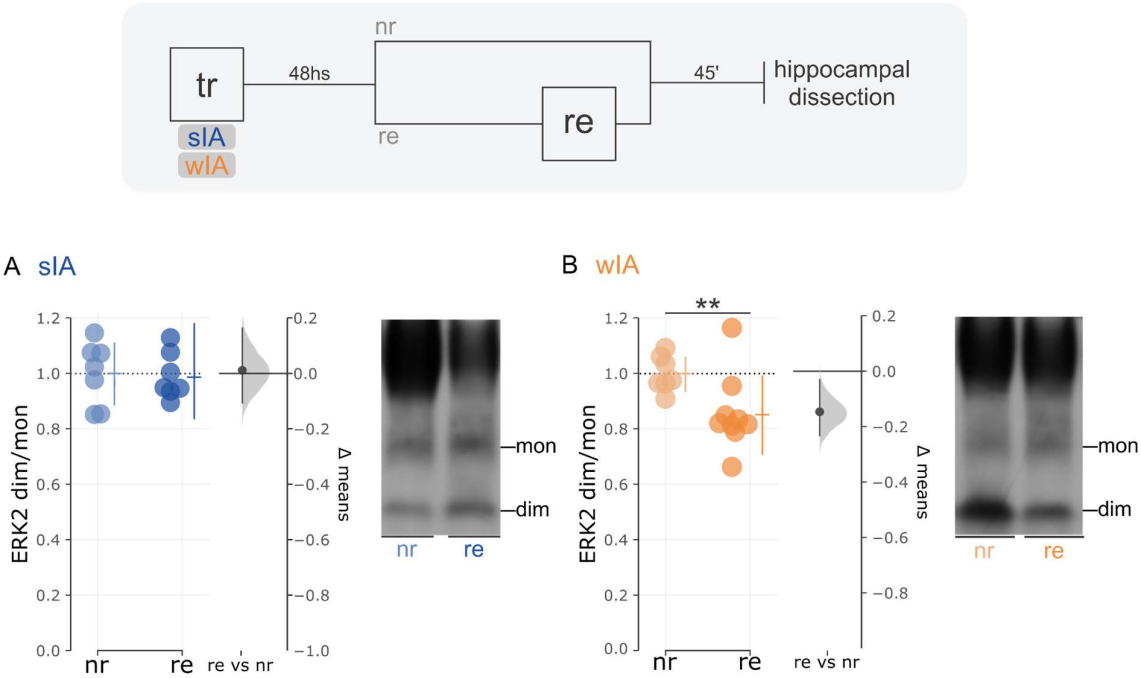


Figure 2. Hippocampal ERK2 dimerization after memory reactivation. **Top)** Schematic experimental design for hippocampal ER2 dimerization assessment. **Bottom)** Hippocampal ERK2 dimerization assessment by native PAGE western blots 45 after either sIA **(A)** or wIA **(B)** memory reactivation. Dim/mon ratios are normalized against nr mean value for each experiment. Color circles indicate individual values and error bars shown on the right of each set depict mean ± s.d. values. Estimation plots shown on the right of each graph represent the difference between means (black dot) and the bootstrapped distribution of means (grey area) with 95% confidence intervals. Representative western blots are also shown (right). *, $p < 0.05$; **, $p < 0.01$ in Tukey comparisons. n = 7-9 / group.

356 Reexposure to the IA context 48 h after strong TR did not induce evident changes in ERK2
357 dimerization levels related to NR control animals 45 min later (fig. 2A; $t_{13} = 0.161$; $p = 0.873$).
358 Meanwhile, wIA memory reactivation showed significant decrease in ERK2 dimerization
359 relative to the NR control group (fig. 2B; $t_{14} = 2.569$; $p = 0.0223$). The observation that memory
360 reactivation induced differential ERK2 dimerization depending on TR strength suggests the
361 possibility that this molecular mechanism plays a role in memory reconsolidation, as well.

362

363 *Post-retrieval hippocampal ERK2 dimerization supports strong IA memories*

364 Given that ERK2 dimerization was differentially regulated upon memory reactivation, we
365 sought to determine whether memory reconsolidation, a process triggered by reactivation, was
366 affected by its inhibition. In consequence, we first evaluated whether ERK2 dimerization was
367 affected by intrahippocampal DEL administration after memory reactivation. For this
368 experiment, all animals were injected either VEH or DEL in both hippocampi 35 min after RE
369 or the corresponding time for NR animals (fig. 3A, upper scheme) and 10 min later animals
370 were euthanized and their hippocampi were removed and homogenized. As shown in figure
371 3A (lower panel), reactivation did not induce changes in ERK2 dimerization levels ($F_{2,21}$ =
372 5.559; $p = 0.0115$; $p = 0.336$ in NR-VEH vs RE-VEH contrast). However, hippocampal DEL
373 injection after reactivation significantly reduced ERK2 dimerization ($p = 0.0102$ in RE-DEL vs
374 NR-VEH contrast), similar to the levels observed following wIA memory reactivation.
375 Next, we asked whether basal ERK2 dimerization levels are necessary for strong IA memory
376 re-stabilization. We aimed to answer this question by administering VEH or DEL 40 min after
377 strong IA memory reactivation and tested retention 24 h later (fig. 3B, upper panel). All groups
378 performed similarly during training and reactivated groups expressed strong retention during
379 reexposure to the training context (fig. 3B, lower panel). However, there was a significant
380 interaction between reactivation and drug treatment on latencies during the test session ($F_{2,28}$=
381 5.291; $p = 0.029$). Thus, while latencies from VEH- and DEL-injected NR animals did not differ
382 statistically ($p = 0.9833$), DEL treatment after memory reactivation induced a significant
383 decrease in latencies compared to the VEH group ($p = 0,0287$). General response levels
384 during TS were similar, as latencies from VEH-injected groups were not statistically different
385 ($p = 0.6847$). Furthermore, this impairment lasted for at least a week (suppl. fig. 1; $p = 0.013$).
386 This long-lasting impairment suggests a specific effect on the reconsolidation process. In
387 consequence, basal ERK2 dimerization levels would be required for strong IA memory
388 reconsolidation.

389

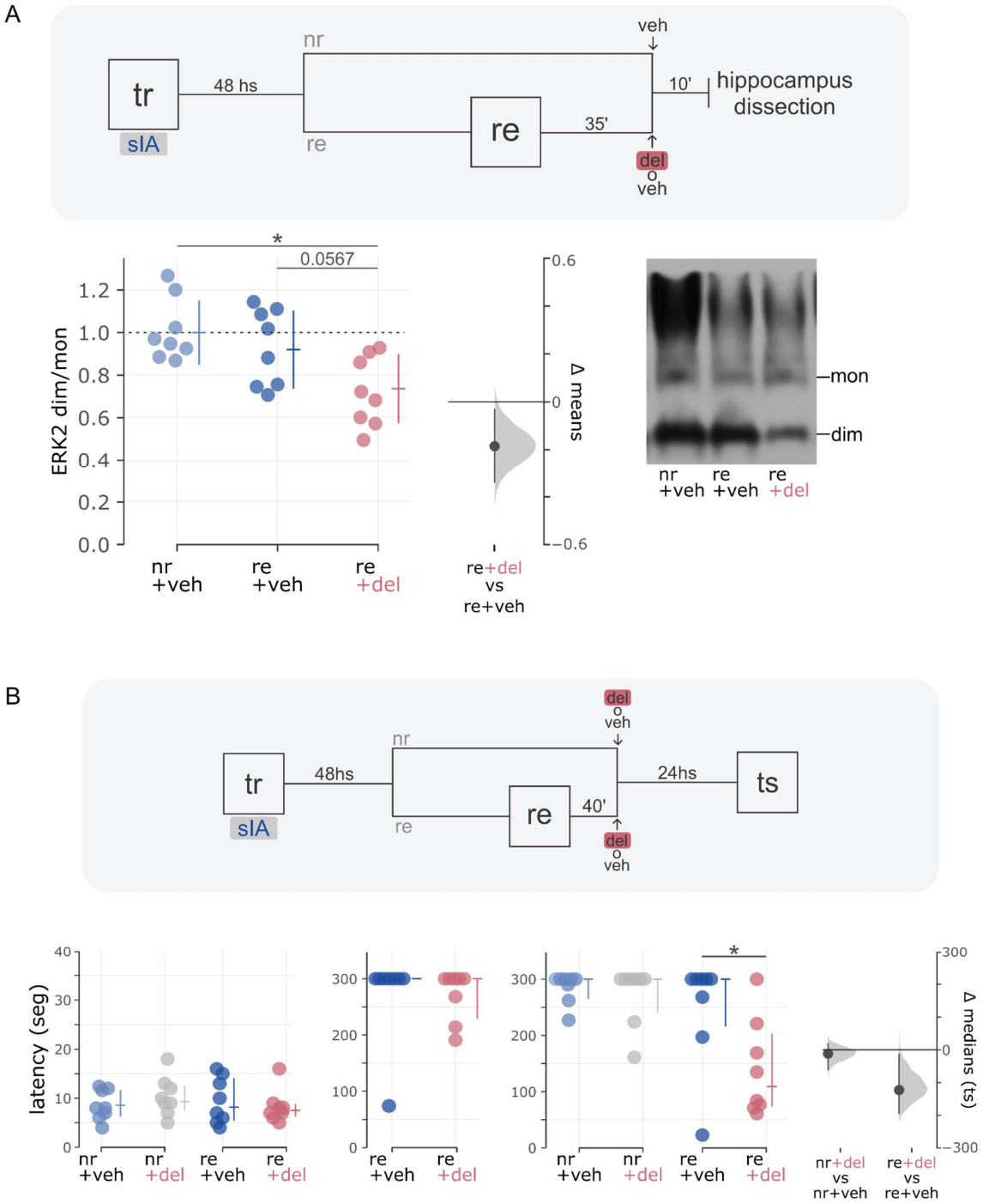


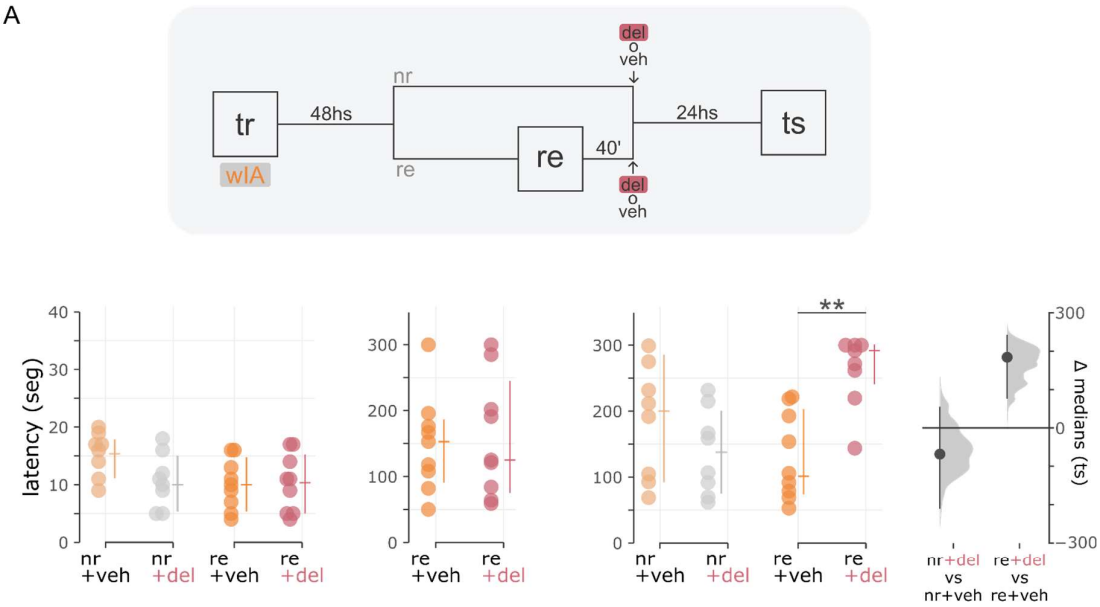
Figure 3. Role of hippocampal ERK2 dimerization after strong IA memory reactivation. **A)** DEL decreases hippocampal ERK2 dimerization after strong IA memory reactivation. Schematic experimental design for hippocampal ERK2 dimerization assessment is shown **(top)**. Hippocampal ERK2 dimerization was assessed 45 min after strong IA memory reactivation and 10 min after VEH or DEL intra-hippocampal administration. Dimer to monomer ratios were normalized against nr mean value for each experiment. Color circles indicate individual values and error bars shown on the right of each dataset depict mean ± s.d. values. Estimation plots shown on the right of each graph represent the difference between means (black dot) and the bootstrapped distribution of means (grey area) with 95% confidence intervals. Representative western blots are also shown (right). *, $p < 0.05$; **, $p < 0.01$ in Tukey adjusted *post hoc* comparisons. n = 8 / group. **B)** Inhibition of hippocampal ERK2 dimerization after strong

IA memory reactivation impairs LTM. Schematic experimental design is shown **(top)**. Color circles indicate individual latencies to step through values and error bars shown on the right of each set depict median ± inter quartile range (IQR). Estimation plots shown on the right of ts graph represent the difference between means (black dot) and the bootstrapped distribution of means (grey area) with 95% confidence intervals. *****, $p < 0.05$; ******, $p < 0.01$ in Tukey adjusted *post hoc* comparisons. n = 8 / group.

Inhibiting hippocampal ERK dimerization after reactivation strengthens weak IA memories

We showed that wIA memory reactivation induced a decrease in ERK2 dim/mon ratio in mice hippocampi (fig. 2B). Consequently, we wondered whether intra-hippocampal DEL administration after wIA reactivation would have any consequence on LTM retention. We trained 4 groups of animals in a wIA task and submitted two of them to memory reactivation (RE) 48 h later. Performance during training was similar in all groups. As expected, RE groups expressed median latencies of ≈150 sec during the reactivation session. One NR and one RE groups of animals were administered VEH and the other two groups were injected DEL in the dorsal hippocampus 40 min after the reactivation session. All groups were tested 24 h later (fig. 4A). Latencies to step through during the test session exhibited a significant interaction between reactivation procedure and drug treatment ($F_{2,30} = 13.254$; $p = 0.001$). Surprisingly, in RE-animals, the DEL-injected group showed significantly higher latencies than the VEH-injected one ($p = 0.005$), and this effect was specific for the reactivated groups as NR animals did not show significant differences in latencies between VEH and DEL treatment during test ($p = 0.379$). Moreover, VEH-treated groups did not differ statistically in their latencies during the test session ($p = 0.273$). Not only did intrahippocampal DEL administration after wIA memory reactivation improve retention, but it also seemed to have drastically increased the proportion of animals exhibiting higher latencies during TS compared to RE (suppl. fig.2, right panel). Notably, a significant positive correlation was observed between RE and TS response levels in DEL-RE animals (suppl. fig. 2; $R^2 = 0.73$; $p = 0.025$), which differed from the VEH-RE group (suppl. fig. 2; $R^2 = 0.42$; $p = 0.270$).

A



B

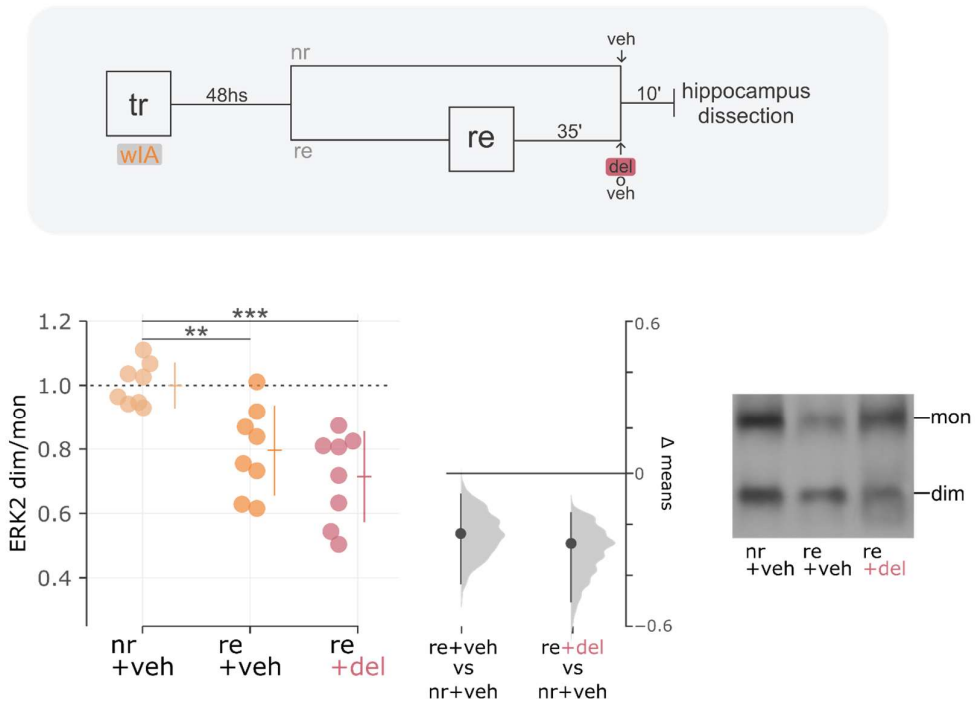


Figure 4. Role of hippocampal ERK2 dimerization after wIA memory reactivation. **A)** Inhibition of hippocampal ERK2 dimerization after strong IA memory reactivation improves LTM. Schematic experimental design is shown on top. Color circles indicate individual latencies to step through values during tr (left), re (center) and ts (right). Error bars shown on the right of each dataset depict median ± IQR. Estimation plots shown on the right of ts graph represent the difference between means (black dot) and the bootstrapped distribution of means (grey area) with 95% confidence intervals. *, $p < 0.05$; **, $p < 0.01$ in Tukey adjusted *post hoc* comparisons. n = 8 - 9 / group. **B)** Hippocampal ERK2 dimerization decreases after wIA memory reactivation. Schematic experimental design is shown on top. Color circles indicate individual values and error bars shown on the right of each dataset depict mean ± s.d. values. Estimation plots shown on the right of each graph represent the difference between means

437 (black dot) and the bootstrapped distribution of means (grey area) with 95% confidence intervals. Representative
438 western blots are also shown (right). *, $p < 0.05$; **, $p < 0.01$ in Tukey adjusted *post hoc* comparisons. n = 8 / group.

439 Finally, ERK2 dimerization levels were significantly affected by reactivation also in VEH/DEL-
440 injected animals as well ($F_{2,21} = 13.254$; $p = 0.0002$). In contrast to what we observed after sIA
441 training, wIA memory reactivation led to a significant decrease in ERK2 dimerization (see fig.
442 2B) which was also observed after VEH-administration in mice hippocampi (fig. 4B; $p =$
443 0.0064). However, DEL injection did not significantly affect ERK2 dimerization levels following
444 memory reactivation ($p = 0.3753$), though these levels were significantly different from those
445 in the control group NR-VEH ($p = 0.0003$).

446

447 **DISCUSSION**

448 In this work, we show for the first time that ERK2 dimerization constitutes a key regulatory step
449 involved in plasticity and memory processes. Several lines of evidence indicate that
450 subcellular localization of a particular intracellular signaling pathway activity is crucial for a
451 certain biological outcome[12,53,54]. In the central nervous system, for example, it was shown that
452 NF-kappaB might be regulating different phenomena related to neural plasticity, memory and
453 learning processes depending on its subcellular localization[55]. Over the past decade,
454 oncological research has advanced the development of drugs targeting key pathways to
455 reverse pathological conditions. Interestingly, many of these pathways have also been
456 implicated in learning and memory processes. However, their regulatory mechanisms within
457 the nervous system often remain poorly understood. ERK signaling serves as a notable
458 example. ERK phosphorylation is well known to be essential for neural plasticity and cognitive
459 processes, and its deregulation often leads to pathological conditions[9]. Interestingly, ERK
460 dimerization has been proposed to regulate the subcellular location of its activity, thereby
461 influencing the phosphorylation of specific targets and ultimately the cellular fate[12,13]. However,
462 despite extensive studies on ERK activation in neural tissue[reviewed in 9], the functional role of
463 ERK dimerization in the nervous system remains largely unexplored.

464 Consequently, as an initial step, we demonstrated that ERK dimerization occurs in neural
465 tissue, both *in vitro* using primary neuronal cultures and *in vivo* within the hippocampus of CF-
466 1 mice. Primary neuronal cultures stimulated with either EGF or a glycine-based cLTP protocol
467 showed increased ERK2 dimerization which was inhibited by DEL pre-treatment (fig. 1A).
468 Moreover, upon EGF stimulation, DEL treatment inhibited *in vivo* hippocampal ERK2
469 dimerization without affecting its phosphorylation (fig. 1B and 1C). Previous research has
470 shown that EGF stimulation induces ERK dimerization in cancer cells[13]. In physiological
471 contexts, the concentration of EGF plays a critical role in determining cellular responses, with
472 variations in EGF dosage influencing the transition between migratory and proliferative
473 states[56]. Thus, low EGF concentration, typical of paracrine signaling in early stages of wound
474 closure, is inadequate to trigger robust proliferative ERK signals in the cytoplasm. However, it
475 leads to pro-migratory signaling, inducing an increase in cell migration dependent on KSR-
476 ERK activation at the plasma membrane[56]. The EGF concentration used in our culture
477 experiments was consistent with that used by Herrero and colleagues[13] for HEK293T cells
478 and with concentrations promoting proliferation in MCF7 cells[56]. In this study, we replicated
479 Herrero and colleagues´ experiments, which also served as a control to evaluate ERK2
480 dimerization in the nervous tissue of rodents (fig. 1A). EGF has been demonstrated to induce

481 rapid and transient ERK phosphorylation in PC12 cells, which promotes cell proliferation[50].
482 We observed similar kinetics in our study. As many other tissues, brain cells express EGF
483 receptors and its activation regulates neurite outgrowth, as well as other outputs related to
484 neuronal homeostasis[57]. However, it is well known that different ligands are capable of
485 inducing different ERK activation kinetics and cellular outputs[58]. Recent findings from research
486 on breast tumors demonstrated that disrupting ERK dimerization, which was ligand-
487 dependent, hinders cellular motility by impairing the molecular machinery responsible for actin
488 cytoskeleton rearrangements[59]. It remains to be determined how ERK dimerization is altered
489 according to ligand specificity and its biological consequences, specifically in neurons.

490 ERK dimers in plasticity

491 We have recently discussed the role of ERK signaling in plasticity[9]. Hippocampal plasticity has
492 been proposed as a cellular correlate of experience-coding leading to memory formation and
493 storage. Then, a plausible hypothesis suggests that ERK dimerization might regulate memory
494 processes. Since the initial work by English and Sweatt[3] showing that ERK inhibition blocked
495 HFS-induced LTP in rat hippocampal area CA1, several authors have provided evidence in
496 favor of its participation in these processes[5,17,60,61] and, as stated before, different stimuli acting
497 on the same intracellular signaling pathway might cause alternative cellular fates depending
498 on the set of scaffolding proteins engaged[12]. As a general hallmark, it has been shown that
499 HFS protocol applied in the Schaffer Collaterals inputs to CA1 area induced ERK2
500 phosphorylation in rodents, and blocking ERK phosphorylation previous to LTP induction
501 prevented longer lasting forms of LTP[3]. In this study, we demonstrated that DEL pre-treatment
502 on acute slices allowed potentiation induction after HFS, but impaired normal hippocampal
503 LTP. In combination with previous work, these findings suggest that both ERK phosphorylation
504 and dimerization are crucial for the maintenance of LTP. Additionally, since ERK dimerization
505 and phosphorylation target different neuronal compartments, it is possible that at least two
506 distinct pathways are triggered during ERK activation. Moreover, memory processes have
507 been shown to involve the activation of multiple receptor systems, which in turn can activate
508 other molecular pathways that influence ERK signaling ultimately affecting its kinetics and
509 downstream targets. In consequence, it can be expected that ERK2 dimerization might also
510 be involved in LTP induction.

511 *ERK dimerization in IA LTM reconsolidation*

512 To this day, ERK signaling has been classically considered a key pathway for LTM
513 consolidation[5–8,27,61]. It has also been proposed as one of the molecules involved in memory
514 labilization and reconsolidation, becoming activated in the same set of neurons previously
515 engaged during conditioning[62]. It is worth noting that ERK's role in plasticity and memory
516 processes has been attributed almost exclusively to phosphorylation, leaving the potential
517 involvement of other post-translational modifications in regulating these processes largely
518 unexplored. Our results show that hippocampal inhibition of ERK dimerization impairs sIA
519 memory reconsolidation. However, the same manipulation of the pathway induced memory
520 enhancement when a wIA task was used, suggesting that the same brain structure might
521 engage opposing mechanisms depending on the strength of the consolidated memory. These
522 results are consistent with the idea that the restabilization process that takes place after either
523 sIA or wIA memory retrieval would be extremely specific. Based on this rationale, memory
524 restabilization might, at some point, quantitatively engage ERK signaling to guide behavioral

output. Consistent with this idea, it has been reported that pharmacological inhibition of ERK dimerization increases its nuclear signaling[11]. Whether this mechanism might lead to memory enhancement is still uncertain. Further investigation will either discard or confirm this possibility. On the other hand, the scenario in which sIA and wIA retrieval/reconsolidation might recruit distinct neural circuits cannot be discarded. Alternatively, different stimuli (e.g. ligand identity and/or concentration) may activate distinct components from ERK pathways which could in turn be transduced in the involvement of spatially distinct ERK pools that regulate different cellular outputs. For instance, a strong stimulus could trigger protein translation via ERK activation and promote gene transcription via ERK nuclear translocation. In this scenario, ERK inhibition would impair memory by disrupting these mechanisms. Conversely, after a weak stimulus, ERK inhibition might enhance memory by promoting protein synthesis through alternative pathways. This could compensate for reduced ERK activation and facilitate memory reconsolidation. Supporting this hypothesis, it was observed that protein synthesis inhibition after reactivation exerted differential effects on memory restabilization of an IA task in mice according to the area infused. Thus, protein synthesis immediately after reactivation was required in the amygdala in order to support memory reconsolidation while in the hippocampus and mPFC it was necessary to enhance retention[63]. Further experiments are necessary in order to support this proposal.

Our experiments also confirmed that the effect of hippocampal ERK dimerization is specific to the reconsolidation process by showing that NR animals´ latencies to step through were not affected by intra-hippocampal DEL injection (figures 3B and 4A). Regarding sIA memory impairment following DEL administration, we observed persistent impairment after one week (suppl. fig. 1), suggesting that there was no spontaneous recovery of the original memory which would have suggested the possibility of the enhancement of an extinction process.

In the present manuscript, we showed for the first time evidence of ERK2 dimerization playing a critical role in the central nervous system. Our findings reveal that ERK2 dimerization is essential for hippocampal plasticity and memory reconsolidation, introducing a novel mechanism of ERK2 regulation in the brain. Future studies will likely elucidate the other molecular players recruited by this mechanism.

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Author Contributions

S.O.R. performed experiments, analyzed data, and contributed to write parts of the manuscript. C.M. performed behavioral experiments, analyzed data, and reviewed the manuscript. M.C.K., J.M. and M.F.A. performed experiments and reviewed the manuscript. M.V.B. performed experiments and contributed to write parts of the manuscript. M.M.B contributed to design the experiments, performed behavioral experiments and interpreted data. A.G.R contributed to data analysis and reviewed the manuscript. F.J.U. & S.O.R.

568 designed and performed electrophysiology experiments in slices, interpreted data. M.F.
569 proposed the original research project, designed experiments and performed biochemical
570 assays, interpreted data, and drafted the manuscript.

571 **Conflicts of Interest**

572 The authors declare no conflicts of interest.

573

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