EJN
European Journal of Neuroscience

European Journal of Neuroscience, Vol. 27, pp. 3009–3019, 2008 doi:10.1111/j.1460-9568.2008.06262.x

Protein degradation, as with protein synthesis, is required during not only long-term spatial memory consolidation but also reconsolidation

Julien Artinian,[1,*] Anne-Marie T. McGauran,[1,*] Xavier De Jaeger,[1] Lionel Mouledous,[2] Bernard Frances[1] and Pascal Roullet[1]
[1]Centre de Recherches sur la Cognition Animale, CNRS 5169, Université Paul Sabatier, Toulouse, France
[2]Institut de Pharmacologie et Biologie Structurale, CNRS-UMR 5089, 205 route de Narbonne, 31077 Toulouse, France

Keywords: CA3, hippocampus, mouse, spatial learning

Abstract

The formation of long-term memory requires protein synthesis, particularly during initial memory consolidation. This process also seems to be dependant upon protein degradation, particularly degradation by the ubiquitin-proteasome system. The aim of this study was to investigate the temporal requirement of protein synthesis and degradation during the initial consolidation of allocentric spatial learning. As memory returns to a labile state during reactivation, we also focus on the role of protein synthesis and degradation during memory reconsolidation of this spatial learning. Male CD1 mice were submitted to massed training in the spatial version of the Morris water maze. At various time intervals after initial acquisition or after a reactivation trial taking place 24 h after acquisition, mice received an injection of either the protein synthesis inhibitor anisomycin or the protein degradation inhibitor lactacystin. This injection was performed into the hippocampal CA3 region, which is specifically implicated in the processing of spatial information. Results show that, in the CA3 hippocampal region, consolidation of an allocentric spatial learning task requires two waves of protein synthesis taking place immediately and 4 h after acquisition, whereas reconsolidation requires only the first wave. However, for protein degradation, both consolidation and reconsolidation require only one wave, taking place immediately after acquisition or reactivation, respectively. These findings suggest that protein degradation is a key step for memory reconsolidation, as for consolidation. Moreover, as protein synthesis-dependent reconsolidation occurred faster than consolidation, reconsolidation did not consist of a simple repetition of the initial consolidation.

Introduction

The formation of long-term memory (LTM) involves a process by which a labile short-term memory (STM) is converted into a stable trace. This process, named memory consolidation, requires synaptic plasticity, which relies upon different molecular mechanisms as an activation of gene transcription (Bailey *et al.*, 1996) and especially *de novo* protein synthesis (Davis & Squire, 1984; Bourtchouladze *et al.*, 1998; Quevedo *et al.*, 1999). To stabilize newly learned information, it seems that there are not only one but two waves of protein synthesis. This process seems general as it has been found in various species and aversive experimental paradigms (Grecksch & Matthies, 1980; Quevedo *et al.*, 1999; Wanisch *et al.*, 2005). This synaptic plasticity also seems to be dependent upon protein degradation, particularly degradation by the ubiquitin–proteasome system (UPS; Hedge *et al.*, 1997; Lopez-Salon *et al.*, 2001). Thus, an up-regulation of the UPS leads to proteolysis-dependent long-term facilitation (LTF) in *Aplysia* (Hedge *et al.*, 1997) and an inhibition of UPS impairs the early consolidation phase of LTF (Chain *et al.*, 1999). In rat, passive avoidance has been strongly linked to the UPS (Foley *et al.*, 2000), while memory consolidation following an inhibitory avoidance task is impaired after protein degradation inhibition in the CA1 (Lopez-Salon *et al.*, 2001).

A number of recent reports have shown that, when reactivated, a previously-consolidated memory becomes temporarily labile again and requires a new stabilization process referred to as reconsolidation (Lewis, 1979; Sara, 2000; Nader, 2003). This memory reconsolidation process requires new protein synthesis, as during the initial memory consolidation (for review, Dudai & Eisenberg, 2004 and Alberini, 2005), although some conflicting results appear in the literature (for review, see Tronson & Taylor, 2007). In these reconsolidation experiments, no studies focus on the temporal dynamics of protein synthesis during reconsolidation. In addition, to our knowledge no studies have investigated the role of protein degradation in memory reconsolidation.

The first objective of the present study was to examine the dynamics of the requirements of protein synthesis in the initial consolidation of spatial information in order to determine whether there is a double wave of protein synthesis, as seen in consolidation of aversive learning. The second aim of this study was to compare the temporal dynamics of protein synthesis in both initial memory consolidation and memory reconsolidation. If reconsolidation is simply another

Correspondence: Dr P. Roullet, as above.
E-mail: roullet@cict.fr

*These authors contributed equally to this work.

Received 19 October 2007, revised 9 April 2008, accepted 11 April 2008

consolidation, we would expect the two processes to have the same temporal dynamics. The third aim was to investigate the implications of protein degradation during initial consolidation of an allocentric learning task and, more importantly, to see whether this protein degradation is also necessary when a well-stabilized trace was reactivated. To test this, anisomycin (ANI) or lactacystin (LAC; respectively, protein synthesis and degradation inhibitors) were injected with various time delays after the acquisition or the reactivation of spatial information learned in the Morris water maze. Injections were performed into the CA3 hippocampal region, which we have shown in precedent experiments to require protein synthesis during the initial phase of consolidation as well as during reconsolidation of spatial information (Artinian *et al.*, 2007).

Materials and methods

Experimental subjects

A total of 239 CD1 male mice (IFFA Credo, Lyon, France) were housed in groups of four in standard breeding cages placed in a rearing room at a constant temperature under diurnal conditions (light–dark: 08.00–20.00 h), with food and water *ad libitum*. At the time of surgery, they were between 90 and 110 days old. They were tested during the first half of the light period and all experiments were performed in strict accordance with the recommendations of the European Union (86/609/EEC) and the French National Committee (87/848) and approved by the Local Ethical Committee.

Surgery

Prior to surgery, animals were anaesthetized with i.p. injection of chloral hydrate (40% in H_20; Sigma, St Quentin-Fallavier, France) and sedation was confirmed by interdigital skin pinch. Bilateral guide cannulae (0.56 mm in diameter) were implanted 1.2 mm above the CA3 region. The following coordinates with lambda and bregma in the same horizontal plane were used: posterior to bregma, −1.7 mm; lateral to midline, ±2.5 mm; and 1.5 mm beneath the skull surface. The subjects were then left in their home cage for a recovery period of 7–8 days.

Hippocampal injection procedure

For the protein synthesis experiments, ANI (Sigma) or NaCl (0.9%) was bilaterally injected in a volume of 0.25 μL/side into the dorsal hippocampus. ANI was initially dissolved in 3 N HCl and the solution was brought to a pH of 7.4 and a final concentration of 100 μg/μL by addition of 3 N NaOH and NaCl. At this dose, ANI inhibits > 90% of protein synthesis in the brain during the first 2 h (Flood *et al.*, 1973; Morris *et al.*, 2006). For the protein degradation experiments, LAC (200 μM; Sigma) dissolved in DMSO (2%), or DMSO (2%) alone, was bilaterally injected into the dorsal hippocampus in the same volume as ANI. A specific inhibitor of peptidase activity in the proteasome, LAC causes irreversible inhibition of this structure (Fenteany *et al.*, 1995). At the dose used in this study, LAC injection produces maximal inhibition of the proteasome activity before reaching a plateau (Fornai *et al.*, 2003). The injector (0.25 mm in diameter) was connected with a polyethylene tube to a 1-μL Hamilton syringe driven by a microinjection pump at a rate of 0.1 μL/min. Mice remained in their cages during the injection time. The injection lasted 2 min 30 s for each side and the needle was left in the cannulae for an additional 60 s to allow diffusion.

Behavioral testing

The apparatus and protocol for evaluating spatial memory were the same as in previous experiments (Florian & Roullet, 2004). Briefly, mice were trained in a massed procedure in the spatial Morris water maze (110 cm diameter). A circular goal platform (9 cm in diameter) was placed in the centre of one quadrant, 15 cm from the wall. Four start positions were located around the perimeter of the pool, dividing its surface into four equal quadrants. The apparatus was surmounted by a video camera connected to a video recorder and a computerized tracking system (Ethovision®; Noldus, Wageningen, the Netherlands).

Mice were individually submitted to a single familiarization session of three consecutive trials with the platform protruding 0.5 cm above the surface of the water. The training phase consisted of four consecutive sessions of three trials with an intersession delay of 15–20 min during which mice were returned to their home cage. During the training phase, the platform was submerged 0.5 cm beneath the surface of the water. Mice were required to navigate to the invisible platform using the spatial cues available in the experimental environment. In each experiment, mice were tested for their long-term memory retention of spatial orientation by giving them a probe test consisting of a 60-s free swim trial without the platform.

Experiment 1: protein synthesis-dependent consolidation

Immediately or 2, 4 or 6 h after the final training session, mice received injections of ANI or NaCl. The probe test took place 24 h after the injections.

Experiment 2: protein synthesis-dependent reconsolidation

Twenty-four hours after training, a reactivation trial consisting of an additional learning trial was performed. Mice were injected just after or 2, 4 or 6 h after the reactivation trial and the probe test took place 24 h later.

Experiment 3: protein degradation-dependent consolidation

Immediately or 3 h after the final training session, mice received injections of LAC or DMSO. The probe test took place 24 h post-injection.

Experiment 4: protein degradation-dependent reconsolidation

Twenty-four hours after training, a reactivation trial, consisting of an additional learning trial, was performed. Mice were injected just after or 3 h after the reactivation trial and the probe test took place 24 h later.

Experiment 5: control of specific effect of ANI and LAC

Mice did not undergo reactivation and were merely left in their home cage. Injections were performed 24 h after the training sessions in the animal room and the probe test performed 24 h later.

Experiment 6: STM post-reactivation

The procedure was the same as for the reconsolidation experiments except that mice were injected with LAC, ANI, DMSO or NaCl immediately after the reactivation trial and the probe test took place 1 h later. All the behavioral groups were independent, i.e. each mouse was tested in one condition only. For the training phase, mean escape latencies (s) were used for each session (three trials per session).

During the probe test, the number of annulus crossings, defined as the number of times a mouse crossed an ideal circle (14 cm diameter) located at the original platform position and the three equivalent areas in each of the other quadrants, were analyzed. The number of annuli crossings could reveal the strategy used whilst searching for the

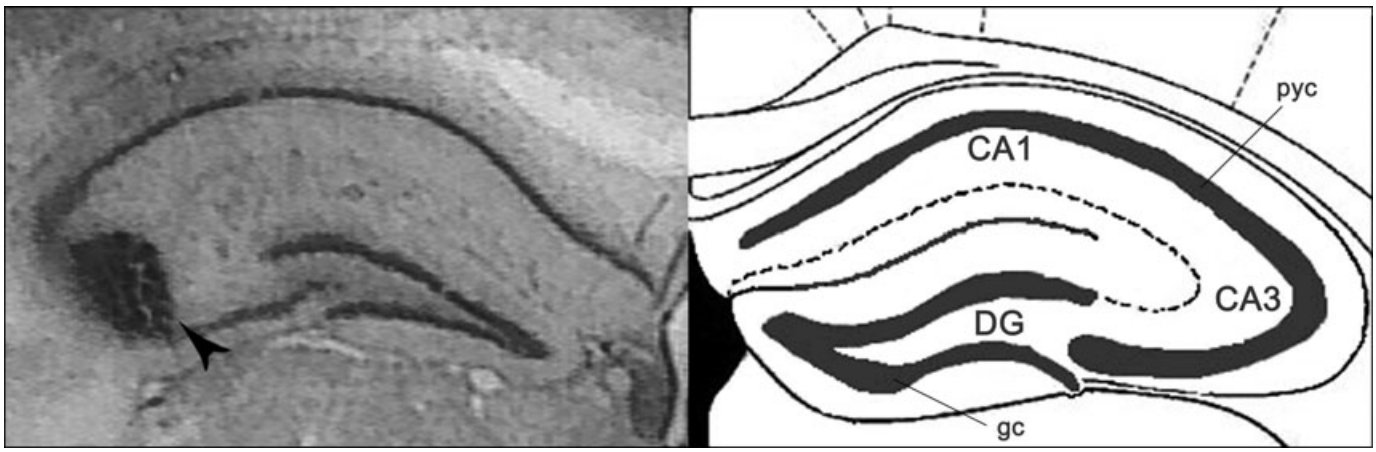

FIG. 1. A representative sample of a thionine-stained brain section showing placement of the tip of the cannulae (black arrows). DG, dentate gyrus; pyc, pyramidal cells; gc, granule cells.

platform, while the number of target annulus crossings would determine whether mice learned the target location.

Cannulae placement verification

At the end of the experiment, each animal was killed with a lethal dose of chloral hydrate and the brain was removed. The cannulae position was determined for each mouse by examination of serial coronal sections (40 μm) stained with thionine. Only mice with both needle tracks terminating within the CA3 hippocampal region were included in the behavioral analysis. The locations of the infusion needle tips are summarized in Fig. 1.

Test of proteasome activity

In order to verify that the injected dose of LAC (200 μM) was sufficient to inhibit proteasome activity at the target area CA3, proteasome activity after injection was measured in a number of control mice. Naïve animals ($n = 8$) received an injection of LAC (200 μM) on one hemisphere and DMSO (2%) on the other, each at a volume of 0.25 μL per side. One hour later, mice received a lethal dose of chloral hydrate (800 mg/kg, i.p.), and the brain was quickly removed and placed into ice-cold physiological fluid (in mM: Hepes, pH 7.3, 10; NaCl, 150; KCl, 2.5; and glucose, 10). Brains were placed into a brain block apparatus, where rostrocaudal slices of 1-mm thickness were cut, centered at the position of the cannulae. Using the end of a Pasteur pipette (1 mm diameter) a 1-mm section of tissue, centered at the CA3 region, was cut out of the slice and placed into a buffer [Tris, 50 mM; EDTA, 1 mM; and protease inhibitor cocktail (Roche, Meylan, France)] and homogenized 2 × 5 times. Protein concentration was measured and equilibrated via Bradford (Bio-Rad, Marnes-la-Coquette, France) analysis. Fifty microlitres of each sample was placed, in duplicate, into a 96-well plate, into which was then added 50 μL of the substrate Leu-Leu-Val-Tyr-7 amino-4-methylcoumarin (Calbiochem) at 400 μM, which allows the detection of chymotrypsin-like catalytic activity by the proteasome. Fluorescence was quantified every 5 min for 1 h 30 min via a spectrofluorometer (FLX-800; Bio-Tek, Colmar, France). The rates of substrate hydrolysis (fluorescence units over time) reflecting proteasome activity were determined from the initial linear portions of curves. The percentage inhibition was determined in each mouse by calculating the ratio of the activity obtained from the LAC-injected side over the activity obtained from the saline-injected side (Craiu *et al.*, 1997).

Statistical analysis

The SYSTAT 9.0 (Erkrath, Germany) statistical software package was used for data analysis. The results are expressed as mean ± SEM and analyzed using one- or two-way ANOVA, or a repeated-measures ANOVA when appropriate. *Post hoc* multiple comparisons were carried out when allowed, using Tukey's honestly significant difference test.

Results

ANI impaired initial spatial memory consolidation if injected immediately or 4 h after acquisition

To investigate the role of protein synthesis during memory consolidation, animals were injected with ANI immediately or 2, 4 or 6 h post-acquisition (Fig. 2A). Concerning the latency to find the hidden platform (Fig. 2B), an ANOVA for repeated measures revealed a significant session effect [$F(3,192) = 25.876$; $P < 0.001$] but no pre-treatment effect [$F(1,64) = 0.006$; $P = 0.940$] and no interaction between these two factors [$F(3,192) = 0.630$; $P = 0.596$]. These data confirmed that before treatment the different groups of mice learned the exact position of the platform and displayed the same level of performance during the four learning sessions. As shown in Fig. 2C and E, ANI injection just after or 4 h after acquisition impaired long-term retention during the probe test performed 24 h later. A two-way ANOVA revealed no significant effect between control and ANI-injected mice [0 h: $F(1,56) = 0.330$, $P = 0.568$; 4 h: $F(1,56) = 0.320$, $P = 0.574$] on the total number of annulus crossings, but a significant quadrant effect [0 h: $F(3,56) = 11.182$, $P < 0.001$; 4 h: $F(3,56) = 22.490$, $P < 0.001$]. There was also a significant treatment × quadrant interaction [0 h: $F(3,56) = 7.355$, $P < 0.001$; 4 h: $F(3,56) = 6.856$, $P < 0.01$] showing that the profile of exploration of the quadrants was different in the two groups of mice. Tukey's test showed that, for the two delays of injection, control mice significantly crossed the target annulus more often than the three other annuli ($P < 0.001$). In contrast, for the ANI group there was no significant difference in the exploration of the four annuli. Moreover, in both cases, control mice crossed the target annulus more often than the ANI mice ($P < 0.01$). For the injection 2 or 6 h after the last training sessions, control animals and ANI-injected mice crossed the annulus located in the target quadrant significantly more often than the remaining three annuli. In these two experiments, the two-way ANOVA showed a significant quadrant effect [2 h: $F(3,56) = 17.111$, $P < 0.001$; 6 h: $F(3,64) = 19.389$, $P < 0.001$], no treatment effect

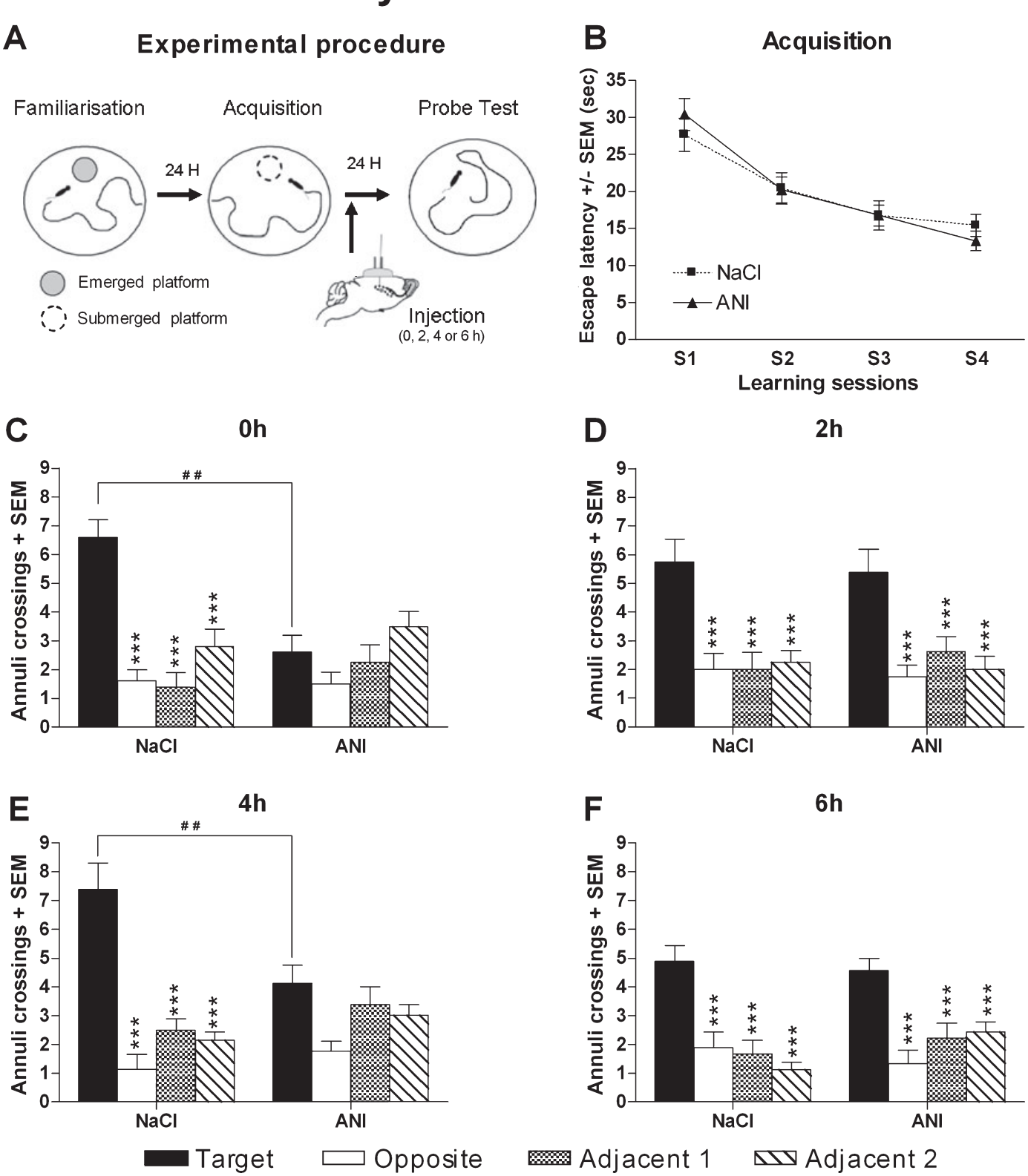

FIG. 2. Memory consolidation of the spatial learning task requires two waves of protein synthesis. (A) The behavioral procedure used for experiment 1. (B) Mean latency in s to find the platform during the training phase of the spatial water maze task. (C) Immediately, (D) 2 h, (E) 4 h or (F) 6 h after the last training session, the mice received injections of ANI or NaCl. (C–F) Number of annuli crossings during the 60-s probe trial. ANI injection in the CA3 region just after and 4 h after acquisition impaired spatial performances; they had no effect if injections were performed at 2 or 6 h. ***$P < 0.001$ vs. target quadrant. $^{\#\#}P < 0.01$, target quadrant NaCl vs. ANI group.

[2 h: F(1,56) = 0.022, $P = 0.882$; 6 h: F(1,64) = 0.006, $P = 0.936$] and no interaction between these two factors (2 h: F(3,56) = 0.302, $P = 0.824$; 6 h: F(3,64) = 0.731, $P = 0.537$). These results demonstrate that performance was impaired if ANI was injected immediately or 4 h after reactivation but not when injected at 2 or 6 h.

ANI impaired spatial memory reconsolidation only if injected immediately after reactivation

To investigate the role of protein synthesis during memory reconsolidation, animals were injected with ANI immediately, 2, 4 or 6 h after reactivation (Fig. 3A). During acquisition (Fig. 3B), a repeated-measures ANOVA revealed a significant session effect [F(3,198) = 19.315, $P < 0.001$] but no pre-treatment effect [F(1,66) = 0.046, $P = 0.830$] and no interaction between these two factors [F(3,198) = 2.170, $P = 0.093$]. Figure 3C shows the effects of immediately post-reactivation ANI and NaCl injections on the number of annulus crossings during the probe test. A two-way ANOVA revealed no significant effect between NaCl- and ANI-injected mice [F(1,64) = 2.426, $P = 0.124$], a significant quadrant effect [F(3,64) = 18.067, $P < 0.001$] and a treatment × quadrant interaction [F(3,64) = 7.063, $P < 0.001$]. The control animals crossed the target annulus more often than the three others ($P < 0.001$) and there was no significant difference between the explorations of the four quadrants for the ANI group. Moreover, the control group crossed the target quadrant more often than the ANI group ($P < 0.001$). For the three other delays (2, 4 and 6 h) there was no effect of ANI injections. In these three experiments, the two-way ANOVA showed a significant quadrant effect [2 h: F(3,60) = 75.220, $P < 0.001$; 4 h: F(3,56) = 45.378, $P < 0.001$; 6 h: F(3,56) = 18.920, $P < 0.001$], no treatment effect [2 h: F(1,60) = 0.007, $P = 0.963$; 4 h: F(1,56) = 0.612, $P = 0.437$; 6 h: F(1,56) = 0.759, $P = 0.387$] and no interaction between these two factors [2 h: F(3,60) = 1.213, $P = 0.313$; 4 h: F(3,56) = 1.248, $P = 0.301$; 6 h: F(3,56) = 1.182, $P = 0.325$]. These results demonstrate that performance was impaired only if ANI was injected immediately after reactivation.

LAC impaired spatial memory consolidation only if injected immediately after training

To investigate the role of protein degradation during memory consolidation, animals were injected with LAC either immediately or 3 h after acquisition (Fig. 4A). A repeated-measures ANOVA on latency to find the platform during training (Fig. 4B) revealed a significant session effect [F(3,96) = 6.389, $P = 0.001$] but no pre-treatment effect [F(1,32) = 0.149, $P = 0.702$] and no interaction effect between the two factors [F(3,96) = 0.381, $P = 0.767$]. This demonstrates that animals successfully acquired the task. Figure 4C demonstrates that injection of LAC immediately post-acquisition impaired animals' ability to remember the location of the platform. An ANOVA test revealed no overall difference in the total number of annulus crossings between LAC- and DMSO-injected mice [F(1,56) = 3.567, $P = 0.065$] but a significant quadrant effect [F(3,56) = 9.196, $P < 0.001$] and an interaction effect [F(3,56) = 6.645, $P = 0.001$]. Tukey *post hoc* comparisons revealed that control mice swam more often at the target annulus than at any of the other three equivalent annuli, while LAC-injected animals did not show any such quadrant preference. For the animals that were injected 3 h post-acquisition, animals in both the control and LAC groups swam more often in the target annulus than in the other three annuli (Fig. 4D). ANOVA revealed that animals in both groups demonstrated a significant quadrant effect [F(3,64) = 20.801, $P < 0.001$], no treatment effect [F(1,64) = 0.125, $P = 0.815$], and no quadrant × treatment interaction effect [F(3,64) = 0.569, $P = 0.861$]. These results demonstrate that performance was not impaired by LAC injected 3 h post-acquisition.

LAC impaired spatial memory reconsolidation only if injected immediately after reactivation

To investigate the role of protein degradation during memory reconsolidation, animals were injected with LAC either immediately or 3 h after reactivation (Fig. 5A). A repeated-measures ANOVA on latency to find the platform during training (Fig. 5B) revealed a significant session effect [F(3,96) = 11.501, $P < 0.001$] but no pre-treatment effect [F(1,32) = 2.214, $P = 0.147$] and no interaction between the two factors [F(3,96) = 0.150, $P = 0.929$]. This demonstrates that animals successfully acquired the task. Figure 5C demonstrates that injection of LAC immediately post-reactivation impaired animals' ability to remember the location of the platform. An ANOVA test revealed no overall difference in the total number of annulus crossings between LAC- and DMSO-injected mice [F(1,60) = 0.300, $P = 0.586$], but a significant quadrant effect [F(3,60) = 9.966, $P < 0.001$] and an interaction effect [F(3,60) = 8.434, $P < 0.001$]. Tukey *post hoc* comparisons revealed that control mice swam more often at the target annulus than at any of the other three equivalent annuli, while LAC-injected animals did not show any quadrant preference. For the animals that were injected 3 h post-reactivation, animals in both the control and LAC groups swam more often in the target annulus than in the other three annuli (Fig. 5D). ANOVA revealed that animals in both groups demonstrated a significant quadrant effect [F(3,60) = 19.942, $P < 0.001$], no treatment effect [F(1,60) = 0.056, $P = 0.814$], and no quadrant × treatment interaction effect [F(3,60) = 0.697, $P = 0.557$]. These results demonstrate that performance was not impaired by LAC injected 3 h post-reactivation.

Control of the specific effect of ANI and LAC (injection without reactivation)

This experiment (Fig. 6A) was conducted to examine the nonspecific effects of CA3 intrahippocampal injections of ANI and LACTA on long term spatial memory.

ANI or NaCl treatment

As shown in Fig. 6B, ANI injection 24 h after initial acquisition and without reactivation did not impair long-term retention during the probe test performed 24 h later. NaCl- and ANI-injected mice crossed the annulus located in the target quadrant significantly more often than the remaining three annuli. The two-way ANOVA revealed a significant quadrant effect [F(3,56) = 20.026; $P < 0.001$], no treatment effect [F(1,56) = 1.745; $P = 0.192$] and no interaction between these two factors [F(3,56) = 0.151; $P = 0.929$].

LAC or DMSO treatment

Figure 6C shows the number of annulus crossings by the two groups during the probe test 24 h after injection without a reactivation trial. The two-way ANOVA revealed a significant quadrant effect [F(3,56) = 17.329; $P < 0.001$], no treatment effect [F(1,56) = 0.054; $P = 0.817$] and no interaction between these two factors [F(3,56) = 0.102; $P = 0.959$]. Therefore, the impairment observed in LTM cannot be attributed to nonspecific drug effects such as neurotoxicity or general impairment.

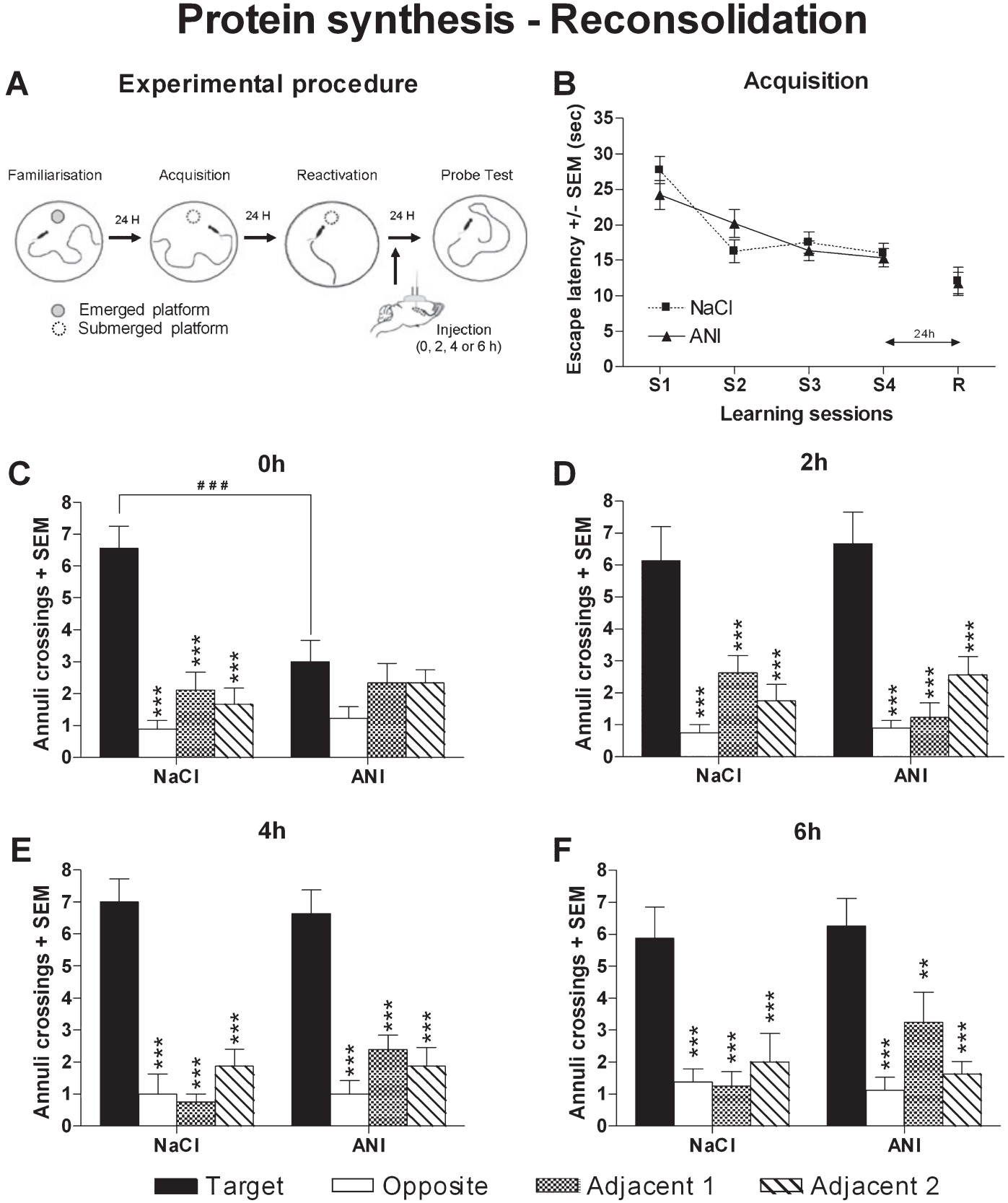

FIG. 3. Memory reconsolidation of the spatial learning task required only one wave of protein synthesis. (A) The behavioral procedure used for experiment 2. (B) Mean latency (s) to find the platform during the training phase and during the reactivation trial of the spatial water maze task. (C) Immediately, (D) 2 h, (E) 4 h or (F) 6 h after the reactivation trial, the mice received injections of ANI or NaCl. (C–F) Number of annuli crossings during the 60-s probe trial. ANI injection in the CA3 region just after the reactivation trial impaired spatial performances and had no effect at the three other intervals. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. target quadrant. $^{\#\#\#}P < 0.001$, target quadrant NaCl vs. ANI group.

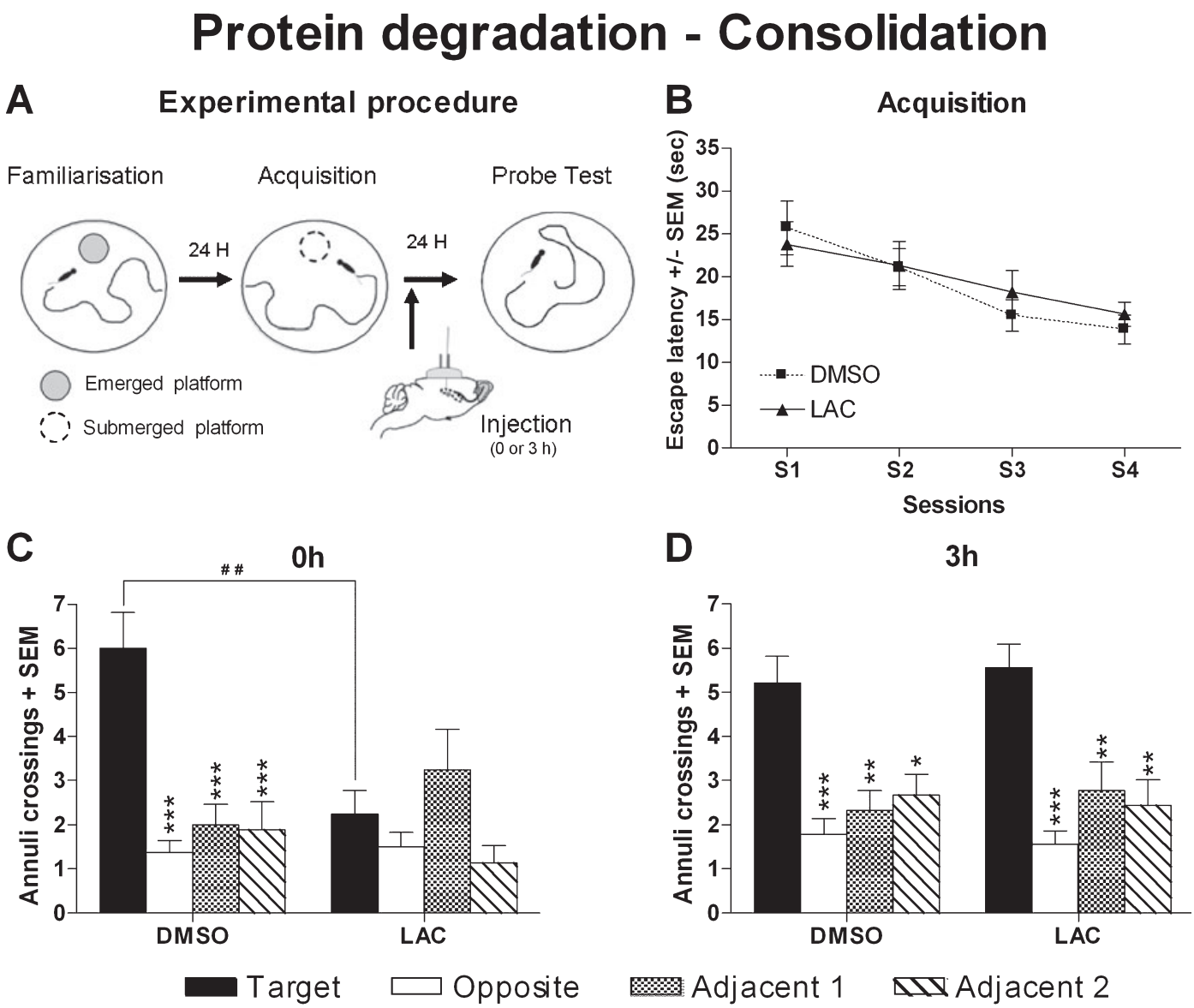

FIG. 4. Memory consolidation of the spatial learning task required only one wave of protein degradation. (A) The behavioral procedure used for consolidation. (B) Mean latency (s) to find the platform during the training phase of the spatial water maze task. (C) Immediately or (D) 3 h after the last training session, the mice received injections of LAC or DMSO. (C and D) Number of annuli crossings during the 60-s probe trial. LAC injection in the CA3 region just after acquisition impaired spatial performances and had no effect if injections were performed at 3 h. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. target quadrant. $^{\#\#}P < 0.01$, target quadrant DMSO vs. LAC group.

Neither ANI nor LAC had an effect on STM

To verify that the effects observed post-reactivation were indeed a reflection on long-term memory and not simply due to an impairment in STM (STM), the effects of post-reactivation injections of ANI or LAC on STM were investigated (Fig. 7A).

ANI or NaCl treatment

During the post-reactivation STM test, all animals crossed the annulus located in the target quadrant significantly more often than the remaining three annuli, regardless of treatment condition (Fig. 7B). A two-way ANOVA revealed a significant quadrant effect [$F(3,56) = 31.504$; $P < 0.001$] and no treatment effect [$F(1,56) = 0.008$; $P = 0.930$]. There was no interaction between these two factors [$F(3,56) = 0.785$; $P = 0.507$].

LAC or DMSO treatment

During the post-reactivation STM test, all animals crossed the annulus located in the target quadrant significantly more often than the remaining three annuli, regardless of treatment condition (Fig. 7C). A two-way ANOVA revealed a significant quadrant effect [$F(3,48) = 16.011$, $P < 0.001$] and no treatment effect [$F(1,48) = 0.530$, $P = 0.470$] There was no interaction between these two factors [$F(3,48) = 0.199$, $P = 0.897$]. Thus, ANI and LAC injections had no effect on STM when infused immediately post-reactivation.

Ex vivo *determination of the rate of inhibition of proteasome activity after LAC injection*

By measuring and comparing the normalized activity between the LAC- and DMSO-treated hemispheres of each sample, the mean

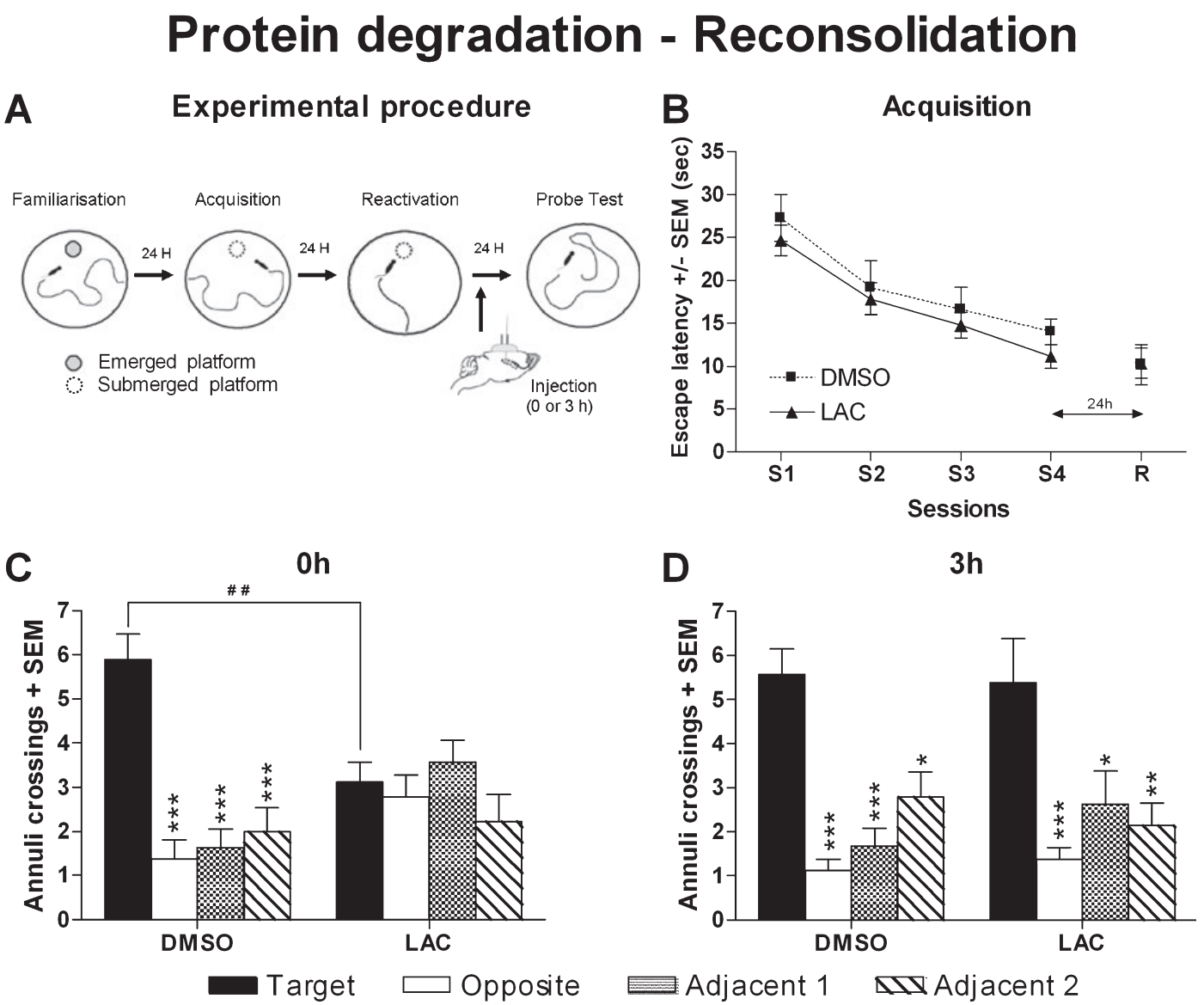

FIG. 5. Memory reconsolidation of the spatial learning task required only one wave of protein degradation. (A) The behavioral procedure used for reconsolidation. (B) Mean latency (s) to find the platform during the training phase of the spatial water maze task. (C) Immediately or (D) 3 h after the reactivation session, the mice received injections of LAC or DMSO. (C and D) Number of annuli crossings during the 60-s probe trial. LAC injection into the CA3 region just after reactivation impaired spatial performances but had no effect if injections were performed at 3 h. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. target quadrant. $^{\#\#}P < 0.01$, target quadrant DMSO vs. LAC group.

percentage inhibition was calculated. DMSO injection resulted in a mean normalized activity slope of 153.81, compared to 108.11 for LAC. A paired-samples *t*-test confirmed that LAC injection resulted in a significant inhibition of proteasomal activity by 30% [t (7) = 3.114, $P = 0.017$].

Discussion

The first series of experiments revealed that an injection of ANI into the CA3 hippocampal subregion prevented memory consolidation if the injection was carried out immediately or 4 h after the acquisition of information, but not 2 or 6 h afterwards. Many studies had already established this fact for simple aversive associative tasks using electric shock, such as fear conditioning or passive avoidance, in various species such as chick, rat or mouse (Grecksch & Matthies, 1980; Bourtchouladze *et al.*, 1998; Quevedo *et al.*, 1999). Nevertheless, these dynamics of protein synthesis during initial memory consolidation had never been tested for a complex learning task, such as allocentric spatial navigation in the water maze. In fact, in order to ensure that only the initial phase of memory consolidation is being worked on, it is important to use one-trial learning or a massed procedure (see Florian & Roullet, 2004 for a discussion). It is very interesting to notice that the double wave of protein synthesis during memory consolidation seems to be found throughout the different experimental paradigms including those with or without electric shock and, especially, simple associative or complex learning.

In the second series of experiments, we found that mice treated with ANI after a reactivation phase similarly displayed a deficit when the injection was performed after the initial consolidation phase. However, Morris *et al.* (2006) showed that, after reactivation in the Morris water

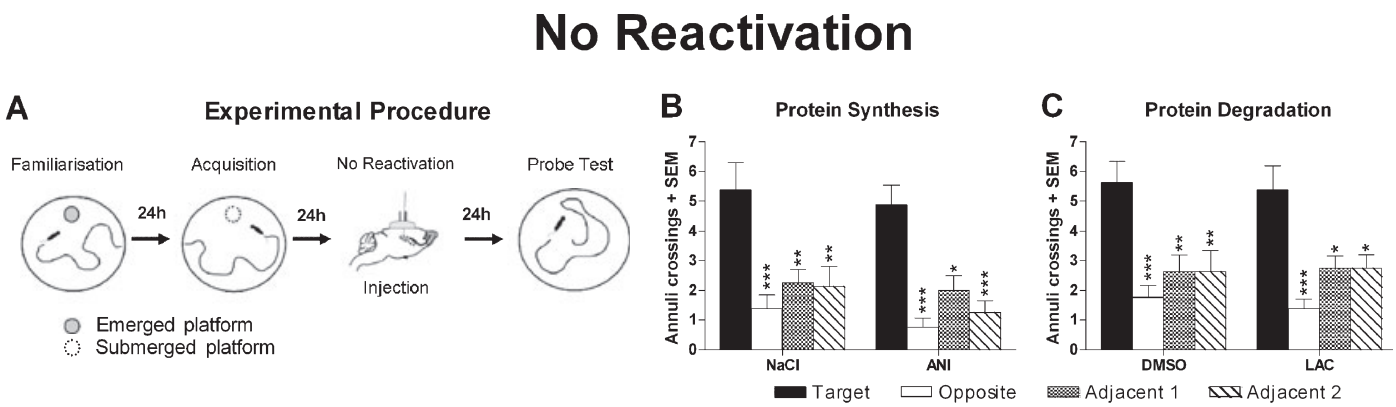

FIG. 6. ANI and LAC did not produce nonspecific effects. (A) The behavioral procedure used for the nonreactivation experiment. Mice did not undergo reactivation and were merely left in their home cage. Injections were performed 24 h after the training sessions in the animal room and the probe test conducted 24 h later. Animals were injected with (B) ANI or NaCl or (C) LAC or DMSO. (B and C) Number of annuli crossings during the 60-s probe trial. Neither ANI nor LAC influenced spatial performances, demonstrating no nonspecific effects of the drugs. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. target quadrant.

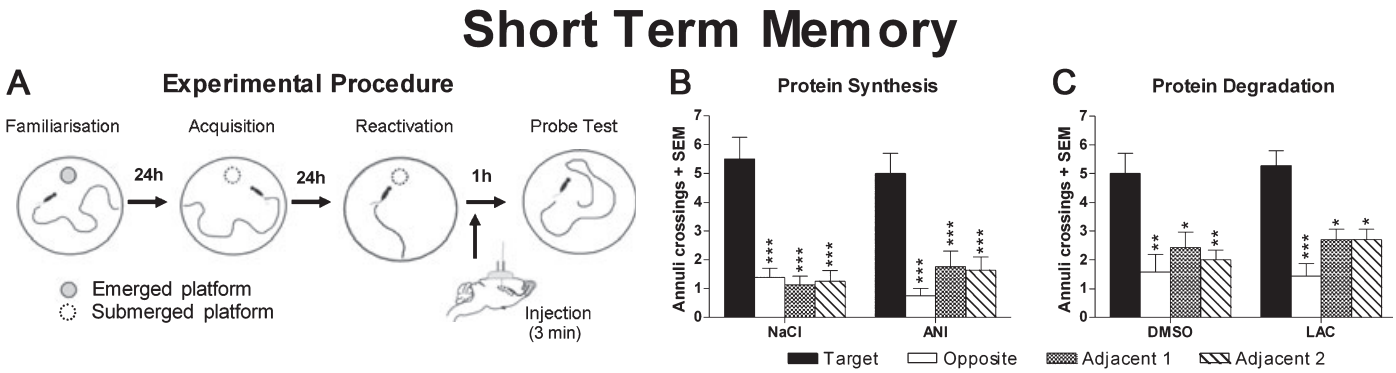

FIG. 7. ANI and LAC did not produce effects on STM. (A) The behavioral procedure used for STM. Immediately post-reactivation, animals were injected with (B) ANI or NaCl or (C) LAC or DMSO. (B and C) Number of annuli crossings during the 60-s probe trial conducted 1 h post-reactivation. Neither ANI nor LAC influenced spatial performances, demonstrating no effects of the drugs in STM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. target quadrant.

maze, memory was not sensitive to ANI in a reference memory task with a distributed procedure over several days but was sensitive in a delayed matching-to-place task procedure. Thus, intrahippocampal ANI injections seemed to affect only rapidly acquired forms of spatial memory or those involving new spatial information every day. In these conditions, a consolidated and stable spatial memory can become labile again after reactivation, and new protein synthesis is then required to maintain the memories that have been reactivated. Importantly, we have demonstrated that ANI caused impairment in long-term memory but not in STM when infused immediately after reactivation and that ANI injection had no effect if performed without a reactivation trial. Thus, the impairments observed in long-term memory reconsolidation cannot be attributed to nonspecific drug effects such as neurotoxicity or general impairment.

The major finding from these initial experiments is that the temporal dynamics of protein synthesis during initial consolidation and reconsolidation differ. Firstly, we demonstrated that initial consolidation requires two distinct waves of protein synthesis whereas reconsolidation requires only one wave, starting immediately after reactivation. This result is consistent with past studies, for example auditive fear conditioning (Nader *et al.*, 2000a) or the conditioned palpebral response (Inda *et al.*, 2005), which show that an injection of ANI 4 or 6 h (respectively) after reactivation is without effect on memory reconsolidation. In addition, Judge & Quatermain (1982) and Anokhin *et al.* (2002) have shown that the sensitive period for the effect of ANI was shorter post-reactivation (5 min) than post-training (2 h and 30 min respectively). Thus, this would suggest memory reconsolidation to be a process which can be accomplished more quickly than initial consolidation. This process would consist of a re-stabilization of pre-existent synaptic networks whereas the initial consolidation rather requires the creation, growth and stabilization of new synapses (Nader *et al.*, 2000b). Thus, memory reconsolidation could be accomplished with the production of a smaller quantity of proteins than those necessary for initial consolidation (Debiec *et al.*, 2002).

The final series of experiments in this study investigated the role of protein degradation in spatial memory consolidation and reconsolidation. In the initial phase of these experiments, we found that LAC injected into the CA3 immediately post-acquisition impaired animals' performance in the probe trial. Thus, protein degradation by the UPS pathway was necessary in the hippocampus during consolidation of a complex associative-learning task, as already shown in simpler associative learning. For example, ubiquitin has been shown to play a role in LTF in *Aplysia* (Hedge *et al.*, 1997) and mammalian LTP (Jiang *et al.*, 1998). In more recent behavioral studies, LAC infusion into the CA1 of the hippocampus impaired inhibitory avoidance in the rat (Lopez-Salon *et al.*, 2001) and place preference in the rat has been shown to be dependent upon the ubiquitin pathway in memory consolidation (Foley *et al.*, 2000). More recently, it was shown that mice deficient in the ubiquitin C-terminal hydrolase L3 have a

significant learning deficit in the Morris water maze and in the radial maze task (Wood *et al.*, 2005). However, with this kind of genetic approach it is not possible to dissociate the roles of UPS in acquisition and in memory consolidation.

While protein degradation inhibition immediately post-acquisition impaired performances in our study, inhibition after a delay of 3 h did not produce any effect. As LAC causes irreversible inhibition of protein breakdown (Craiu *et al.*, 1997), injection 1 h before the known onset of this second wave of protein synthesis allowed us to cover the possibility of degradation occurring slightly before synthesis whilst maintaining inhibition throughout the second wave. The idea that proteasome-dependent degradation is required only immediately after learning has been previously suggested (Hu *et al.*, 2003) but contradictory findings also exist, suggesting that proteasome activity persists until 7 h after learning (Lopez-Salon *et al.*, 2001).

The final phase of our experiments demonstrated that the inhibition of protein degradation in the hippocampus during reconsolidation also provokes amnesia. These experiments offer the first evidence that protein degradation is necessary during memory reconsolidation or, more precisely, when a memory trace is reactivated. Importantly, LAC injection did not impair STM and had no effect if performed without a reactivation trial. Thus, the impairments observed in long-term reconsolidation memory cannot be attributed to nonspecific drug effects. Moreover, protein degradation in reconsolidation follows a similar pattern to consolidation, with one single wave of activity taking place immediately after reactivation. At first glance, it seems that protein degradation-related consolidation and reconsolidation display the same profile. However, whether or not they share the same functional mechanisms is less certain. Two hypotheses are generally applied to explain the role of the UPS system in LTM (see Merlo & Romano, 2007; for details). First, there are some specific inhibitory proteins that need to be degraded for the onset of synaptic plasticity. Second, the UPS itself could function as an inhibitory constraint on synaptic strengthening. In both cases, protein degradation could act as a regulatory process whereby inhibitory constraints are removed in order to allow memory consolidation (Kandel, 2001). As LAC injection 3 h after learning has no effect on memory performances, these inhibitory constraints clearly act only during the initial phases of memory consolidation rather than before all waves of protein synthesis.

For reconsolidation, protein degradation could act in the same way. But, in addition, when a memory is reactivated it undergoes a destabilization process whereby it becomes labile once again (Nader, 2003; Stickgold & Walker, 2005). LAC could act directly on this destabilization process. However, the presence of a post-reactivation-LTM deficit in the absence of post-reactivation-STM deficits suggests that the effect is more in restabilization than in destabilization of the memory trace. In fact, if LAC blocked the destabilization of the memory trace, this trace would remain stable and the animals should have a good memory performance 24 h later. That was not the case in our experiments. In a very recent study, Lee *et al.* (2008) show that postsynaptic proteins are degraded by the UPS in the CA1 hippocampus after retrieval of contextual fear conditioning but that injection of β-LAC into this region after acquisition or retrieval did not affect performances in this task. Interestingly, in this experiment the same β-LAC injection after retrieval, but not after acquisition, prevented ANI-induced memory impairment. Thus, LAC only produced an effect on reconsolidation, not on consolidation. For the authors, these results show that protein degradation is important for the destabilization of pre-existing memories rather than for the restabilization process. Combined with our results, these two studies show that protein degradation underlies the reconsolidation process, but more experiments are needed to determine whether protein degradation is implicated only in destabilization, only in restabilization or in both. On the other hand, in our study, if LAC was injected after a delay of 3 h the memory would have already undergone destabilization and restabilization with protein synthesis. Thus, as the memory trace would be, at this time, insensitive to disruption, memory reconsolidation would not be affected and spatial performance 24 h later would be normal.

In this study, we have focused on the hippocampal CA3 region because this area is essential in the treatment of spatial information (Kesner *et al.*, 2000; Florian & Roullet, 2004) and different computational theories consider the CA3 region to be an autoassociative recurrent network (McNaughton & Morris, 1987; Treves & Rolls, 1992). To build this associative network, it is suggested that perforant path–CA3 synapses and/or recurrent fibre synapses are modified during initial encoding (McNaughton & Morris, 1987; Treves & Rolls, 1992) and this long-term synaptic plasticity necessitates protein synthesis. Moreover, during the early phase of memory consolidation there is a structural remodelling in both the dendritic and axonal synaptic fields in this hippocampal region (Stewart *et al.*, 2005). Thus, there is synaptic plasticity in the CA3 region supporting initial consolidation but also supporting memory reconsolidation even if this latter process seems to need less protein synthesis in this hippocampal region. Of course, other cerebral structures or other hippocampal regions, such as the CA1 region, could be implicated in the spatial memory reconsolidation process and it will be interesting to compare the temporal dynamics of protein synthesis and degradation during reconsolidation in these different structures.

To conclude, memory reactivation seems to induce a destabilization of the neural networks supporting the memory trace. Some authors have postulated that this event allows a certain plasticity, in particular to update the memory or to link new information to a reactivated memory (Nader *et al.*, 2000b; Nader, 2003). Reactivation is thus a key event in memory formation during which the memory can be modified and then reconsolidated to allow the stabilization of this information. Moreover, memory reconsolidation seems to be a process which can be accomplished more quickly than initial consolidation and, thus, does not consist of a simple repetition of the initial consolidation.

Acknowledgements

This study was supported by CNRS ANR program (ANR-06-Neuro-027-03), by the University Paul Sabatier Toulouse 3 and by a CNRS grant to A.M.M.G. We thank Elodie Billhot for technical assistance.

Abbreviations

ANI, anisomycin; LAC, lactacystin; LTF, long-term facilitation; LTM, long term memory; STM, short-term memory; UPS, ubiquitin–proteasome system.

References

Alberini, C.M. (2005) Mechanisms of memory stabilization: are consolidation and reconsolidation similar or distinct processes? *Trends Neurosci.*, **28**, 51–56.

Anokhin, K.V., Tiunova, A.A. & Rose, S.P.R. (2002) Reminder effects – reconsolidation or retrieval deficit? Pharmacological dissection with protein synthesis inhibitors following reminder for a passive-avoidance task in young chicks. *Eur. J. Neurosci.*, **15**, 1759–1765.

Artinian, J., De Jaeger, X., Fellini, L., de Saint Blanquat, P. & Roullet, P. (2007) A reactivation with a simple exposure to the experimental environment is sufficient to induce reconsolidation requiring protein synthesis in the hippocampal CA3 region in mice. *Hippocampus*, **17**, 181–191.

Bailey, C.H., Bartsch, D. & Kandel, E.R. (1996) Toward a molecular definition of long-term memory storage. *Proc. Natl Acad. Sci. USA*, **93**, 13445–13452.

Bourtchouladze, R., Abel, T., Berman, N., Gordon, R., Lapidus, K. & Kandel, E.R. (1998) Different training procedures recruit either one or two critical periods for contextual memory consolidation, each of which requires protein synthesis and PKA. *Learn. Mem.*, **5**, 365–374.

Chain, D.G., Casadio, A., Schacher, S., Hedge, A.H., Valbrun, N., Yamamoto, N., Goldberg, A.L., Bartsch, D., Kandel, E.R. & Schwartz, J.H. (1999) Mechanisms for generating the autonomous cAMP-dependent protein kinase required for long-term facilitation in *Aplysia*. *Neuron*, **22**, 147–156.

Craiu, A., Gaczynska, M., Akopian, T., Gramm, C.F., Fenteany, G., Goldberg, A.L. & Rock, K.L. (1997) Lactacystin and clasto-lactacystin beta-lactone modify multiple proteasome beta-subunits and inhibit intracellular protein degradation and major histocompatibility complex class I antigen presentation. *J. Biol. Chem.*, **16**, 3437–3445.

Davis, H.P. & Squire, L.R. (1984) Protein synthesis and memory. A review. *Psychol. Bull.*, **96**, 518–559.

Debiec, J., Ledoux, J.E. & Nader, K. (2002) Cellular and systems reconsolidation in the hippocampus. *Neuron*, **36**, 527–538.

Dudai, Y. & Eisenberg, M. (2004) Rites of passage of the engram: reconsolidation and the lingering consolidation hypothesis. *Neuron*, **44**, 93–100.

Fenteany, G., Standaert, R.F., Lane, W.S., Choi, S., Corey, E.J. & Schreiber, S.L. (1995) Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. *Science*, **268**, 726–731.

Flood, J.F., Rosenzweing, M.R., Bennet, E.L. & Orme, A.E. (1973) The influence of duration of protein synthesis inhibition on memory. *Physiol. Behav.*, **10**, 555–562.

Florian, C. & Roullet, P. (2004) Hippocampal CA3-region is crucial for acquisition and memory consolidation in Morris water maze tasks in mice. *Behav. Brain Res.*, **154**, 365–374.

Foley, A.G., Hartz, B.P., Gallagher, H.C., Ronn, L.C., Berezin, V., Bock, E. & Regan, C.M. (2000) A synaptic peptide ligand of neural cell adhesion molecule (NCAM) IGI domain prevents NCAM internalization and disrupts passive avoidance learning. *J. Neurochem.*, **74**, 2607–2613.

Fornai, F., Lenzi, P., Gesi, M., Ferrucci, M., Lazzeri, G., Carla, L., Busceti, C.L., Ruffoli, R., Soldani, P., Ruggieri, S., Alessandrı, M.G. & Paparelli, A. (2003) Fine structure and biochemical mechanisms underlying nigrostriatal inclusions and cell death after proteasome inhibition. *J. Neurosci.*, **23**, 8955–8966.

Grecksch, G. & Matthies, H. (1980) Two sensitive periods for the amnesic effect of anisomycin. *Pharmacol. Biochem. Behav.*, **12**, 663–665.

Hedge, A.N., Unokuchi, K., Pei, W., Casadio, A., Ghirardi, M., Chain, D.G., Martin, K.C., Kandel, E.R. & Schwartz, J.H. (1997) Ubiquitin C-terminal hyrdolase is an immediate-early gene essential for long-term facilitation in *Aplysia*. *Cell*, **89**, 115–126.

Hu, Z.A., Tan, Y.L., Luo, J., Li, H.D. & Li, X.C. (2003) Relationship between hippocampal long term potentiation induction and activity of 26S proteasome. *Sheng Li Xue Bao*, **55**, 648–652.

Inda, M.C., Delgado-Garcia, J.M. & Carrion, A.M. (2005) Acquisition, consolidation, reconsolidation, and extinction of eyelid conditioning responses require de novo protein synthesis. *J. Neurosci.*, **25**, 2070–2080.

Jiang, Y.H., Armstrong, D., Albrecht, U., Atkins, C.M., Noebels, J.L., Eichele, G., Sweatt, J.D. & Beaudet, A.L. (1998) Mutation of the angelman ubiquitin ligase in mice causes increased cytoplasmic p53 and deficits of contextual learning and long-term potentiation. *Neuron*, **21**, 799–811.

Judge, E.J. & Quatermain, D. (1982) Characteristics of retrograde amnesia following reactivation of memory in mice. *Physiol. Behav.*, **28**, 585–590.

Kandel, E.R. (2001) The molecular biology of memory storage: a dialogue between genes and synapses. *Science*, **294**, 1030–1038.

Kesner, R.P., Gilbert, P.E. & Wallenstein, G.V. (2000) Testing neural network models of memory with behavioral experiments. *Curr. Opin. Neurobiol.*, **10**, 260–265.

Lee, S.H., Choi, J.H., Lee, N., Lee, H.R., Kim, J.I., Yu, N.K., Choi, S.L., Lee, S.H., Kim, H. & Kaang, B.K. (2008) Synaptic protein degradation underlies destabilization of retrieved fear memory. *Science*, **319**, 1253–1256.

Lewis, D.J. (1979) Psychobiology of active and inactive memory. *Psychol. Bull.*, **86**, 1054–1083.

Lopez-Salon, M., Alonso, M., Vianna, M.R.M., Viola, H., Mello e Souza, T., Izquierdo, I., Pasquini, J.M. & Medina, J.H. (2001) The ubiquitin-proteasome cascade is required for mammalian long-term memory formation. *Eur. J. Neurosci.*, **14**, 1820–1826.

McNaughton, N. & Morris, R.G. (1987) Hippocampal synaptic enhancement and information storage within a distributed memory system. *Trends Neurosci.*, **10**, 408–415.

Merlo, E. & Romano, A. (2007) Long-term memory consolidation depends on proteasome activity in the crab chasmagnathus. *Neuroscience*, **147**, 46–52.

Morris, R.G., Inglis, J., Ainge, J.A., Olverman, H.J., Tulloch, J., Dudai, Y. & Kelly, P.A. (2006) Memory reconsolidation: sensitivity of spatial memory to inhibition of protein synthesis in dorsal hippocampus during encoding and retrieval. *Neuron*, **50**, 479–489.

Nader, K. (2003) Memory traces unbound. *Trends Neurosci.*, **26**, 65–72.

Nader, K., Schafe, G.E. & Ledoux, J.E. (2000a) Fear memories require protein synthesis in synthesis in the amygdala for reconsolidation after retrieval. *Nature*, **406**, 722–726.

Nader, K., Schafe, G.E. & LeDoux, J.E. (2000b) The labile nature of consolidation theory. *Nat. Rev. Neurosci.*, **1**, 216–219.

Quevedo, J., Vianna, M.R., Roesler, R., de-Paris, F., Izquierdo, I. & Rose, S.P. (1999) Two time windows of anisomycin-induced amnesia for inhibitory avoidance training in rats: protection from amnesia by pertaining but not pre-exposure to the task apparatus. *Learn. Mem.*, **6**, 600–607.

Sara, S.J. (2000) Retrieval and reconsolidation: toward a neurobiology of remembering. *Learn. Mem.*, **7**, 73–84.

Stewart, M.G., Davies, H.A., Sandi, C., Kraev, I.V., Rogachevsky, V.V., Peddie, C.J., Rodriguez, J.J., Cordero, M.I., Donohue, H.S., Gabbott, P.L.A. & Popov, V.I. (2005) Stress suppresses and learning induces plasticity in CA3 of rat hippocampus: a three-dimensional ultrastructural study of thorny excrescences and their postsynaptic densities. *Neuroscience*, **131**, 43–54.

Stickgold, R. & Walker, M.P. (2005) Memory consolidation and reconsolidation: what is the role of sleep? *Trends Neurosci.*, **28**, 408–415.

Treves, A. & Rolls, E.T. (1992) Computational constraints suggest the need for two distinct input systems to the hippocampal CA3 network. *Hippocampus*, **2**, 189–200.

Tronson, N.C. & Taylor, J.C. (2007) Molecular mechanisms of memory reconsolidation. *Nat. Neurosci.*, **8**, 262–275.

Wanisch, K., Tang, J., Mederer, A. & Wotjak, C.T. (2005) Trace fear conditioning depends on NMDA receptor activation and protein synthesis within the dorsal hippocampus of mice. *Behav. Brain Res.*, **157**, 63–69.

Wood, M.A., Kaplan, M.P., Brensinger, C.M., Guo, W. & Abel, T. (2005) Ubiquitin C-terminal hydrolase L3 (Uchl3) is involved in working memory. *Hippocampus*, **15**, 610–621.