

1 **Two spaced training trials induce associative ERK-dependent long-**  
2 **term memory in *Neohelice granulata*.**

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15 ABSTRACT

16 **Memory formation depends upon several parametric training conditions. Among**  
17 **them, trial number and inter-trial interval (ITI) are key factors to induce long-term**  
18 **retention. However, it is still unclear how individual training trials contribute to**  
19 **mechanisms underlying memory formation and stabilization. Contextual conditioning**  
20 **in *Neohelice granulata* has traditionally elicited associative long-term memory (LTM)**  
21 **after 15 spaced (ITI = 3 min) trials. Here, we show that LTM in crabs can be induced**  
22 **after only two training trials by increasing the ITI to 45 min (2t-LTM) and maintaining**  
23 **the same training duration as in traditional protocols. This new LTM observed was**  
24 **preserved for at least 96 h, exhibited protein synthesis dependence during**  
25 **consolidation and reconsolidation as well as context-specificity. Moreover, we**  
26 **demonstrate that 2t-LTM depends on inter-trial and post-training ERK activation**  
27 **showing a faster phosphorylation after the second trial compared to the first one. In**  
28 **summary, we present a new training protocol in crabs with reduced number of trials**  
29 **that shows associative features similar to traditional spaced training. This novel**  
30 **protocol allows intra-training manipulation and the assessment of individual trial**  
31 **contribution to LTM formation.**

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34 Keywords: CRAB, SPACING EFFECT, CONSOLIDATION, RECONSOLIDATION, PD  
35 98059, MAPK.

36

37 Abbreviations:

38 2t-LTM, two trial-long term memory; CHX, Cycloheximide; CPC, contextual pavlovian  
39 conditioning; CS, conditioned stimulus; CT, control group, ERK, extracellular signal-  
40 regulated kinase; ITI, inter-trial interval; LTM, long term memory; LTS, long-term  
41 sensitization; MAPK, mitogen-activated protein kinase; PD, PD 98059; pERK, phosphor-  
42 ERK; tERK, total ERK; TR, trained group; US, unconditioned stimulus; VEH, vehicle.

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## 45 1. INTRODUCTION

46 Memory processes require the activation of several transduction pathways that lead  
47 to post-translational modifications of proteins and gene expression regulation (Dudai, 2012),  
48 thus promoting the stabilization of the memory trace. Training protocols that integrate  
49 multiple trials spaced over time are more effective in the induction of long-term memory  
50 (LTM) than protocols involving massed presentation of training trials. This ubiquitous  
51 behavioral occurrence known as spacing effect was first described by Ebbinghaus (1885),  
52 and has since been observed in different species (Aziz et al., 2014; Bello-Medina et al.,  
53 2013; Pagani et al., 2009; Philips et al., 2007; Toda et al., 2009; Vlach et al., 2008). LTM  
54 formation has been shown to be induced by specific time intervals between trials in very  
55 different learning and memory animal paradigms including vertebrates (Bolding & Rudy,  
56 2006; Klapdor & van der Staay, 1998; Williams et al., 1991) as well as invertebrates (Carew  
57 et al., 1972; Gerber et al., 1998; Lukowiak et al., 1998; Maldonado et al., 1997; Rogers et al.,  
58 1994; Tully et al., 1994). However, while the spacing effect has been thoroughly  
59 characterized from a behavioral standpoint very little is known about the molecular and  
60 synaptic mechanisms that underlie this phenomenon. Work addressing this question in the  
61 mollusk *Aplysia californica* showed that there is a narrow permissive window (45 min) on the  
62 spacing of two-trial training in order to induce long term sensitization (LTS) (Philips et al.,  
63 2007, 2013). This memory process seems to be mediated by extracellular signal-regulated  
64 kinase/mitogen-activated protein kinase (ERK/MAPK) phosphorylation and nuclear  
65 translocation. Interestingly, activation of ERK by the first trial was not sufficient to induce LTS  
66 and the second trial was necessary to prompt it, suggesting that the molecular machinery  
67 recruited by the first trial is required to interact with that triggered by the second trial in order  
68 to elicit LTS.

69 In line with this notion, work in *Drosophila melanogaster* proposed that spaced  
70 training protocols generate repetitive waves of MAPK activation defined by the duration of

71 inter-trial intervals (ITIs), while massed training induced only one peak of MAPK activation  
72 after the last stimulus (Pagani et al., 2009). Accordingly, spaced training induced MAPK-  
73 dependent activation of c-Fos and CREB, two known ERK targets involved in LTM  
74 consolidation (Cammarota et al., 2000; Dash et al., 1995), but not after massed training  
75 (Miyashita et al., 2018). These results support the idea that spaced training is more effective  
76 than massed training due to recruitment of specific molecular and cellular mechanisms  
77 shown to support LTM (Naqib et al., 2012).

78 A large body of work has extensively studied the memory processes and their  
79 molecular basis on the crab *Neohelice granulata* (formerly *Chasmagnathus granulatus*) (Feld  
80 et al., 2005, 2008; Frenkel et al., 2002; Freudenthal & Romano, 2000; Locatelli & Romano,  
81 2005; María Eugenia Pedreira et al., 2004; María Eugenia Pedreira & Maldonado, 2003;  
82 Arturo Romano et al., 2006; Tomsic et al., 2003). The behavioral approach to study LTM of  
83 the crab *Neohelice* takes advantage of the crab's innate escape response elicited by the  
84 presentation of a visual danger stimulus (unconditioned stimulus, US). Training sessions  
85 typically consist of 15 trials of CS-US presentation spaced by 3 min ITI (Fustiñana et al.,  
86 2013; Maldonado, 2002) and LTM is robustly evidenced 24 h later by a decrease on the  
87 escape response that can last for up to 5 days. LTM is protein synthesis-dependent, context-  
88 specific, sensitive to labilization/reconsolidation and extinction. However, the number of trials  
89 needed and the relatively short ITI pose a complication when studying the individual trial  
90 contribution to molecular mechanisms. Here, we present a newly developed training protocol  
91 consisting of only two conditioning trials spaced by 45 min that elicits robust LTM and offers  
92 a valuable tool for studying the single contribution of individual trial to memory formation. We  
93 further show this memory to be context-specific, protein synthesis-dependent and mediated  
94 by ERK activation. Memory reactivation upon a unique CS presentation 24 h after training  
95 session suggest two-trial elicited LTM (2t-LTM) can be rendered labile again and must  
96 undergo reconsolidation to restabilize while protein synthesis inhibition blocked memory  
97 expression 24 h afterwards. Finally, we assessed 2t-LTM ERK kinetics and discussed the  
98 molecular implications of the spacing effect observed in different tasks, including learning

99 and memory in *Neohelice*. Altogether, our results show that 2t-LTM improves the possibility  
100 of pharmacological manipulations during the 45 min ITI and provides insight on individual  
101 trial input to unravel the molecular mechanisms behind the spacing effect.

## 102 2. MATERIAL AND METHODS

### 103 2.1. Animals

104 Adult male *Neohelice granulata* measuring 2.7–3.0 cm across the carapace and  
105 weighing an average of  $14.97 \pm 0.47$ g were collected from narrow coastal inlets of San  
106 Clemente del Tuyú, Argentina, and transported to the laboratory where they were housed in  
107 plastic tanks (32 × 46 × 20 cm, 20 animals per tank) filled to a depth of 1 cm with 12 ‰  
108 diluted seawater (prepared from Cristalsea Marinemix salts, USA).

109 The holding and experimental rooms were kept on a 12 h light-dark cycle (lights on  
110 from 08:00 am to 20:00 pm) and the temperature was set on a range of 22-24 °C.  
111 Experiments were carried out within the first week after capture and each crab was used in  
112 only one experiment. Animals were maintained and experiments were conducted in  
113 accordance with the National Research Council Guide for the Care and Use of Laboratory  
114 Animals (USA) as well as Argentinean guidelines for ethical use of laboratory animals.

115

### 116 2.2. Drugs and injection procedure

117 Dimethyl sulfoxide (DMSO, Anedra, Argentina) was used as vehicle (VEH). The VEH  
118 or drug solutions were injected through the right side of the dorsal cephalotoracic-abdominal  
119 membrane using a Hamilton syringe with a needle fitted with a plastic cannula to control the  
120 penetration depth to 4 mm, ensuring that the desired solution is injected into the pericardial  
121 sac. The total volume of haemolymph in a crab has been estimated in 5 ml (30 % of the  
122 body weight; Gleeson and Zubkoff, 1977) and 10ul of VEH or drug were injected, resulting in  
123 a dilution of approximately 500-fold for DMSO of injected drugs.

124 The following drugs were used:

125 A stock solution (74.8 mM) of the MEK inhibitor 2-(2-amino-3-methoxyphenyl)-4H-1-  
126 benzopyran-4-one (PD98059, PD hereafter, Sigma-Aldrich, Argentina) was conserved at -20  
127 °C and freshly diluted in DMSO to the desired concentration (11.22 mM, final dose of 1.765  
128 µg/g) the day of the experiment. The protein synthesis inhibitor Cycloheximide (CHX, Sigma-

129 Aldrich, Argentina) was diluted in DMSO on the day of the experiment and 10  $\mu$ l were  
130 injected (40  $\mu$ g/crab, a dose commonly used at our lab, Pérez-Cuesta & Maldonado, 2009).

131

### 132 **2.3. Experimental device**

133 The experimental device used to train and to test animals, called actometer, consists  
134 of a bowl-shaped plastic container where one crab is placed. The unconditioned stimulus  
135 (US) consists of an opaque rectangular screen that moves horizontally over the animal and  
136 provokes the crab's innate running response (Maldonado et al., 1997). Two light sources  
137 allow changing the context by illuminating the actometer from above (upper light) or below  
138 (lower light) the container. Vibrations produced by the animal movement were registered by  
139 four microphones attached to the base of the container. This signal was amplified, integrated  
140 during the entire trial and translated into arbitrary numerical units ranging from 0 to 22,500  
141 by a computer.

142

### 143 **2.4. Behavioral procedures**

144 Each crab was placed in a container and initially adapted to it during 13 min with  
145 lower light. Each trial started with an illumination switch from lower light to upper light (CS),  
146 18 sec prior to the screen movement. Two successive events of horizontal cyclical screen  
147 movement drawing a 90° angle and lasting 9 sec defined the US ending together with a new  
148 light switch from upper to lower. During intertrial intervals (ITIs), the actometers remained  
149 illuminated with the lower light. Trial sequences, illumination, duration and ITI were  
150 programmed and controlled by the registering computer. The experimental room contained  
151 forty experimental devices separated from each other by partitions and allowed training or  
152 testing of 40 crabs simultaneously.

153 Training: Each training session consisted of two trials and a 45 min ITI, unless stated  
154 otherwise. In all experiments one group was trained (TR) while the other group underwent  
155 the same manipulation but didn't receive any US (control group, CT). In drug administration

156 experiments two pairs of CT and TR groups were injected with either drug or VEH. Animals  
157 were randomly assigned to the experimental groups.

158       Reactivation: Twenty-four hours after training animals were placed in the  
159 experimental device and after the adaptation period the upper lights turned on for 27 sec,  
160 without US presentation. This protocol has been found effective to trigger labilization of the  
161 consolidated LTM (Fustiñana et al., 2013).

162       Testing: Testing for LTM began 24 h after training or reactivation. During the testing  
163 session animals were placed in the actometer for 10 min adaptation and later received three  
164 spaced trials (ITI: 153 sec).

165

## 166 **2.5. Procedure for ERK/MAPK phosphorylation determination**

167 Animals were anaesthetized by immersion in iced marine water for five minutes and central  
168 brain dissection was performed. Each central brain was homogenized with 15 strokes in a  
169 Dounce Tight homogenizer with 50 µl of Buffer A (10 mM Hepes pH 7.9; 1,5 mM MgCl<sub>2</sub>; 10  
170 mM KCl; 1 mM DTT; 1 µg/ml Pepstatin A; 10 µg/ml Leupeptin; 0,5 mM PMSF; 10 µg/ml  
171 Aprotinin; 1 mM sodium orthovanadate and 50 mM sodium fluoride). The homogenate was  
172 centrifuged for 15 min at 16000 x g and the supernatant was aliquoted and kept at -20 °C  
173 until used. All the extraction protocol was performed at 4 °C.

174 Fifteen µl of sample were run in 12.5% SDS-PAGE gels at 100V for 2 h and then blotted to  
175 0.45 µm low-fluorescence PVDF membrane (Millipore-Merck, USA) at 100V for 50 min.  
176 Membranes were then blocked in 5% nonfat dry milk in Tris Buffered Saline-0.1 % Tween 20  
177 (TTBS) and incubated with primary antibodies against phospho-ERK (pERK, Santa Cruz  
178 Biotechnology, sc-7383, 1:500) and total ERK (ERK, Cell Signaling Technologies, cat.  
179 #9102, 1:1000) in TTBS at 4 °C. Detection was performed using IRDye secondary  
180 antibodies (Li-Cor, USA) at a 1:10,000 dilution, using LI-COR Odyssey Imaging System.  
181 Secondary antibodies used were 800CW Donkey anti-Mouse IgG (cat. 926-32212) and  
182 680RD Donkey anti-Rabbit IgG (cat. 926-68073).



## 183 **2.6. Data Analysis**

184 LTM retention criterion was established as a statistically lower normalized response of the  
185 trained group compared against control groups in the first trial of the testing session. The  
186 escape response during each trial was normalized within animal, against each animal's  
187 maximum escape response. Therefore, the escape response during testing varied between  
188 the values 0 and 1. This procedure was done to account for differences between animals  
189 due to factors other than training (e.g. weight, stamina, overall health).

190 Since behavioral data violated normality and heteroscedasticity assumptions Mann-Whitney  
191 U test or Kruskal-Wallis test were used followed by Dunn's Multiple Comparison post-hoc  
192 tests. Unless stated otherwise, median +/- interquartile ranges of normalized escape  
193 response is shown.

194 In ERK/MAPK phosphorylation assays, Relative Optical Density (ROD) was quantified using  
195 NIH ImageJ v1.51j8 software (Schneider et al., 2012). Statistical analysis of the data  
196 consisted of ANOVA followed by Holm-Sidak's multiple *post hoc* comparisons between TR  
197 and CT groups.

198 All data analysis was performed using GraphPad Prism 8 software.

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## 200 3. RESULTS

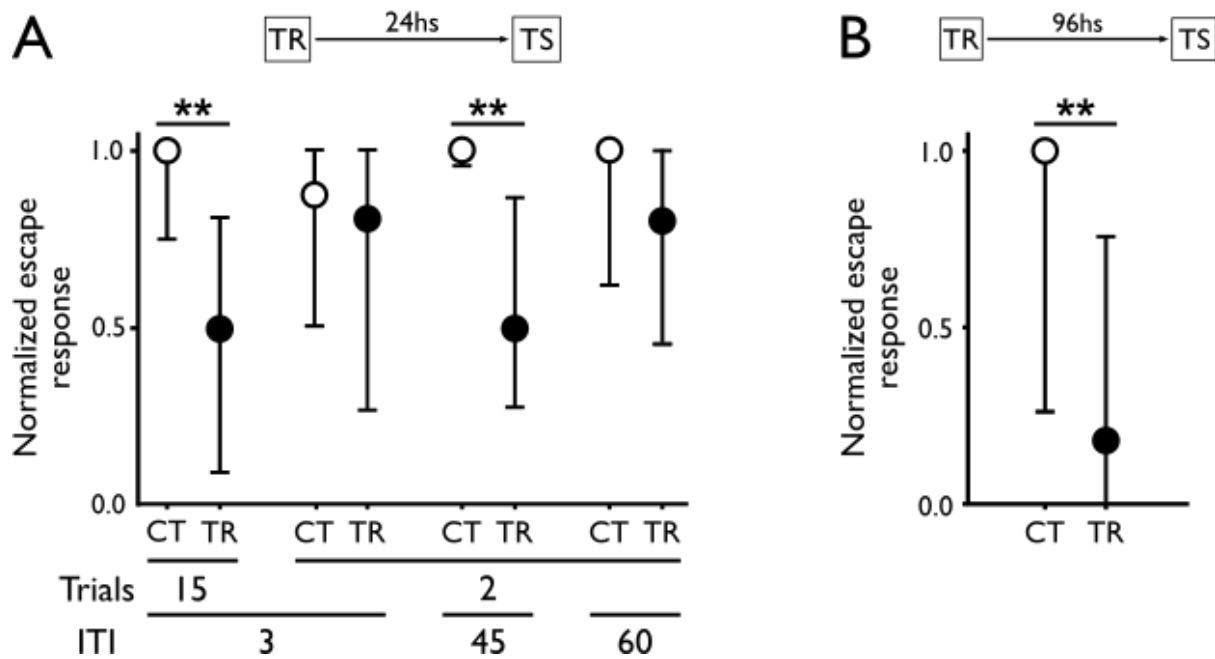
### 201 3.1. Two training trials spaced by 45 min elicit LTM formation

202 The traditional training protocol of contextual pavlovian conditioning (CPC) in *Neohelice*  
203 *granulata* involves 15 trials spaced by 3 min (Fustiñana et al., 2013). Compelling evidence  
204 supports the notion that given the adequate ITI, training protocols involving fewer trials would  
205 be as effective as higher numbers of stimuli to elicit LTM formation (Philips et al., 2007).  
206 Thus, we addressed the question whether two trials were enough to induce LTM in  
207 *Neohelice granulata*. Given the drastic reduction in the number of training trials, we  
208 examined whether increasing the ITI would lead to LTM formation, as described in previous  
209 studies. Therefore, we trained groups of animals with two trials (CS-US presentation) using  
210 three different ITIs (3, 45 and 60 min). We also trained a CPC group with 15 trials and 3 min  
211 ITI that served as a control for LTM (Fig. 1A). All trained groups (TR groups) were run in  
212 parallel to a control group (CT groups), subjected to the same experimental conditions but  
213 without US presentation. All groups were tested 24 h after training for LTM.

214 In line with earlier work from our lab (Fustiñana et al., 2013), CPC-trained animals (15 trials,  
215 3 min ITI) showed memory retention during the testing session, evidenced by the significant  
216 lower escape response observed between this group and its respective control (Fig. 1A; CT  
217 vs TR,  $p = 0.0016$ ). Strikingly, two-trial training were sufficient to elicit LTM when the ITI was  
218 45 min (CT vs TR,  $p = 0.0110$ ; from now on referred to as 2t-LTM) but not when they were  
219 spaced by 3 (CT vs TR,  $p > 0.9999$ ) or 60 min (CT vs TR,  $p = 0.9770$ ), consistent with  
220 previous findings on spacing effect.

221 To address the stability of the 2t-LTM, we tested memory retention 96 h after training.  
222 Interestingly, LTM was preserved 96 h after training (Fig. 1B,  $p = 0.0061$ ), suggesting 2t-  
223 LTM strength is conserved in spite of the reduction in trial number.

224



225

226 **Figure 1: Two trials spaced by 45 min induce LTM lasting for at least 96 h.** **A)** Normalized escape response  
227 in testing session (24 h after TR) from animals stimulated (TR groups, filled circles) or not (CT groups, empty  
228 circles) with the US using four different protocols: 15 trials with 3 min ITI or 2 trials with different ITIs (3; 45 or 60  
229 min). Kruskal-Wallis ( $H = 23.38$ ,  $df = 7$ ,  $p = 0.0015$ ,  $n = 16 - 20$  animals per group) followed by post hoc Dunn's  
230 multiple comparisons test.  $**p < 0.01$ . **B)** Normalized escape response in testing session (96 h after TR) from  
231 animals trained with 2 trials and 45 min ITI. Mann-Whitney test ( $U = 68$ ,  $**p < 0.01$ ;  $n = 16 - 18$  animals per group).

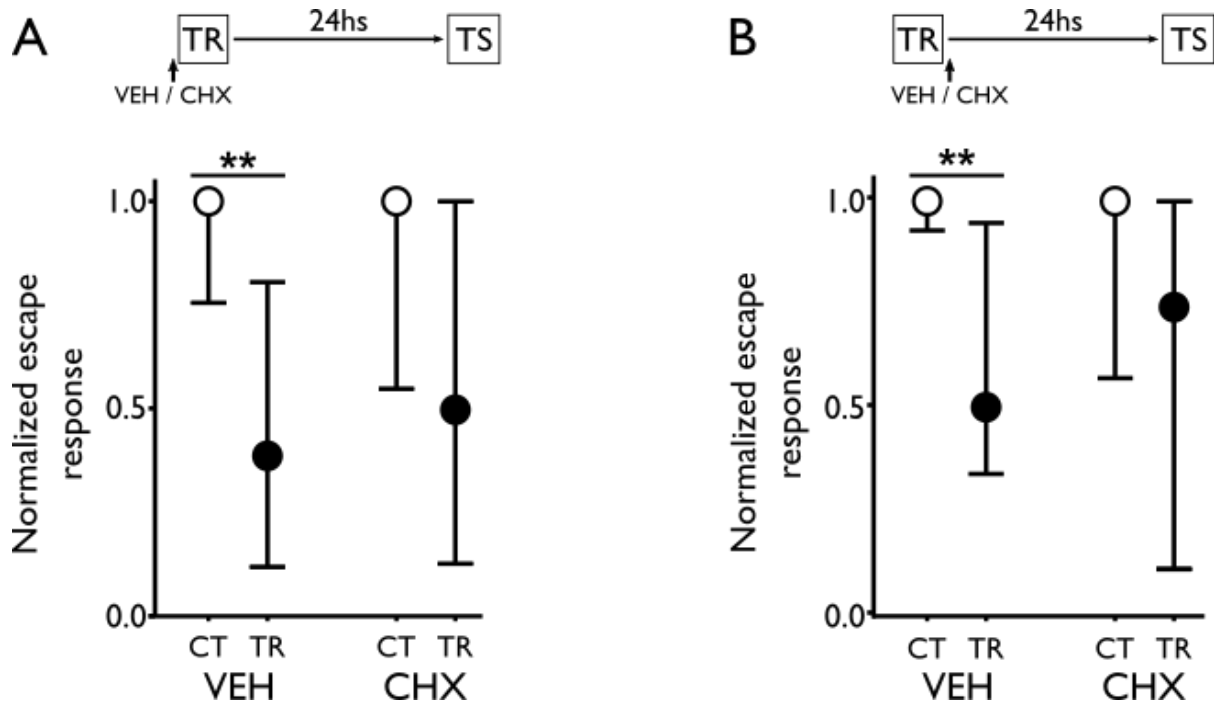
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### 233 3.2. 2t-LTM consolidation depends on protein synthesis

234 It is well established that LTM consolidation depends on protein synthesis in invertebrates as  
235 well as in vertebrates (Alberini, 2008; Pedreira et al., 1996). Thus, we tested whether protein  
236 synthesis is also required for 2t-LTM consolidation. Pairs of TR and CT groups were  
237 systemically administered with cycloheximide (CHX, a eukaryotic protein synthesis inhibitor)  
238 immediately before (Fig. 2A) or after (Fig. 2B) training. Both experiments included a pair of  
239 CT and TR groups injected with vehicle (VEH) at the same time points. While both pairs of  
240 VEH.TR groups showed robust memory retention ( $p = 0.0051$  and  $p = 0.0027$  for CT vs TR  
241 post hoc comparisons from pre- or post-TR injection, respectively), CHX administration  
242 impaired 2t-LTM formation when injected both before ( $p = 0.239$  for CT vs TR post hoc test)  
243 or after ( $p = 0.214$  for CT vs TR post hoc test) the training session (Fig. 2). These results

244 indicate that, comparable with training protocols of 15 trials with 3 min ITI, 2t-LTM is protein  
245 synthesis dependent.

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249 **Figure 2: 2t-LTM depends on protein synthesis.** CHX or VEH were administered immediately pre-TR ( $H =$   
250  $13.28$ ,  $df = 3$ ,  $p = 0.0041$ ,  $n = 18 - 20$  animals per group, **A**) or post-TR ( $H = 14.47$ ,  $df = 3$ ,  $p = 0.0023$ ,  $n = 17 -$   
251  $18$  animals per group, **B**) and animals were tested 24hs after TR. Kruskal-Wallis followed by post hoc Dunn's  
252 multiple comparisons test. TR groups, filled circles; CT groups, empty circles; \*\* $p < 0.01$ .

253

### 254 3.3. Retrieval of 2t-LTM induces the labilization/reconsolidation process

255 Associative LTMs are capable of being reactivated and labilized upon retrieval (Alberini &  
256 Ledoux, 2013). Labile memories are susceptible to disruption or updating (Forcato et al.,  
257 2011; Krawczyk et al., 2015; Fustiñana et al., 2014). If no disrupting process follows  
258 labilization, the memory becomes restabilized through a process called reconsolidation  
259 (Nader et al., 2000; Przybylski & Sara, 1997). Moreover, LTM reconsolidation has been  
260 demonstrated to be protein synthesis dependent in many animal species (Cai et al., 2012;  
261 Fustiñana et al., 2013; Nader et al., 2000; María Eugenia Pedreira et al., 2002).

262 In order to assess whether 2t-LTM is labilized after retrieval, we inhibited protein synthesis  
263 after memory reactivation. Twenty four hours after the training session, two pairs of TR and  
264 CT groups of animals were subjected to memory reactivation 20 min after injection of VEH or  
265 CHX. A third pair of TR and CT groups was injected and kept in the holding containers, to  
266 control for protein synthesis inhibition without reactivation (see schematic experimental  
267 design in Fig. 3). Twenty four hours after retrieval session, all groups were tested for LTM  
268 retention (Fig. 3). TR animals injected with VEH before re-exposure to the same context  
269 (SC.VEH) showed LTM retention when compared with the respective CT group (CT vs TR;  
270  $p = 0.006$ ; Fig. 3, SC.VEH), indicating that VEH injection did not affect memory  
271 reconsolidation. In contrast, CHX-injected animals re-exposed to the training context did not  
272 show significant differences in normalized escape response during testing session (CT vs  
273 TR;  $p = 0.187$ ; Fig. 3, SC.CHX). Furthermore, CHX administration without context re-  
274 exposure did not affect performance (CT vs TR;  $p = 0.0021$ ; Fig. 3, NR.CHX), suggesting  
275 that 2t-LTM reconsolidation process can be triggered specifically after CS-induced memory  
276 reactivation.

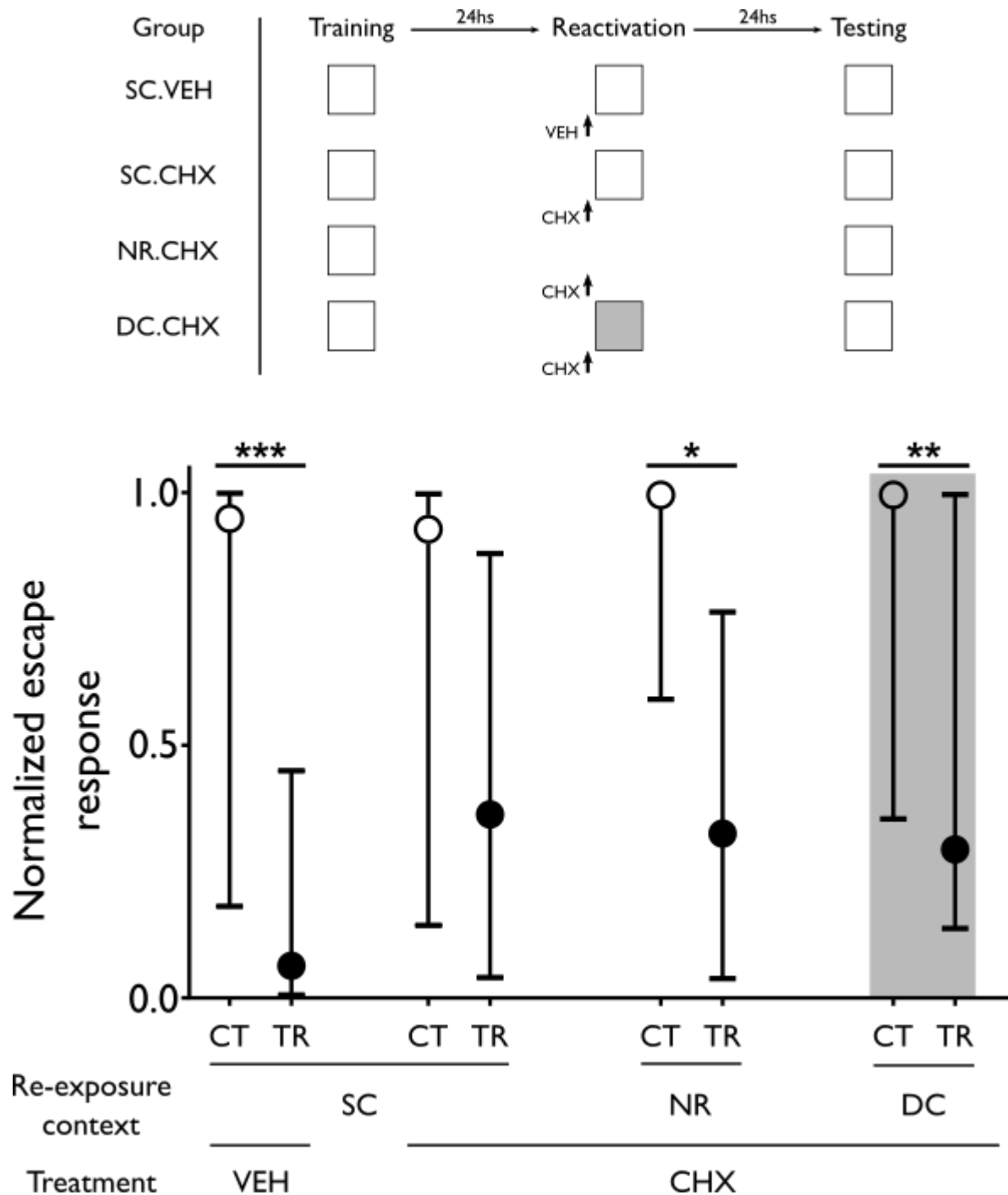
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### 278 **3.4. 2t-LTM is context-specific**

279 Several studies have shown that contextual memories can undergo  
280 labilization/reconsolidation only when subjects are briefly re-exposed to the training context,  
281 while exposure to a different context fails to reactivate the associative memory (de la Fuente  
282 et al., 2011; Fustiñana et al., 2013; María Eugenia Pedreira et al., 2002; Suzuki et al., 2004).  
283 Given that 2t-LTM can be labilized by re-exposing animals to the training context (Fig. 3,  
284 SC.CHX groups), this opens the question whether reactivation of this memory is specific to  
285 the TR context where the original CS-US association was established. To address this  
286 question, we included a fourth pair of CT and TR groups in the previous experiment which  
287 were re-exposed to a different context (DC) 20 min after CHX administration (Fig. 3,  
288 DC.CHX groups). We predicted that if 2t-LTM entails a specific association between the  
289 context and the US, exposure of animals to a different context 24 h after TR would not

290 labilize the original memory, and CHX injection would not have any effect on memory  
291 reconsolidation.

292 Consistent with our hypothesis that 2t-LTM reactivation is context-specific, memory was not  
293 impaired in DC.CHX pair of groups. Normalized escape response from TR group was  
294 significantly lower than the corresponding CT group when tested 24 h after being placed in a  
295 different context under the effect of the protein synthesis inhibitor ( $p = 0.043$ ; Fig 3,  
296 CHX.DC). Together these results show that 2t-LTM can undergo labilization/reconsolidation  
297 only after re-exposure to the same TR context while placement in a different CS (different  
298 context) failed to trigger these processes, further supporting context-specificity. Moreover, it  
299 discards possible spurious interactions between CS exposure and drug administration.



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**Figure 3: 2t-LTM is context-specific and its reconsolidation depends on protein synthesis.** CHX or VEH was administered 20 min before memory reactivation in the same context (SC). Pairs of CT and TR animal groups were injected with CHX and 20 min afterwards either subjected to the same manipulation but in a different context (DC) or not re-exposed to any context at all (NR). Schematic experimental design is shown in upper panel. Kruskal-Wallis ( $H = 42.88$ ,  $df = 7$ ,  $p < 0.0001$ ,  $n = 33 - 40$  animals per group) followed by post hoc Dunn's multiple comparisons test. TR groups filled circles; CT groups, empty circles; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

### 309 **3.5. 2t-LTM depends on ERK/MAPK pathway**

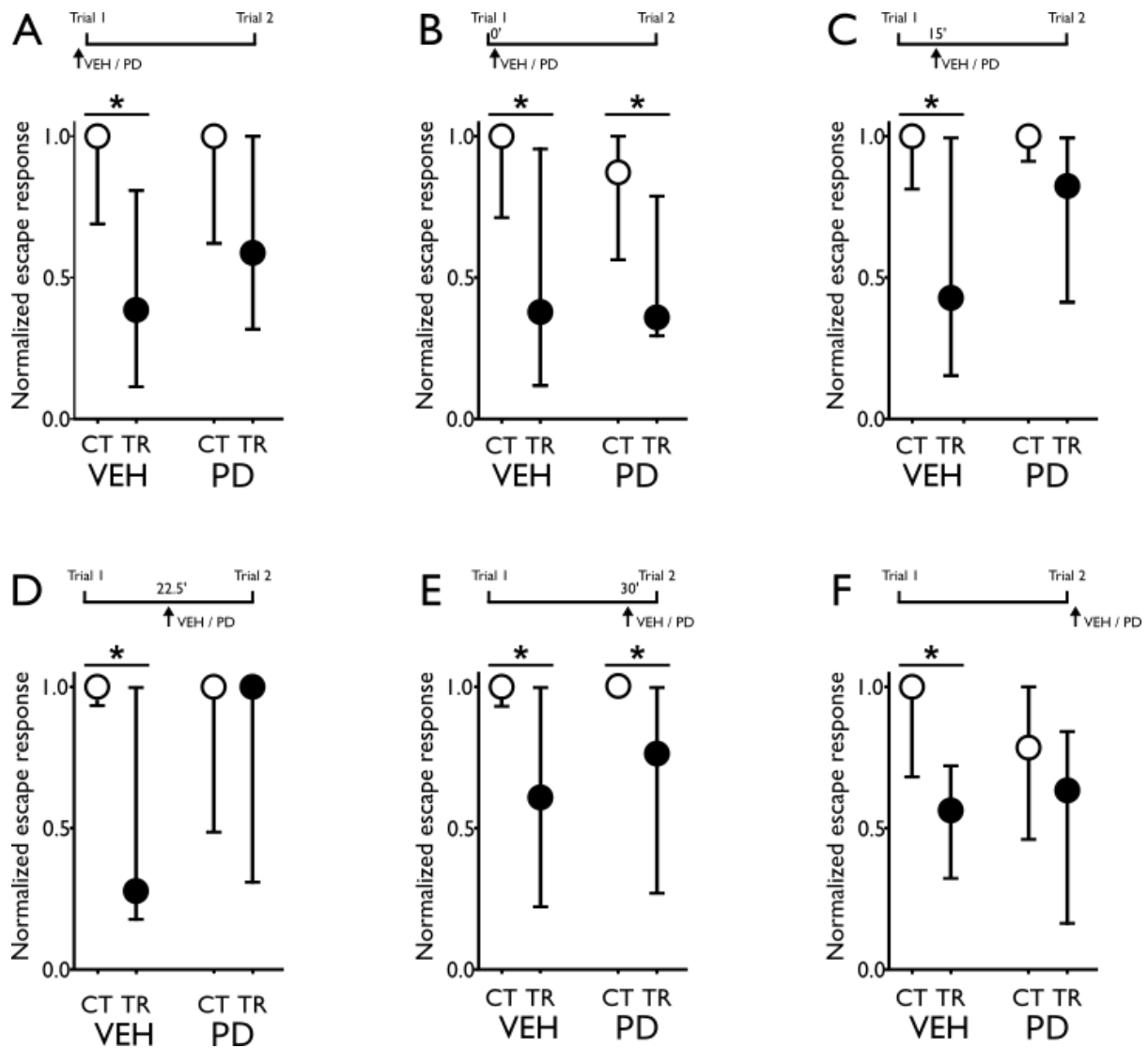
310 LTM in invertebrates has previously been linked to the activation of the MAPK pathway  
311 (Crow et al., 1998; Purcell et al., 2003; Sharma et al., 2003). Specifically, ERK involvement  
312 in LTM formation has already been described in *Neohelice* (Feld et al., 2005). According to  
313 previous reports, 2-trial/45 min-ITI memory in *Aplysia* (Philips et al., 2013) depends on a  
314 specific ERK activation kinetics. Consequently, we aimed at dissecting this signaling  
315 pathway participation in 2t-LTM.

316 In order to assess ERK contribution to 2t-LTM formation, we administered PD 98059 (PD), a  
317 MEK (ERK kinase) inhibitor, at different times before, during and after training (Fig. 4) at  
318 concentrations that had previously been established to efficiently inhibit ERK activity and to  
319 block LTM consolidation in *Neohelice granulata* (Feld et al., 2005).

320 Administration of PD both immediately before (Fig. 4A; CT.VEH vs TR.VEH,  $p = 0.011$ ;  
321 CT.PD vs TR.PD,  $p = 0.098$ ) and after training (Fig. 4F; CT.VEH vs TR.VEH  $p = 0.032$ ;  
322 CT.PD vs TR.PD  $p = 0.32$ ) had an amnesic effect, supporting ERK requirement during 2t-  
323 LTM formation.

324 However, PD administration at different times during ITI showed distinct effects on memory  
325 retention. It impaired LTM when administered at 15 (Fig. 4C; CT.VEH vs TR.VEH,  $p = 0.010$ ;  
326 CT.PD vs TR.PD,  $p = 0.355$ ) or 22.5 min (Fig. 4D; CT.VEH vs TR.VEH,  $p = 0.010$ ; CT.PD vs  
327 TR.PD,  $p > 0.999$ ), but not when injected immediately (Fig. 4B; CT.VEH vs TR.VEH,  $p =$   
328  $0.007$ ; CT.PD vs TR.PD,  $p = 0.02$ ) or 30 min (Fig. 4E; CT.VEH vs TR.VEH,  $p = 0.02$ ; CT.PD  
329 vs TR.PD,  $p = 0.007$ ) after the first trial. Thus, these results are in line with the hypothesis of  
330 tight temporal regulation of ERK activation during 2t-LTM formation.





331

332 **Figure 4: 2t-LTM depends on ERK/MAPK activation at specific timepoints.** PD 98059 (PD, a MEK inhibitor)  
 333 or VEH were administered immediately before ( $H = 14.4$ ,  $df = 3$ ,  $p = 0.0024$ ; **A**) or after ( $H = 10.29$ ,  $df = 3$ ,  $p =$   
 334  $0.0163$ ; **F**) training; or immediately after ( $H = 16.69$ ,  $df = 3$ ,  $p = 0.0008$ ; **B**); 15 min ( $H = 11.93$ ,  $df = 3$ ,  $p = 0.0076$ ;  
 335 **C**); 22.5 min ( $H = 8.12$ ,  $df = 3$ ,  $p = 0.039$ ; **D**) or 30 min ( $H = 16.38$ ,  $df = 3$ ,  $p = 0.0009$ ; **E**) after the first training trial  
 336 and 24 h later animals were tested for memory retention. Kruskal-Wallis followed by post hoc Dunn's multiple  
 337 comparisons test. TR groups, filled circles; CT groups, empty circles; \* $p < 0.05$ ; \*\* $p < 0.01$ ;  $n = 14-20$  animals per  
 338 group.

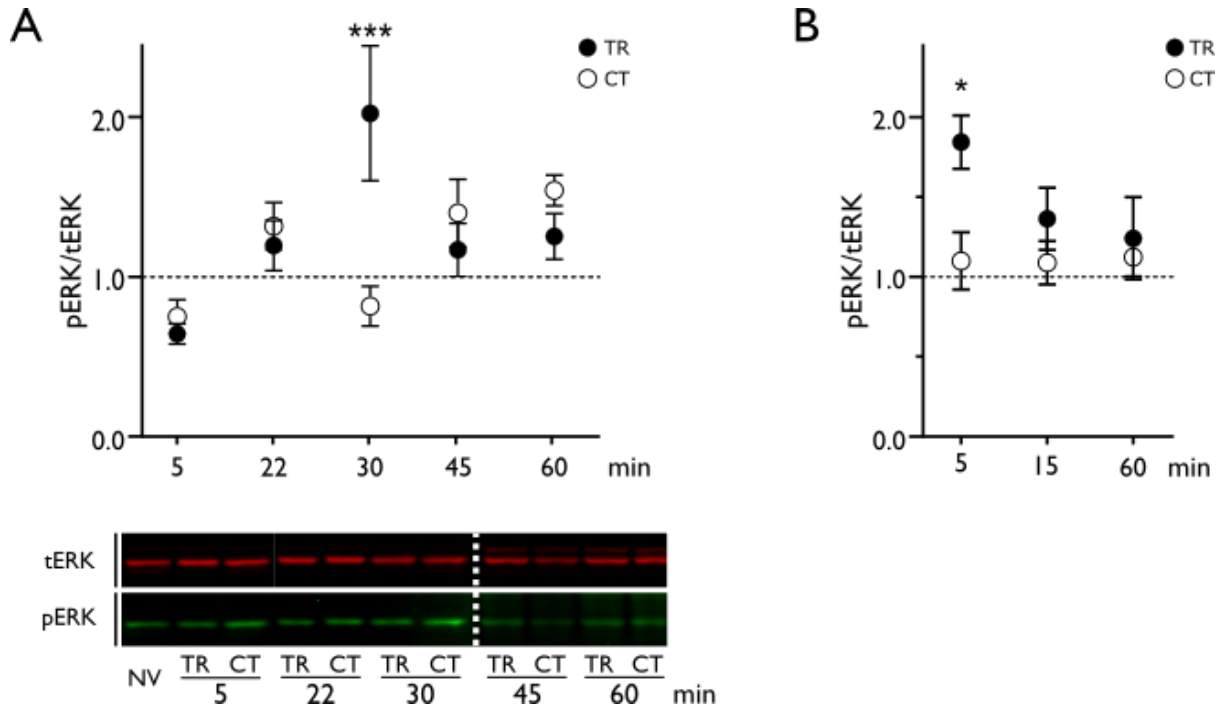
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### 341 3.6. ERK/MAPK pathway activation dynamics in 2t-LTM

342 In order to understand ERK dynamics in 2t-LTM formation, we assessed ERK activation in  
 343 protein extracts from the central brain of *Neohelice* obtained at different times during and

344 after training. We measured the phosphorylation levels of ERK by immunoblotting against  
 345 phosphorylated ERK (pERK) and total ERK independently of its phosphorylation state  
 346 (tERK). Then, pERK/tERK ratios were calculated and normalized against *naïve* (NV)  
 347 activation levels.



348  
 349 **Figure 5: 2t-LTM formation ERK/MAPK activation kinetics.** Animals were euthanized at different time points  
 350 after first TR trial ( $F_{10, 86} = 4.46$ ,  $p < 0.0001$ ; **A**) or after TR completion ( $F_{6, 57} = 2.30$ ,  $p = 0.046$ , **B**). Mean  $\pm$  SEM  
 351 of Naïve-relativized ERK activation calculated as pERK / totalERK optical density from control (CT, empty  
 352 circles); trained (TR, filled circles) and naïve (NV, dotted line) animals are shown. Representative tERK and  
 353 pERK blots are shown (white dotted line separates different membranes). One-way ANOVA followed by Holm-  
 354 Sidak's post hoc comparisons test CT vs TR. \* $p < 0.05$ ; \*\*\* $p < 0.001$ ;  $n = 7-10$  animals per group.

355  
 356 ERK was significantly activated 30 min after the first trial (Fig. 5A, CT vs TR,  $p = 0.0004$ ).  
 357 ERK phosphorylation from TR groups at other time points during ITI were not significantly  
 358 different from CT or NV groups, suggesting a small outflow from basal levels. CT groups did  
 359 not differ significantly from NV groups. These results, together with the memory impairment  
 360 observed by intra-TR PD administration (Fig. 4C and D), support a transient ERK activation  
 361 window necessary for memory formation after the first trial.

362 Surprisingly, a second training trial induced significant ERK activation only at 5 min after  
363 training completion (Fig. 5B, CT vs TR,  $p = 0.013$ ), while later time points were not  
364 significantly different from CT or NV groups ( $p > 0.05$ ). This result supports the requirement  
365 of this kinase activation for consolidation of 2t-LTM in *Neohelice*.

366 In summary, our data suggest that after the first trial, ERK activity is primed for a second trial  
367 to induce a faster dynamic, necessary for 2t-LTM formation. Our novel LTM-inducing training  
368 protocol entails a useful tool for intra-trial interventions and enables further insight into the  
369 molecular mechanisms that regulates the spacing effect and memory formation.

#### 370 4. Discussion

371 Here we showed that *Neohelice* associative aversive memory can be unveiled by a training  
372 protocol involving 2 trials spaced by 45 min, achieving memory retention for at least 96 h (2t-  
373 LTM).

374 In *Neohelice*, as in many different species, the temporal distribution of training trials  
375 drastically affects memory retention (Pereyra et al., 2000). It has been shown that 15 or  
376 more spaced trials can induce LTM. Moreover, previous studies in this invertebrate showed  
377 a requirement of at least 10 spaced trials to induce 24-h retention. Furthermore, experiments  
378 exploring the effect of reducing the duration of the ITI indicated that massed training (e.g. ITI  
379 shorter than 27 sec) did not allow associative LTM expression even when the number of  
380 trials was significantly increased (e.g. 120; 300 or 1000) as to allow a total stimulation time  
381 that equals spaced training duration (Maldonado, 2002). Additionally, it was later shown that  
382 4 or 5 trials with ITI of 3 min did not induce memory retention either (Fustiñana et al., 2013;  
383 Romano et al., 1996). Consistently with previous results, 2 trials with an ITI of 3 min did not  
384 induce LTM (Fig. 1). However, a small number of trials had never been combined with ITIs  
385 longer than 3 min, leading to the idea of a boundary condition for LTM formation  
386 (Maldonado, 2002) in this model. Results presented here support the notion that fewer trials  
387 are able to induce long-term retention (up to 96 h) as long as the adequate ITI (e.g. 45 min)  
388 is applied, suggesting this event to be constrained by or contingent upon the presence of a  
389 specific molecular environment. Such protocol would allow unraveling the mechanisms  
390 associated with the LTM formation during the training.

391 Protein synthesis dependency has been shown for different memory paradigms in diverse  
392 animal species, and for a variety of learning tasks (Davis & Squire, 1984). 2t-LTM shares  
393 parametric features with other well established LTM protocols: it lasts for at least 96 h; it  
394 requires protein synthesis to be consolidated; retrieval of consolidated memory after context  
395 exposure renders memory labile again and, finally, a change in context during retrieval

396 precludes memory labilization. These findings support an associative nature of the memory  
397 trace induced by this new training protocol. Moreover, the ITI used provides a temporal  
398 window to pharmacologically dissect the contribution of individual trials to LTM formation and  
399 study the spacing effect. Current attempts to explain the spacing effect hypothesize there is  
400 a refractory period between trials, such that additional stimuli are ineffective for learning  
401 improvement (Smolen et al., 2016). One of these hypotheses proposes that time is  
402 necessary for the neural circuitry and molecular machinery to adequately record an event.  
403 Thus, time between stimuli would allow the system to regain its ability to respond. Another  
404 hypothesis argues that the molecular processes occurring towards the end of training allow  
405 the formation of a LTM. Thus, the first trials of a spaced training would have a 'priming' effect  
406 on synapses, so that subsequent stimulation act to reinforce learning. These hypotheses are  
407 not mutually exclusive and further molecular studies will lead to better distinguish between  
408 these possibilities. Here, we described a new associative memory protocol, using the same  
409 trial structure as in CPC, but reducing to a minimum the number of trials while maintaining  
410 the same total training duration. Thus, in *Neohelice* CPC 15 trials are delivered in a 45 min  
411 training session, while in 2t-LTM training only 2 trials are applied during the same time lapse,  
412 leaving a period of almost 45 min in between with no stimulation. Although these memories  
413 appear indistinguishable, our results are not conclusive about what is the contribution of  
414 trials between 1 and 15 or whether these are functionally different memories. Ongoing  
415 experiments will help clarify this interrogation.

416 ERK activation has already been shown to be necessary for different phases of memory  
417 (Bekinschtein et al., 2008; Besnard et al., 2014; Krawczyk et al., 2015, 2016; Merlo et al.,  
418 2018) in different species (Alonso et al., 2002; Atkins et al., 1998; Blum et al., 1999; Crow et  
419 al., 1998; Feld et al., 2005; Purcell et al., 2003; Sharma et al., 2003). Among invertebrates,  
420 ERK phosphorylation has been linked to LTM in invertebrates such as *Aplysia* (Philips et al.,  
421 2007, 2013), *Drosophila* (Miyashita et al., 2018) and *Hermisenda* (Crow et al., 1998). ERK  
422 activation in crab's nervous system had also been demonstrated to be required for LTM

423 formation in *Neohelice* (Feld et al., 2005) using a similar protocol to CPC, but without light  
424 changes. In these conditions, ERK is activated in the crab's central brain 1 h after the  
425 training session, while massed training or no stimulation control elicit immediate ERK  
426 activation. Inhibition of the pathway 45 min (but not immediately or 1 h) after training impairs  
427 performance 24 h later, but not at 4 h, supporting a specific requirement of the pathway in  
428 memory consolidation. However, intra-training activation or effect of inhibition on memory  
429 performance was difficult to assess using 3-min ITIs.

430 Using a two-trial training paradigm in *A. californica*, Philips et al. (Philips et al., 2007, 2013)  
431 dissected the role of each trial. In *Aplysia* LTS pERK peaks at 45 min after the first trial, just  
432 at the same time the second trial is applied. The model posits that the first trial would start  
433 the activation of cascades that establish a narrow time window during which a favorable  
434 molecular environment allows LTM formation to be triggered by a second trial (Philips et al.,  
435 2013). The data presented here is consistent with this proposal. However, during 2t-LTM  
436 training, ERK phosphorylation increases significantly by 30 min after the first trial and 15 min  
437 before second trial is applied. Supporting its requirement for 2t-LTM formation,  
438 pharmacological inhibition using PD effectively impaired behavioral performance only when  
439 the drug was injected 15 or 22.5 min (but not 30 min) after the first trial, a few minutes  
440 previous to ERK peak activity. Furthermore, PD injection after training also impaired memory  
441 retention, suggesting ERK activation 5 min after the second trial is required for LTM  
442 formation. Noteworthy, ERK activity showed a specific temporal pattern elicited during and  
443 after training. This kinetics profile argues in favor of the importance of the second trial in  
444 order to induce LTM formation, while providing support for using longer ITI, as ERK  
445 activation 30 min after the first trial enables a second trial to trigger LTM. It is still uncertain  
446 what other mechanisms are triggered after ERK activation at this time point. We cannot  
447 discard the fact that we are using total protein extraction to determine ERK activation, while  
448 activity might be localized in a particular subcellular compartment and consequently is being  
449 underestimated (Salles et al., 2015). Consistent with this possibility, the first trial memory

450 trace might be clustered in a restrained group of neurons (Kastellakis et al., 2015), which  
451 would make differences difficult to detect with these techniques. Experiments are in progress  
452 to assess the role of ERK downstream targets in 2t-LTM. But the strong matching between  
453 ERK kinetics and pharmacological alteration of behavior strengthens the idea that ERK  
454 would be a key factor in 2t-LTM consolidation and/or acquisition.

455 ERK activation has been shown to be necessary not only for synaptic plasticity, but also for  
456 several forms of learning and memory (Thomas & Huganir, 2004). Studies on dendritic spine  
457 formation in cultured hippocampal neurons showed that only repeated depolarization  
458 induced sustained activation of ERK that correlated with and was essential for spine  
459 formation (Wu et al., 2001). Moreover, multiple cytosolic ERK targets have also been found  
460 to be relevant for different plastic processes (Ahn, 2009; Earnest et al., 1996; Gong & Tang,  
461 2006; Kelleher et al., 2004; Kneussel & Wagner, 2013; Schrader et al., 2006), supporting a  
462 role for this cascade throughout the neuron. At the molecular level, the increase in  
463 effectiveness that is observed behaviorally after spaced training can be translated as a  
464 greater effectiveness in recruiting the molecular pathways relevant for memory formation  
465 (Naqib et al., 2012). In this sense, it has been found that spaced training is more effective in  
466 the recruitment of CREB activation than massed training (Genoux et al., 2002; Josselyn et  
467 al., 2001). This protein is essential in the regulation of gene expression and has a key role in  
468 plasticity and memory formation processes (Kandel, 2012; Kida & Serita, 2014) and its  
469 function in specific areas from mice brain has been shown to influence the probability that  
470 individual neurons are recruited into a memory trace (Han et al., 2007). Furthermore, using  
471 aversive olfactory association in flies, Miyashita and coworkers recently demonstrated that  
472 LTM requires repeated training trials with rest intervals between them in order to induce  
473 pERK-dependent transcriptional cycling between c-Fos and CREB (Miyashita et al., 2018).  
474 In their report, they also show that training trials suppress ERK activity by recruiting ERK  
475 phosphatases (calcineurin and protein phosphatase 1), consistent with the previous proposal  
476 that length interval is determined by the activity of SHP2 (protein tyrosine phosphatase 2),

477 associated with increased activity of ERK/MAPK (Pagani et al., 2009). Our results slightly  
478 differ from the ones described in *Drosophila* and *Aplysia*, as ERK activity showed a  
479 significant increase between trials, suggesting different phosphatase kinetics. However, the  
480 nature and kinetics of the hypothetical phosphatase activity remain to be elucidated.

481 This body of results suggests that the spacing effect can be unraveled through assessment  
482 of LTM. Contrary to what we had expected, only two trials (instead of 15) and a longer ITI  
483 (45min instead of 3min) maintaining the same training duration induced robust LTM  
484 formation and allowed intra-training manipulations without impairing behavioral performance.  
485 As shown in different experiments, vehicle administration during ITI did not alter memory  
486 expression or impair performance during tests. Moreover, this training protocol allows  
487 assessing individual trial contribution to LTM formation processes. This represents a great  
488 advantage compared to previous protocols involving a larger number of trials and a shorter  
489 ITI. Simultaneously, it contributes to the understanding of relevant trial structure for LTM  
490 induction and disentangling the mechanisms associated with LTM formation.

491

492



493 Acknowledgements

494 We thank Angel Vidal for the invaluable technical support. We are in debt with María  
495 Eugenia Pedreira and María Sol Fustiñana for their thorough reading of the manuscript.

496

497 Funding

498 This work was supported by the following grants: ANPCYT (PICT2016 0296 and PICT2015  
499 1199), CONICET (PIP 2014-2016 No. 11220130100519CO) and UBACYT (2018-2021 -  
500 20020170100390BA and 2014-2017 - 20020130200283BA), Argentina.

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