HIPPOCAMPUS 24:1549–1561 (2014)

Calcineurin Phosphatase as a Negative Regulator of Fear Memory in Hippocampus: Control on Nuclear Factor-κB Signaling in Consolidation and Reconsolidation

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ABSTRACT: Protein phosphatases are important regulators of neural plasticity and memory. Some studies support that the Ca^{2+}/calmodulin-dependent phosphatase calcineurin (CaN) is, on the one hand, a negative regulator of memory formation and, on the other hand, a positive regulator of memory extinction and reversal learning. However, the signaling mechanisms by which CaN exerts its action in such processes are not well understood. Previous findings support that CaN negatively regulate the nuclear factor kappaB (NF-κB) signaling pathway during extinction. Here, we have studied the role of CaN in contextual fear memory consolidation and reconsolidation in the hippocampus. We investigated the CaN control on the NF-κB signaling pathway, a key mechanism that regulates gene expression in memory processes. We found that post-training intrahippocampal administration of the CaN inhibitor FK506 enhanced memory retention one day but not two weeks after training. Accordingly, the inhibition of CaN by FK506 increased NF-κB activity in dorsal hippocampus. The administration of the NF-κB signaling pathway inhibitor sulfasalazine (SSZ) impeded the enhancing effect of FK506. In line with our findings in consolidation, FK506 administration before memory reactivation enhanced memory reconsolidation when tested one day after re-exposure to the training context. Strikingly, memory was also enhanced two weeks after training, suggesting that reinforcement during reconsolidation is more persistent than during consolidation. The coadministration of SSZ and FK506 blocked the enhancement effect in reconsolidation, suggesting that this facilitation is also dependent on the NF-κB signaling pathway. In summary, our results support a novel mechanism by which memory formation and reprocessing can be controlled by CaN regulation on NF-κB activity.

KEY WORDS: calcineurin; NF-κB; fear conditioning; consolidation; reconsolidation; hippocampus

INTRODUCTION

Animals are surrounded by a large number of diverse and changing stimuli. In spite of this, only salient stimuli are perceived and centrally processed, and salience usually depends on both external factors and on the internal individual state. Accordingly, animals do not remember every perceived stimulus, nor make associations between all the stimuli around them. Thus, for a memory to be formed, stimuli should be relevant for the subject. In that sense, there must be cellular and molecular mechanisms that constrain memory formation (Genoux et al., 2002; Silva and Josselyn, 2002). It has been proposed that inhibitory interneurons might play an important role in regulating activation of circuits that will encode memory (Ehrlich et al., 2009; Letzkus et al., 2011). Moreover, excitatory neurons that will participate in the memory trace might have cellular mechanisms that constrain its neural plasticity as well (Genoux et al., 2002).

Consolidation is the process by which memories are stabilized and encoded in neuronal circuits. In the case of pavlovian fear memories, animals associate an aversive stimulus with some cues of the context in which the stimulus takes place. If some time later a reminder, such as contextual cues, is presented to the animal, memory can become labile again and a process of reconsolidation takes place for memory restabilization. It is well accepted that mechanisms underlying memory formation involve changes in the synaptic connections between some neurons, which is referred to as synaptic plasticity (Martin et al., 2000).

One of the most studied molecular mechanisms for controlling synaptic plasticity and memory is the balance between the phosphorylation and dephosphorylation of specific substrates. Previous studies support that protein kinases positively regulate synaptic plasticity and memory, whereas phosphatases are in general negative regulators (revised in Malleret et al., 2001). Among phosphatases, the protein calcineurin (CaN), also known as phosphatase 2B, is of particular interest for the neurobiology community, as it is directly activated by Ca^{2+}/calmodulin (Klee et al., 1998) and it is present in dendritic spines (Kuno et al., 1992). A body of evidence strongly suggests that CaN constrains memory consolidation (Ikegami and Inokuchi, 2000; Malleret et al., 2001;

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Grant sponsor: ANPCyT, Argentina; Grant number: PICT 2049 and PICT 1482; Grant sponsor: Universidad de Buenos Aires; Grant number: X198; Grant sponsor: CONICET; Grant number: PIP5466.
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Accepted for publication 14 July 2014.
DOI 10.1002/hipo.22334
Published online 17 July 2014 in Wiley Online Library (wileyonlinelibrary.com).

Gerdjikov and Beninger, 2005; Havekes et al., 2006, 2008; Baumgartel et al., 2008), and that it also has an active role in reversal learning (Havekes et al., 2006). Furthermore, it has been shown that its activity is necessary for memory extinction (Lin et al., 2003b, c; Havekes et al., 2008). Extinction entails a temporary inhibition of the original memory expression and it is considered not an unlearning of the previous formed association but rather a new memory (Myers and Davis, 2007). In previous work of our group, we found that hippocampal CaN plays an important role in fear extinction, triggering a transcription factor switch after the extinction session. In fact, CaN inhibited nuclear factor kappaB (NF-κB) and activated nuclear factor of activated T cells (NFAT) (de la Fuente et al., 2011) during extinction. In regard to other forms of synaptic plasticity, it has been shown that inhibiting CaN facilitates long-term potentiation both in vitro and in vivo (Wang and Kelly, 1996; Winder et al., 1998; Malleret et al., 2001), whereas CaN is necessary for long-term depression (Mulkey et al., 1994), a neural plasticity model which could be involved in extinction memory formation (Lin et al., 2003a).

Gene transcription regulation is a key mechanism for long-term memory formation as it allows the expression of proteins involved in neuronal function and morphology (Alberini, 2009). Several studies support that NF-κB pathway activation is necessary for memory consolidation (Freudenthal and Romano, 2000; Yeh et al., 2002; Merlo et al., 2002; Levenson et al., 2004; Freudenthal et al., 2005; Ahn et al., 2008) and reconsolidation (Merlo et al., 2005; Boccia et al., 2007; Lubin and Sweatt, 2007; de la Fuente et al., 2011; Yang et al., 2011; Si et al., 2012; Lee and Hynds, 2013). It has been demonstrated that for NF-κB activation to occur, the balance between kinases and phosphatases should favor kinases, as phosphorylation of different proteins belonging to its signaling cascade enhances its transcriptional activity. For instance, phosphorylation of the IκB regulatory subunit determines its ubiquitination and subsequent degradation by proteasome, unmasking the NLS signal of the NF-κB dimer and thus allowing it to enter to the nucleus for transcription (reviewed in Kaltschmidt et al., 2005; Meffert and Baltimore, 2005). Moreover, it also has been shown that phosphorylation of the p65 NF-κB subunit by cAMP-dependent protein kinase A (PKA) stimulates it transactivation activity (Zhong et al., 1997, 1998, 2002; Dong et al., 2008).

In this context, here we studied the effect of CaN inhibition in the hippocampus during fear conditioning consolidation and reconsolidation, and assessed whether NF-κB signaling pathway is negatively regulated by this phosphatase.

MATERIALS AND METHODS

Animals

C57BL/6 male mice, 8–10 weeks old, weighting 25–30 g, were used (La Plata University animal facilities, La Plata, Argentina). The animals were individually caged and singly housed after the time of stereotaxic surgery, with water and food ad libitum under a 12 h light/dark cycle (lights on at 8:00 A.M.) at a temperature of 21–22°C. Experiments were performed during the light cycle (between 9.00 A.M. and 16.00 P.M.). Procedures were performed with the approval of the University of Buenos Aires Institutional Animal Care and Use Committee (CICCUAL N°29/2014) and were designed in accordance with regulations of the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH publication 80-23/96), USA. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Apparatus and Behavioral Procedures

The conditioning chamber was made of transparent acrylic (24.5 cm wide × 24.5 cm length × 42 cm height) in a white wooden box with a clear front lid. The floor of the chamber consisted of parallel stainless steel grid bars, each measuring 0.3 mm diameter and spaced 0.8 mm apart. The grid was connected to a device to deliver the foot-shocks and tone presentations. Before training, the animals were handled once a day for two days. The standard training consisted of placing each mouse in the conditioning chamber and allowing a two min adaptation period. After this period, the mice received three trials (with an intertrial interval of one min) of a tone presentation (10 s, 80 dB) which coterminated with a foot-shock (0.6 mA, 1 s). After the final trial, the mice remained in the chamber for an additional min and were returned to their home cages. The weak training consisted in the presentation of one single tone-shock trial after the two min acclimatization period instead of three tone-shock trials.

Contextual fear conditioning was evaluated at different times after training by placing the mice in the training environment for five min in the absence of the foot-shock and the tone. Each test was videotaped to calculate freezing. Freezing was defined as the absence of all movements except those related to breathing, and was scored according to an instantaneous time-sampling procedure in which each animal was observed every 5 s in a 300 s testing period (de la Fuente et al., 2011). Memory was assessed and expressed as the percentage of time that the mice spent freezing, which is commonly used as an index of fear in mice (Blanchard and Blanchard, 1969). In consolidation studies, tests took place 24 h and 2 weeks after training. In reconsolidation studies, the mice were re-exposed to the training context for 5-min 24 h after training (i.e. re-exposure session). In that case, further contextual tests were performed 48 h and 2 weeks after training.

Surgery and Intrahippocampal Injections

Mice were deeply anesthetized (ketamine 160 mg/kg and xylazine 8 mg/kg injected intraperitoneally) and placed in a stereotaxic frame. After exposing the skull, two 23 gauge guide cannulae were implanted 1 mm dorsally to the dorsal hippocampus, at coordinates AP −1.9, ML ± 1.2, DV–1.2, from bregma/skull surface, in accordance with the atlas of Franklin and Paxinos (Franklin and Paxinos, 2001) and personal

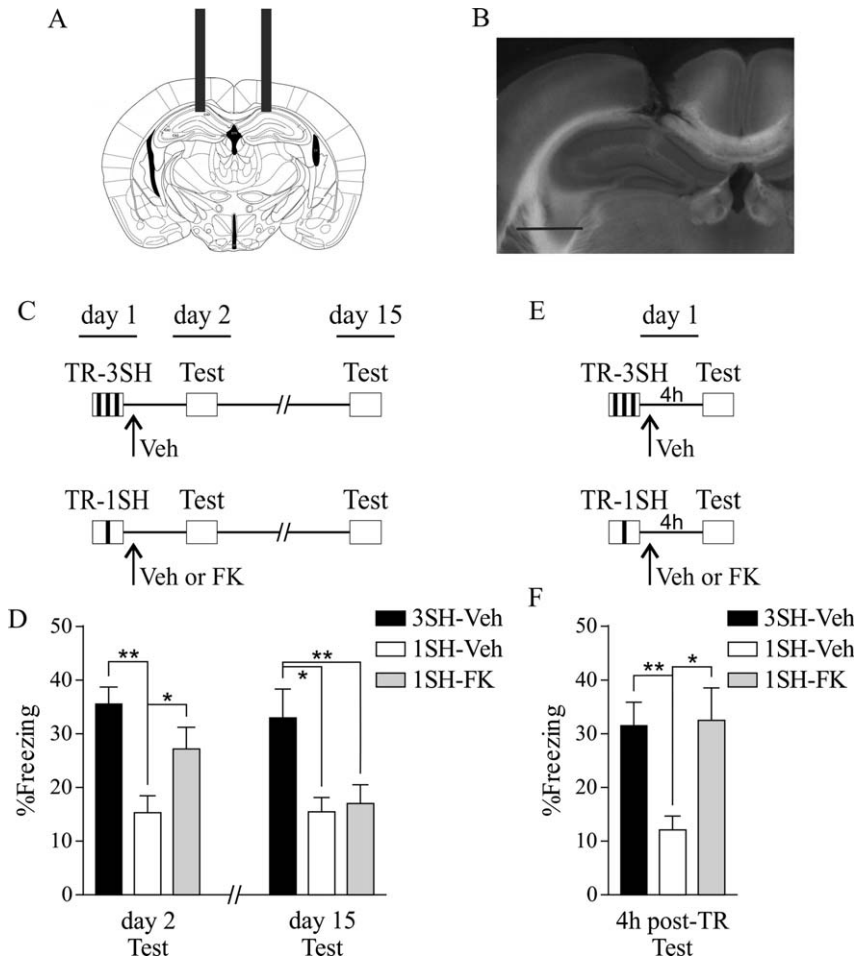

FIGURE 1. CaN inhibition in dorsal hippocampus enhances short- and long-term contextual fear memory. A: Schematic representation of a mouse brain section indicating the intended site of drug injection (−1.94 mm from bregma). Reproduced with permission from Franklin and Paxinos, The Mouse Brain in Stereotaxic Coordinates, 2001, 97, c Elsevier. B: Photograph of a representative coronal brain slice seen with a magnifying glass to verify cannulae position. The bar represents 1 mm. C: Diagram outlining the experimental design. One group of mice received a standard fear conditioning training and other two groups received a weak training. After training, mice were injected into the dorsal hippocampus with vehicle or FK (3SH-Veh; $n = 9$; 1SH-Veh, $n = 7$; 1SH-FK, $n = 10$). Tests took place on days 2 and 15. TR: training session; Test: contextual test. D: Graph represents the mean percentage of freezing for each group ± SEM. E: Same as C, except that test took place 4 h after training (3SH-Veh, $n = 10$; 1SH-Veh, $n = 8$; 1SH-FK, $n = 10$). F: Same as D, except that test took place 4 h after training. Statistical analysis in Material and Methods. * $P < 0.05$, ** $P < 0.01$.

adjustments. (see Fig. 1A) Guide cannulae were fixed to the skull with dental acrylic containing calcium hydroxide. Experiments were performed following animal recovery and injections were administered without anaesthesia. The injection device consisted of a 30 gauge cannula connected to a 5 µl Hamilton syringe by tubing. Initially, the injection device was filled with distilled water and a small air bubble was sucked into the injection cannula, followed by the injection solution. The air bubble allowed for visual inspection of the injection progress. The injection cannula was inserted into the guide cannula with its tip extending beyond the guide by 1 mm to reach the dorsal hippocampus. The injections were administered during 30 s, and operated by hand. The injection cannula was removed after 60 s to avoid reflux and to allow the diffusion of drugs. The volume of each intrahippocampal injection was 0.5 µl/hemisphere. Different injection devices were used for drugs and vehicle. After behavioral procedures, the animals were killed and their brains were placed in 4% paraformaldehyde for one day followed by 30% sucrose in PBS for an additional 24 h. To verify cannulae placement, brains were sliced using a vibratome and analyzed with a magnifying glass (a representative image is shown in Fig. 1B). The deepest position of the needle was superimposed on serial coronal maps. Only data from animals with cannulae located in the intended sites were included in the analysis.

Drugs

The CaN inhibitor FK506 [(3S, 4R, 5S, 8R, 9E, 12S, 14S, 15R, 16S, 18R, 19R, 26aS)−5, 6, 8, 11, 12, 13, 14, 15, 16,

17, 18, 19, 24, 25, 26, 26a-Hexadecahydro-5,19-dihydroxy-3-[(1E)-2-[(1R,3R,4R)-4-hydroxy-3-methoxycyclohexyl]-1-methylethenyl]-14, 16-dimethoxy-4, 10, 12, 18-tetramethyl-8-(2-propen-1-yl)-15, 19-epoxy-3H-pyrido[2,1-c][1,4] oxaazacyclotricosine-1, 7, 20, 21(4H,23H)-tetrone, monohydrate; Sigma-Aldrich, St Louis, MO; F4679] was administered at a concentration of 10 μg/μl to deliver 5 μg per hemisphere (de la Fuente et al., 2011). To inhibit NF-κB signalling pathway we used sulfasalazine (5-[4-(2-Pyridylsulfamoyl)phenylazo]salicylic acid, SSZ; Sigma-Aldrich, S0883), which was freshly dissolved at a final concentration of 2.5 mM (1 μg/μl) to deliver 0.5 μg per hemisphere (Boccia et al., 2007). All the drugs were diluted in dimethyl sulfoxide (DMSO) which was used as vehicle.

Nuclear-Enriched Protein Extraction

Mice were killed by cervical dislocation 45 min after training, which corresponds to the temporal point when an activation of NF-κB was previously observed (see Results). The brains were rapidly removed, and the hippocampus was dissected according to the method described by Glowinski and Iversen (1966). As injections were aimed to the dorsal hippocampus, extracts were performed discarding the ventral portion, and pooling dorsal hippocampus of two mice. To obtain nuclear extracts, tissues were immediately homogenized in 250 μl of buffer A (10mM HEPES pH 7.9, 10mM KCl, 1.5mM $MgCl_2$, 1 mM DTT, 1 μg/ml pepstatin A, 10 μg/ml leupeptin, 0.5mM PMSF, and 10 μg/ml aprotinin) with eight strokes in a Dounce homogenizer, type B pestle. The homogenates were centrifuged at 1000*g* for 15 min. The pellets were resuspended in 30 μl of buffer B (20 mM HEPES pH 7.9, 1200 mM KCl, 1.5 mM $MgCl_2$, 0.4 mM EDTA, 0.5 mM DTT, 50% glycerol, 1 μg/ml pepstatin A, 10 μg/ml leupeptin, 0.5 mM PMSF, and 10 μg/ml aprotinin) and incubated for 20 min on ice. A centrifugation for 15 min at 10,000*g* was then performed. The supernatants (nuclear extracts) were stored at −80°C until used. The entire extraction protocol was performed at 4°C.

Determination of NF-κB DNA-Binding Activity by Gel Shift Assay (EMSA-blot)

DNA binding activity of NF-κB in nuclear fractions was assessed using gel shift assays [electrophoretic mobility shift assay, EMSA (de la Fuente et al., 2011)]. DNA-protein binding was performed containing 20 mM HEPES pH 7.9, 120 mM KCl, 0.4 mM EDTA, 0.5 mM DTT, 25% glycerol, 0.3 μg polidIdC, and 10 μg of protein extract. The samples were incubated for 40 min at 0°C and 35 ng of oligonucleotide DNA probe (double-stranded DNA oligonucleotide containing the NF-κB binding site, AGTTGAG**GGGACTTTCC**CAGGC; binding site in bold; Promega, Madison, WI) was added followed by incubation for another 40 min at 0°C. The reaction mixture was then electrophoresed on a 6% nondenaturing polyacrylamide gel in 0.25% TBE (22.3 mM Tris, 22.3 mM boric acid, 0.5 mM EDTA) for 2 h at 160 V. The gel was electrotransfered to polyvinylidene difluoride (PVDF) membrane for immunoblotting (30 min at 100 V) using transfer buffer with 10% methanol, as described by A. Salles and R. Freudenthal (unpublished observations).

Western blot protocol consisted on activating the EMSA-PVDF membranes in 100% methanol, rinsing in distilled water, and incubating for one hour in blocking buffer [TTBS (0.1% Tween 20, 50 mM Tris-base, 150 mM NaCl, pH7.6) and 4% w/v nonfat dry milk] at room temperature. Membranes were then incubated O.N. at 4°C with anti-NF-κB p65 antibody in blocking buffer (Santa Cruz Biotechnology; sc-320; at 1:1000 dilution). This was followed by three washes in TTBS (5–10 min each time) and incubation for 1 h with HRP-conjugated secondary antibody in blocking buffer (Santa Cruz Biotechnology, sc-2030, at 1:5000 dilution) at room temperature. The membranes were washed twice with TTBS and once with TBS. The detection was performed using a luminol chemiluminescence kit (Amersham Biosciences, Piscataway, NJ; RPN2235) following the manufacturer's protocol, and signals were digitized using an imaging device (Fuji Intelligent Dark Box) and LAS-1000 software. The relative optic density was estimated using NIH ImageJ 1.29x software. The two bands observed after the EMSA-blot technique corresponding to p65 containing-NF-κB complexes were used for the analyses. All measurements were made with exposures within the linear range. Protein contents of the extracts were measured in triplicate by BCA assay method (Pierce Biotechnology, Rockford, lL; 23227).

Data Analyses

Behavioral experiments

The results are presented as mean percentage of freezing ± SEM. When more than one behavioral test was performed to the same animals, data were analyzed by Repeated Measures Two-Way ANOVA, with "behavioral test" as within-subjects factor and "treatment" as between-subjects factor (mixed model ANOVA). Within each behavioral test, the treatments were compared (simple effects between treatments) and corrected for multiple comparisons. If the protocol included two treatments, Holm-Sidak's post hoc test was used. If the protocol included more than two treatments, Newman-Keuls post hoc test was used. Conversely, when one behavioral test was performed and if the protocol included three treatments, data were analyzed by one-way ANOVA corrected for multiple comparisons using the Newman-Keuls post hoc test. If the protocol included two treatments, data were analyzed by unpaired two-tailed Student's *t* test. A significance level of $P < 0.05$ was used for all behavioral analyses. Where stated, NS means no significant differences.

EMSA-blot experiments

The optical densities of the EMSA-blot bands corresponding to each subject from each group were normalized to the mean value of the optical density of the corresponding 1SH-Veh group, which was considered as 100%. Then the mean values of the optical density ± SEM were calculated and were compared against the mean value of optical density of 1SH-Veh group. Data were analyzed by unpaired two-tailed Student's *t* test corrected for multiple comparisons (Bonferroni; $\alpha_{(corrected)} = 0.05/2$), as the data

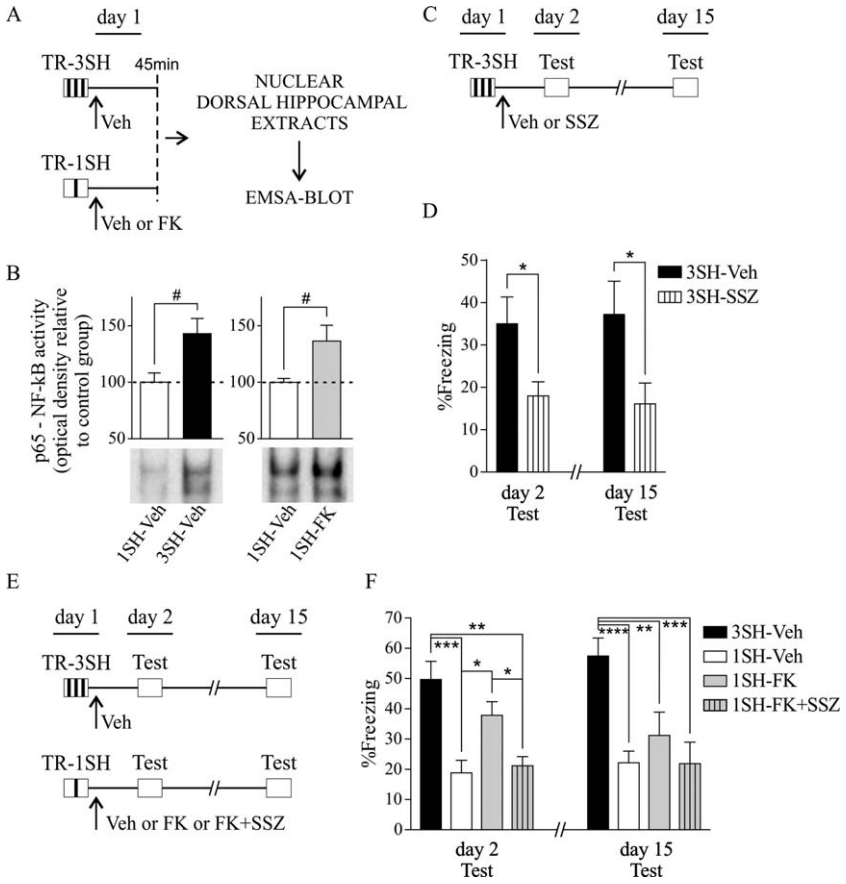

FIGURE 2. A: The hippocampal inhibition of CaN induces NF-κB activation in hippocampus. Diagram outlining the experimental design. One group of mice received a standard fear conditioning training and other two groups received a weak training. After training, mice were injected into the dorsal hippocampus with vehicle or FK. Forty-five minutes after training, mice were killed by cervical dislocation and dorsal hippocampal nuclear extracts were prepared. p65 κB binding activity was estimated by EMSA-blot. B: Graphs comparing mean values of p65 κB binding activity from each group. Left: Mean values of optical density corresponding to 3SH-Veh group, normalized and compared with 1SH-Veh control group, whose extracts were run on the same gel (1SH-Veh, $n = 10$; 3SH-Veh, $n = 9$). Right: Mean values of optical density corresponding to 1SH-FK group, normalized and compared to 1SH-Veh control group, whose extracts were run on the same gel (1SH-Veh, $n = 10$; 1SH-FK, $n = 10$). Representative p65 EMSA-blot bands are shown. C: NF-κB inhibition in dorsal hippocampus after fear conditioning training disrupts long-term memory formation. Diagram outlining the experimental design. Two groups of mice received a standard fear conditioning training and after training were injected into the dorsal hippocampus with vehicle or SSZ (3SH-Veh, $n = 9$; 3SH-SSZ, $n = 9$). Tests took place on days 2 and 15. TR: training session; Test: contextual test. D: Graph represents the mean percentage of freezing for each group ± SEM. E: Long-term memory enhancement induced by CaN inhibition depends on NF-κB. Diagram outlining the experimental design. One group of mice received a standard fear conditioning training and other two groups received a weak training. After training mice were injected with vehicle, FK, or FK + SSZ (3SH-Veh, $n = 11$; 1SH-Veh, $n = 11$; 1SH-FK, $n = 10$; 1SH-FK+SSZ, $n = 10$). Tests took place on days 2 and 15. TR: training session; Test: contextual test. F: Graph represents the mean percentage of freezing for each group ± SEM. Statistical analysis in Material and Methods. $^{\#}P < 0.025$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

from 1SH-Veh group (Fig. 2B left and right) come from the same samples run on different gels.

RESULTS

The Inhibition of CaN in Dorsal Hippocampus Enhances Memory Consolidation

The aim of the first experiment was to analyze the putative role of CaN in the dorsal hippocampus as a negative regulator of contextual fear memory formation. We hypothesized that memory enhancement should be observed after the intrahippocampal administration of the CaN inhibitor FK506. To test this hypothesis, we used a contextual fear conditioning paradigm with two types of trainings, a standard and a weak training, which differed only in the number of training trials: three trials of foot-shocks for the standard training and one trial for the weak training. The standard training was proved to induce a long-term contextual fear memory (de la Fuente et al., 2011), whereas the weak one was expected to induce a weak memory. This distinction between the two trainings should allow us to evaluate if memory enhancement occurs when CaN

is inhibited after a weak training session, which could be established by an increase in the freezing response. Two groups of cannulated mice were trained with one foot-shock, and immediately post-training, they were bilaterally injected into the dorsal hippocampus with either the vehicle DMSO or FK506 (1SH-Veh and 1SH-FK, respectively). A third group trained with three foot-shocks and injected with vehicle (3SH-Veh) was used as a positive control for memory retention at testing. Contextual memory was assessed one day and then two weeks after training (days 2 and 15, respectively; Fig. 1A,B,C). Repeated measures ANOVA revealed a main effect of "treatment" ($F_{(2,23)} = 11.69$, $P = 0.0003$) with no "behavioral test" effect ($F_{(1,23)} = 1.61$, $P = 0.22$) and no "treatment" × "behavioral test" interaction ($F_{(2,23)} = 0.92$, $P = 0.42$). Post hoc comparisons indicated that, in the first test, mice injected with FK506 displayed higher levels of freezing than 1SH-Veh and similar levels to the positive control 3SH-Veh (Newman-Keuls post hoc test: 3SH-Veh vs. 1SH-Veh, $P < 0.01$; 3SH-Veh vs. 1SH-FK, NS; 1SH-Veh vs. 1SH-FK, $P < 0.05$; Fig. 1D). Conversely, at the second test, the 1SH-FK group showed similar levels of freezing to 1SH-Veh and significantly lower levels than 3SH-Veh (3SH-Veh vs. 1SH-Veh, $P < 0.05$; 3SH-Veh vs. 1SH-FK, $P < 0.01$; 1SH-Veh vs. 1SH-FK, NS; Fig. 1D). These results indicate that CaN inhibition by FK506 during consolidation induced long-term memory enhancement. However, such enhancement did not have the same properties as the standard training, as the generated memory was less persistent.

Considering that short-term memory formation requires activation of kinases (revised in Sweatt, 2003), we wondered if inhibiting CaN after acquisition, and therefore altering the kinase-phosphatase balance, would also enhance short-term contextual fear memory. In the following experiment we evaluated the effect of CaN inhibition by evaluating memory after a short period of time, before memory consolidation takes place. The experimental protocol was the same as in Figure 1C but the test was performed 4 h after training instead of 24 h (Fig. 1E). Mice that received a weak training and were bilaterally injected into the dorsal hippocampus with FK506 (1SH-FK) showed higher levels of freezing than those injected with vehicle (1SH-Veh) and similar to the positive control 3SH-Veh (ANOVA: $F_{(2,25)} = 5.27$, $P = 0.012$; Newman-Keuls post hoc test: 3SH-Veh vs. 1SH-Veh, $P < 0.01$; 3SH-Veh vs. 1SH-FK, NS; 1SH-Veh vs. 1SH-FK, $P < 0.05$; Fig. 1F). This indicates that CaN inhibition after training enhanced short-term memory.

The Hippocampal Inhibition of CaN Induces NF-κB Activation in Hippocampus

Results of the former section, as well as previous reports, support that CaN acts as a negative element for short- and long-term memory formation. However, few data are available about the downstream molecular mechanisms that are directly regulated by this phosphatase in memory consolidation. In previous work, we found that CaN impedes NF-κB activation during the formation of extinction memory (de la Fuente et al., 2011). We then asked if the reinforcement observed when CaN is inhibited during consolidation is indeed dependent on NF-κB activation. In other words, we asked whether CaN normally restrains NF-κB signaling pathway when regulating long-term memory formation. To test the purported activation of NF-κB after FK506 administration, we measured NF-κB activity after strong and weak training, particularly when CaN was inhibited. When we previously studied the dynamics of NF-κB activation after training on a fear conditioning task, we found a differential activation between strong-trained animals and naïve animals only when the former were sacrificed 45 min after being trained (unpublished data). Thus, for the following experiment, which was aimed at studying the effect of CaN inhibition in NF-κB signaling pathway, we chose the sacrifice time point as the one at which the activation of NF-κB was previously observed.

Two groups of mice were weakly trained and immediately post-training were bilaterally injected into the dorsal hippocampus with either vehicle or FK506 (1SH-Veh and 1SH-FK, respectively). A third group was standard-trained and injected with vehicle (3SH-Veh). Mice were sacrificed 45 min after training and their dorsal hippocampus were dissected. Nuclear enriched extracts were prepared, and NF-κB activity was measured using the EMSA-blot technique (Fig. 2A). Standard-trained animals (3SH-Veh) showed higher levels of NF-κB activity compared to weakly trained animals (1SH-Veh; $t_{(17)} = 2.79$, $P = 0.013$; Fig. 2B, left), indicating that NF-κB is activated after strong training. Weakly trained animals injected with FK506 (1SH-FK) also showed higher levels of NF-κB activity compared to weakly trained animals injected with vehicle (1SH-Veh; $t_{(18)} = 2.53$, $P = 0.021$; Fig. 2B, right). These results suggest that CaN effectively constrains the NF-κB signaling pathway during memory formation.

On the base of these findings, the following series of experiments were aimed at studying the relationship between CaN and NF-κB, analyzing the effect of simultaneous pharmacological inhibition of both proteins in the hippocampus on the consolidation and reconsolidation processes.

The Hippocampal Inhibition of NF-κB Impedes FK506-Mediated Memory Enhancement

We designed an experiment based on the possibility to inhibit simultaneously CaN and NF-κB. Thus, it was important to use an NF-κB inhibitor that could be dissolved in the same solution as FK506. The drug sulfasalazine (SSZ), which can be dissolved in DMSO, is a specific inhibitor of IκB kinase (IKK), the protein kinase necessary for the canonical NF-κB activation (Wahl et al., 1998; Weber et al., 2000). Sulfasalazine was found to impair memory consolidation in phylogenetically distant species like crabs (Merlo et al., 2002) and rodents (Freudenthal et al., 2005; but see Lee and Hynds, 2013). In particular, it was shown that when injected bilaterally into mice dorsal hippocampus at a dose of 0.5 μg per side, SSZ induced amnesia in an inhibitory avoidance task (Freudenthal et al., 2005). In the following experiment, the same dose of

SSZ was injected bilaterally into the dorsal hippocampus after standard fear conditioning training to test its amnesic effect. After two groups of cannulated animals received a standard training, one group was bilaterally injected into hippocampus with vehicle (3SH-Veh) and the other group with SSZ (3SH-SSZ). Contextual memory was assessed one day and then two weeks after training (days 2 and 15, respectively; Fig. 2C). Repeated measures ANOVA revealed a main effect of "treatment" ($F_{(1,16)} = 7.18$, $P = 0.016$) with no "behavioral test" effect ($F_{(1,16)} = 0.002$, $P = 0.97$) and no "treatment" × "behavioral test" interaction ($F_{(1,16)} = 0.23$, $P = 0.64$). Post hoc comparisons indicated that, in the first test, mice injected with SSZ showed a lower level of freezing than those injected with vehicle (Holm-Sidak's post hoc test: $P < 0.05$; Fig. 2D), suggesting that inhibition of the IKK/NF-κB pathway during memory consolidation causes memory impairment. Similar results were found in the second test, indicating that the impairment was long lasting (Holm-Sidak's post hoc test: $P < 0.05$; Fig. 2D).

Next, we evaluated the effect of FK506 and SSZ cocktail administration on memory consolidation. Three groups of cannulated mice were trained with one foot-shock and were bilaterally injected into the dorsal hippocampus with either vehicle, FK506 or FK506 + SSZ cocktail (1SH-Veh, 1SH-FK, and 1SH-FK + SSZ, respectively; Fig. 2E). A fourth group trained with three foot-shocks and injected with vehicle (3SH-Veh) was used as a positive control for memory retention at testing. Contextual memory was assessed one day and then two weeks after training (days 2 and 15, respectively). Repeated measures ANOVA revealed a main effect of "treatment" ($F_{(3,38)} = 11.80$, $P < 0.0001$) with no "behavioral test" effect ($F_{(1,38)} = 0.17$, $P = 0.68$) and no "treatment" × "behavioral test" interaction ($F_{(3,38)} = 0.98$, $P = 0.41$). Consistent with results of Figure 1D, post hoc comparisons indicated that in the first test, the 1SH-FK group displayed similar levels of freezing to 3SH-Veh and significantly higher than 1SH-Veh (Newman-Keuls post hoc test: 3SH-Veh vs. 1SH-Veh, $P < 0.001$; 3SH-Veh vs. 1SH-FK, NS; 1SH-Veh vs. 1SH-FK, $P < 0.05$; Fig. 2F). However, mice injected with FK506 + SSZ displayed similar levels of freezing to 1SH-Veh and significantly lower than both 3SH-Veh and 1SH-FK (1SH-FK + SSZ vs. 1SH-Veh, NS; 1SH-FK + SSZ vs. 3SH-Veh, $P < 0.01$; 1SH-FK + SSZ vs. 1SH-FK, $P < 0.05$; Fig. 2F). Thus, no memory enhancement was observed when both CaN and NF-κB were inhibited. These findings support that the long-term memory enhancing effect of CaN inhibition depends on NF-κB activation. In the second test, in agreement with the results of Figure 1D, the 1SH-FK group displayed similar levels of freezing to 1SH-Veh and significantly lower levels than 3SH-Veh (Newman-Keuls post hoc test: 3SH-Veh vs. 1SH-Veh, $P < 0.0001$; 3SH-Veh vs. 1SH-FK, $P < 0.01$; 1SH-Veh vs. 1SH-FK, NS; Fig. 2F). Moreover, mice injected with FK506 + SSZ continue not to exhibit any significant difference from either the 1SH-Veh group or the 1SH-FK group (1SH-FK + SSZ vs. 1SH-Veh, NS; 1SH-FK + SSZ vs. 3SH-Veh, $P < 0.001$; 1SH-FK + SSZ vs. 1SH-FK, NS; Fig. 2F).

The Hippocampal Inhibition of CaN After Retrieval Enhances Memory Reconsolidation

Treatments that interfere with consolidation, such as the administration of protein synthesis inhibitors, have been effective in disrupting a reactivated memory (Nader et al., 2000; Pedreira and Maldonado, 2003). Memory reactivation can be achieved by the presentation of a reminder of the learning event. Such conditions determine that the memory trace becomes transiently labile, followed by a process of reconsolidation to restabilize the memory trace. Several studies in the last years have demonstrated that reconsolidation partially recapitulates consolidation, in terms of the anatomical and molecular substrates involved (Nader, 2003; Alberini, 2005; Von Hertzen and Giese, 2005). Regarding its functional value, it was proposed that reconsolidation provides an opportunity for memory updating and/or reinforcement (Lee, 2009; Forcato et al., 2011; Inda et al., 2011). The following experiments were aimed at testing whether CaN inhibition near retrieval enhances postreactivated long-term memory. Thus, we evaluated if the memory generated by a weak training could be strengthened during memory reconsolidation. Three groups of cannulated mice were trained: one using the standard protocol, and the other two using the weak protocol. One day after training, standard-trained animals were bilaterally injected with vehicle (3SH-R-Veh), and weakly trained animals were injected into the dorsal hippocampus with either vehicle or FK506 (1SH-R-Veh and 1SH-R-FK, respectively; Fig. 3A). Fifteen min after injections, all animals were re-exposed for five min to the training context, which constitutes a reminder that induces memory labilization-reconsolidation (de la Fuente et al., 2011). Further tests took place one day after re-exposure and then two weeks after training (days 3 and 15, respectively). Repeated measures ANOVA revealed a main effect of "treatment" ($F_{(2,21)} = 6.89$, $P = 0.005$), a main effect of "behavioral test" ($F_{(2,42)} = 4.42$, $P = 0.02$) and a "treatment" × "behavioral test" interaction ($F_{(4,42)} = 3.36$, $P = 0.02$). As expected, post hoc comparisons indicated that only standard-trained animals displayed high levels of freezing on the re-exposure day (Newman-Keuls post hoc test: 3SH-Veh vs. 1SH-Veh, $P < 0.01$; 3SH-Veh vs. 1SH-FK, $P < 0.01$; 1SH-Veh vs. 1SH-FK, NS; Fig. 3B). However, in the first test after re-exposure the FK506-injected mice displayed similar levels of freezing to standard-trained animals, indicating that memory was reinforced after retrieval (3SH-Veh vs. 1SH-Veh, $P < 0.05$; 3SH-Veh vs. 1SH-FK, NS; 1SH-Veh vs. 1SH-FK, $P < 0.01$; Fig. 3B). Surprisingly, memory facilitation was also observed at the second test after re-exposure (3SH-Veh vs. 1SH-Veh, $P < 0.05$; 3SH-Veh vs. 1SH-FK, NS; 1SH-Veh vs. 1SH-FK, $P < 0.01$; Fig. 3B). To ensure that the former experiment involves reconsolidation-like mechanisms, we analyzed the behavior of non-re-exposed mice bilaterally injected into the dorsal hippocampus with either vehicle or FK506 (3SH-NR-Veh and 3SH-NR-FK, respectively) 24 h after training (Fig. 3C). No memory enhancement was observed if animals were not re-exposed to the training context after the intrahippocampal injections (ANOVA: $F_{(2,21)} = 6.98$, $P = 0.005$;

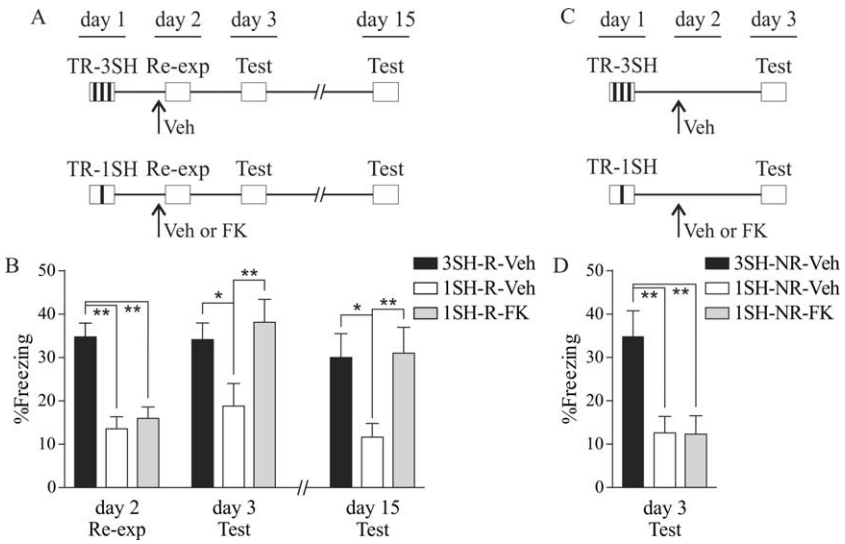

FIGURE 3. CaN inhibition in dorsal hippocampus enhances contextual fear memory reconsolidation. A: Diagram outlining the experimental design. One group of mice received a standard fear conditioning training and other two groups received a weak training. One day after training (day 2), mice were injected with vehicle or FK (3SH-R-Veh, $n = 7$; 1SH-R-Veh, $n = 7$; 1SH-R-FK, $n = 10$). Fifteen min later, all mice were re-exposed to the training context for 5 min. Further tests took place 1 day after re-exposure (day 3) and 2 weeks after training (day 15). TR: training session; Re-exp: 5-min re-exposure session; Test: contextual test. B: Graph represents the mean percentage of freezing for each group ± SEM. C: Diagram outlining the experimental design. Groups are as in A except that animals were not re-exposed to the training context on day 2. Instead, 24 h after training, animals were injected either with vehicle or FK (3SH-NR-Veh, $n = 7$; 1SH-NR-Veh, $n = 7$; 1SH-NR-FK, $n = 10$). D: Graph represents the mean percentage of freezing for each group ± SEM. Statistical analysis in Materials and Methods. * $P < 0.05$; ** $P < 0.01$.

Newman-Keuls post hoc test: 3SH-NR-Veh vs. 1SH-NR-Veh, $P < 0.01$; 3SH-NR-Veh vs. 1SH-NR-FK, $P < 0.01$; 1SH-NR-Veh vs. 1SH-NR-FK, NS; Fig. 3D). Taken together, these experiments demonstrated that the weak training generated a weak memory, which is reactivated by the reminder and can eventually be enhanced during the re-stabilization phase.

Inhibition of NF-κB After Retrieval Impedes FK506-Induced Memory Enhancement

The results obtained in the consolidation experiments led us to investigate whether memory enhancement induced by CaN inhibition after retrieval is also dependent on the NF-κB signaling pathway. Several works indicate that SSZ administration disrupts reconsolidation in different species and memory paradigms (Merlo et al., 2005; Lubin and Sweatt, 2007; Boccia et al., 2007; Si et al., 2012; Lee and Hynds, 2013). Thus, we first verified that the hippocampal injection of SSZ at the same dose used in consolidation (Fig. 2B) was also amnesic in reconsolidation. The time point of injection had to be the same as in the experiment where the effect of FK506 on reconsolidation was evaluated (Fig. 3B), as we planned to further perform a cocktail experiment. Two groups of cannulated animals were trained with a standard protocol. One day after training, mice were bilaterally injected into the dorsal hippocampus with either vehicle or SSZ (3SH-R-Veh and 3SH-R-SSZ, respectively) and 15 min later were re-exposed to the training chamber for five min. Further tests took place one day after re-exposure and then two weeks after training (days 3 and 15, respectively; Fig. 4A). Repeated measures ANOVA revealed a main effect of "treatment" ($F_{(1,24)} = 20.82$, $P = 0.0001$) with no "behavioral test" effect ($F_{(1,24)} = 0.17$, $P = 0.69$) and no "treatment" × "behavioral test" interaction ($F_{(2,24)} = 0.11$, $P = 0.75$). Post hoc comparisons indicated that, in the first test after re-exposure, mice injected with SSZ displayed lower levels of freezing than those injected with vehicle (Holm-Sidak's post hoc test: $P < 0.001$; Fig. 4B), indicating that inhibition of the IKK-NF-κB pathway during memory reconsolidation causes memory impairment. These results are in agreement with Boccia et al. (2007), who used the same dose of SSZ in mice, albeit in a different paradigm. In the second test after re-exposure two alternative results could be expected: (a) memory impairment due to the amnesic effect of the drug or (b) memory retention if extinction was initially enhanced by the drug and then the freezing reappeared by spontaneous recovery. Memory impairment was actually found, suggesting that the impairment was long lasting (Holm-Sidak's post hoc test: $P < 0.001$; Fig. 4B) and ruling out the possibility of extinction enhancement. To confirm that the former experiment involves reconsolidation-like mechanisms, we analyzed the behavior of non-re-exposed cannulated mice bilaterally injected into the dorsal hippocampus with either vehicle or SSZ (3SH-NR-Veh and 3SH-NR-SSZ, respectively) 24 h after training (Fig. 4C). No memory impairment was observed if animals were not re-exposed to the training context after the intra-hippocampal injections ($t_{(14)} = 0.60$, $P = 0.56$; Fig. 4D).

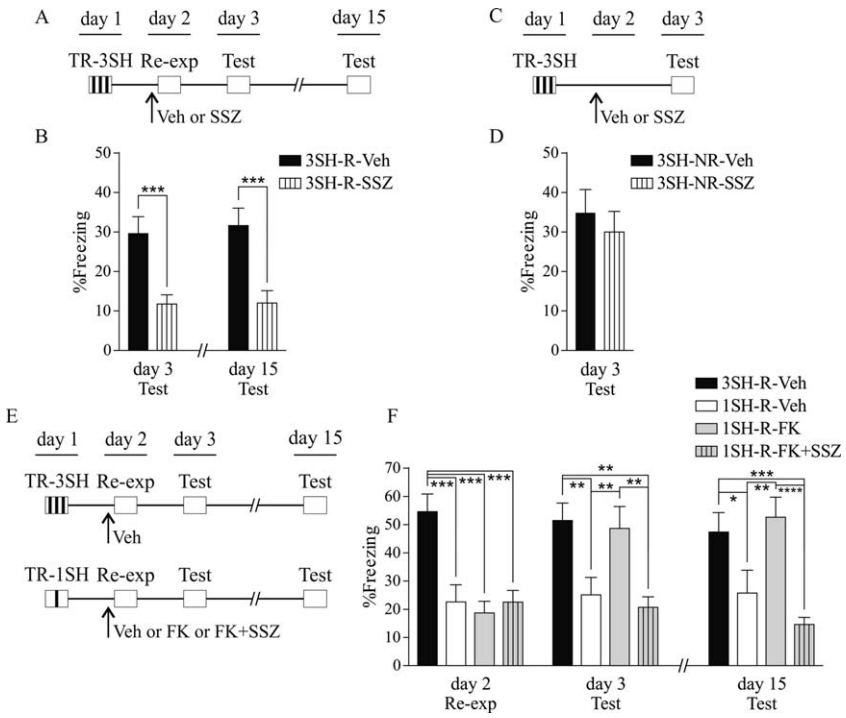

FIGURE 4. A: NF-κB inhibition in dorsal hippocampus after retrieval disrupts memory reconsolidation. Diagram outlining the experimental design. Two groups of mice received a standard fear conditioning training. One day after training (day 2), mice were injected with vehicle or SSZ (3SH-R-Veh, $n = 12$; 3SH-R-SSZ, $n = 14$). Fifteen min later, all mice were re-exposed to the training context. Further tests took place 1 day after re-exposure (day 3) and 2 weeks after training (day 15). TR: training session; Re-exp: 5-min re-exposure session; Test: contextual test. B: Graph represents the mean percentage of freezing for each group ± SEM. C: Diagram outlining the experimental design. Groups are as in A except that animals were not re-exposed to the training context on day 2. Instead, 24 h after training, animals were injected either with vehicle or SSZ (3SH-NR-Veh, $n = 7$; 3SH-NR-SSZ, $n = 9$). D: Graph represents the mean percentage of freezing for each group ± SEM. E: Long-term memory enhancement induced by CaN inhibition after retrieval depends on NF-κB. Diagram outlining the experimental design. One group of mice received a standard fear conditioning training and three groups received a weak training. One day after training (day 2), mice were injected with vehicle, FK, or FK + SSZ (3SH-R-Veh, $n = 9$; 1SH-R-Veh, $n = 9$; 1SH-R-FK, $n = 9$; 1SH-R-FK + SSZ, $n = 11$). Fifteen min later, all mice were re-exposed to the training context. Further tests took place 1 day after re-exposure (day 3) and 2 weeks after training (day 15). TR: training session; Re-exp: 5-min re-exposure session; Test: contextual test. F: Graph represents the mean percentage of freezing for each group ± SEM. Statistical analysis in Materials and Methods. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

In the next experiment we tested whether SSZ was able to block memory enhancement induced by FK506. Four groups of cannulated mice were trained: one with the standard protocol, and the other three with the weak protocol. One day after training, standard-trained animals were bilaterally injected into the dorsal hippocampus with vehicle (3SH-R-Veh), and weakly-trained animals were injected with either vehicle, FK506, or FK506 + SSZ cocktail (1SH-R-Veh, 1SH-R-FK, and 1SH-R-FK + SSZ, respectively; Fig. 4E). Fifteen min after injections, animals were re-exposed to the training chamber for five min. Further tests took place one day after re-exposure and then two weeks after training (days 3 and 15, respectively; Fig. 4E). Repeated measures ANOVA revealed a main effect of "treatment" ($F_{(3,34)} = 11.04$, $P < 0.0001$) and a "treatment" × "behavioral test" interaction ($F_{(6,68)} = 5.06$, $P = 0.0002$), with no "behavioral test" effect ($F_{(2,68)} = 2.50$, $P = 0.09$). As expected, post hoc comparisons indicated that only standard-trained animals displayed high levels of freezing on the re-exposure day (Newman-Keuls post hoc test: 3SH-Veh vs. 1SH-Veh, $P < 0.001$; 3SH-Veh vs. 1SH-FK, $P < 0.001$; 3SH-Veh vs. 1SH-FK + SSZ, $P < 0.001$; Fig. 4F). In the first test after re-exposure, and in agreement with the results shown in Figure 3B, the 1SH-FK group displayed similar levels of freezing to 3SH-Veh and significantly higher than 1SH-Veh (3SH-Veh vs. 1SH-Veh, $P < 0.01$; 3SH-Veh vs. 1SH-FK, NS; 1SH-Veh vs. 1SH-FK, $P < 0.01$; Fig. 4F). However, mice injected with FK506 + SSZ displayed similar levels of freezing to 1SH-Veh and significantly lower than both 3SH-Veh and 1SH-FK (1SH-FK + SSZ vs. 1SH-Veh, NS; 1SH-FK + SSZ vs. 3SH-Veh, $P < 0.01$; 1SH-FK + SSZ vs. 1SH-FK, $P < 0.01$; Fig. 4F). These results indicate that memory enhancement induced by FK506 administration before retrieval was impaired by the inhibition of the IKK/NF-κB pathway, supporting that the enhancing effect of CaN inhibition before retrieval depends on

NF-κB activation. In the second test, the 1SH-FK group displayed similar levels of freezing to 3SH-Veh and significantly higher levels than 1SH-Veh (3SH-Veh vs. 1SH-Veh, $P < 0.05$; 3SH-Veh vs. 1SH-FK, NS; 1SH-Veh vs. 1SH-FK; $P < 0.01$; Fig. 4F). Moreover, mice injected with FK506 + SSZ continue to show no significant differences with the 1SH-Veh group, but displayed lower levels of freezing than both 1SH-FK and 3SH-Veh (1SH-FK + SSZ vs. 1SH-Veh, NS; 1SH-FK + SSZ vs. 3SH-Veh, $P < 0.001$; 1SH-FK + SSZ vs. 1SH-FK, $P < 0.0001$; Fig. 4F).

DISCUSSION

The results of this work support the role of CaN as a negative regulator of contextual fear memory consolidation and reconsolidation in hippocampus. Post-training hippocampal CaN inhibition both enhanced long-term fear contextual memory and increased NF-κB activity in hippocampus 45 min after training, like the standard training did. Memory enhancement was impeded when NF-κB was inhibited, a finding that strongly suggests that the regulation of memory by CaN is achieved by the restraining of NF-κB signaling pathway.

The post-training CaN hippocampal inhibition also produced an enhancement of contextual fear memory 4 h after training. This facilitation of short-term memory is in agreement with the experiments of Malleret et al. (2001), in which novel object recognition memory was enhanced by CaN transgenic inhibition. Short and long-term memories require different molecular mechanisms. Previous results indicate that short-term memory does not depend on transcriptional mechanisms as long-term memory does (for a review see Kandel, 2001), but instead hinge on post-translational modifications of different proteins. Therefore, CaN role in both short and long-term memory enhancements might be different. CaN could be restraining short-term memory formation by dephosphorylating protein effectors like neurotransmitter receptors. Furthermore, it could also dephosphorylate other substrates involved in long-term memory formation like transcription factors and/or components of their signalling pathway.

When CaN was inhibited during the reconsolidation of a weak memory, post-reactivated long term fear memory was also enhanced. Memory enhancement after reconsolidation was also dependent on NF-κB activity, as its inhibition blocked the post-retrieval enhancement induced by CaN hippocampal inhibition. These results support that CaN phosphatase actually acts constraining not only memory formation but also memory reprocessing after retrieval.

Strikingly, the tests performed 2 weeks after training revealed differences in memory enhancement between consolidation and reconsolidation. The enhancement of contextual memory by post-training CaN inhibition was less persistent than the one observed when CaN was inhibited near retrieval. In the last case, memory retention was still present 2 weeks after training. On the one hand, these results show that other mechanisms rather than just an imbalance of phosphorylation levels during consolidation are necessary for long-lasting memory formation. Accordingly, recent findings support the requirement of NF-κB-dependent chromatin acetylation for persistent memory retention (Federman et al., 2013). Further experiments are required to elucidate whether CaN inhibition promotes NF-κB-dependent histone acetylation during fear memory reconsolidation. On the other hand, the disparities found here between the effect of the CaN inhibitor FK506 on memory enhancement in consolidation and reconsolidation can be attributed to differences in the signaling pathways involved in both processes. This idea was previously reported regarding neural substrates involved (Alberini, 2005) and the temporal course of different molecular mechanisms (Anokhin et al., 2002; Lee et al., 2004; Boccia et al., 2007; Lee and Hynds, 2013). Regarding the latter, it is important to note that the cellular and molecular context in which consolidation occurs might be remarkably different than when reconsolidation takes place. At the time of memory retrieval and reconsolidation, consolidation has already occurred and, consequently, a different cellular and molecular context has been created. Evidence is the fact that NF-κB is activated at different times during consolidation and reconsolidation, In this work, EMSA-blotting revealed differences in hippocampal NF-κB activity between standard-trained mice and weak-trained mice, the former displaying more activity than the latter. In line with this, we have found similar differences when comparing NF-κB activity between standard-trained animals and naïve, only when the trained mice were sacrificed 45 min after fear conditioning training (unpublished observations). Similar results were previously found using the inhibitory avoidance paradigm in mice (Freudenthal et al., 2005). Conversely, in memory reconsolidation the activation of NF-κ B was found 15 min after context re-exposure using either the fear conditioning task or the inhibitory avoidance task (Boccia et al., 2007; de la Fuente et al., 2011). In accordance, NF-κB inhibitors have been proved to impair memory consolidation and reconsolidation of fear memories (Merlo et al., 2002, 2005; Yeh et al., 2002; Levenson et al., 2004; Freudenthal et al., 2005; Boccia et al., 2007; Lubin and Sweatt, 2007; Ahn et al., 2008; de la Fuente et al., 2011; Yang et al. 2011; Si et al., 2012). However, Lee and Hynds (2013) found that SSZ only impairs contextual fear reconsolidation when using a fear conditioning task in rats, having no effect on contextual fear consolidation. The authors further found molecular evidence of the involvement of IKK/NF-κB signaling pathway in reconsolidation, but not in consolidation, thus postulating the activation NF-κB signaling pathway as a specific mechanism underlying reconsolidation. The different species, time points for IKK activation and the doses of SSZ used in these studies could account for the dissimilar results.

Like other protein phosphatases, CaN has many targets in the central nervous system, which are involved in signalling pathways that allow for integration of internal and external information. In particular, CaN interacts with the cAMP

cascade, which is of special interest as PKA is involved in many neuronal processes like memory formation and LTP. Indeed, CaN and PKA antagonistically regulate the phosphorylation state and function of several proteins, like the NMDA (Tong et al., 1995; Raman et al., 1996), GluR1 (Price et al., 1999; Beattie et al., 2000) and GluR6 (Traynelis and Wahl, 1997) glutamate receptors, the inhibitor I-1 (Mulkey et al., 1994; El-Armouche et al., 2006), and the transcription factors NFAT (Sheridan et al., 2002) and CREB (Schwaninger et al., 1995; Bito et al., 1996). CaN also dephosphorylate the RII regulatory subunit of PKA (Blumenthal et al. 1986), and inhibits an isoform of adenylyl cyclase (Paterson et al., 1995). Regarding memory processes and transcription factors, Baumgartel et al. (2008) found a correlation between CaN protein decrease and ZIF268 protein increase in cued fear conditioning in amygdala. In fact, Zif268 overexpression together with CaN transgenic inhibition strengthens memory, and makes memory more resistant to extinction. In turn, Lin et al. (2003c) showed a decrement in pCREB in amygdala after fear extinction in rats. This CREB inhibition was dependent on CaN activity. However, to our knowledge, there are no studies regarding which CaN signaling mechanisms related to long-term memory regulation are involved in the hippocampus. Our data support that NF-κB pathway is regulated by CaN during memory formation in the hippocampus. Moreover, this study is the first to postulate that CaN has a role as a memory regulator in reconsolidation, as its post-retrieval inhibition enhances memory. In the light of our results, and taking into account both the Baumgartel et al. (2008) report and the studies which suggest than NF-κB controls ZIF268 transcription (Carayol et al., 2006; Lubin and Sweatt, 2007), NF-κB could be the mediator between CaN and Zif268 expression.

The relationship between CaN and NF-κB signaling pathway has been previously investigated in other physiological systems. Some data support a positive regulation by CaN on NF-κB (Frantz et al., 1994; Biswas et al., 2003; Kim et al., 2004), whereas others support that CaN inhibition enhances NF-κB activity (Alexanian and Bamburg, 1999; Pons and Torres-Aleman, 2000; Fernandez et al., 2007). The direction of the relationship seems to depend on the cell type and the nature of the stimulation (Fernandez et al., 2007). It was reported that some CaN substrates like the transcription factors of the NFAT family have two consensus sequences for CaN recognition (P-x-I-x-IT and L-x-V-P; Liu et al., 2009). However, the direct interaction between CaN and NF-κB has not yet been described. Although there are studies suggesting that CaN dephosphorylates IκB and thus inactivates the NF-κB complex (Pons and Torres-Aleman, 2000; Fernandez et al., 2007), we found that none of the mice proteins immediately upstream to NF-κB (IκBα, IκBβ, IKKα, IKKβ, and IKKγ) or even the components of the NF-κB dimer (p65, p50, and cRel) has the aforementioned consensus sequences for CaN interaction. Nevertheless, it has also been shown that the consensus sequence per se is not determinant, but also the chemical features and the tertiary structure near the phosphorylated residues (Donella-Deana et al., 1994). Ongoing experiments are aimed at testing if CaN interact with proteins belonging to NF-κB signaling pathway, or NF-κB subunits as well.

In summary, our results support the hypothesis that negative constrains like CaN need to be removed to activate neural plasticity mechanisms, such as the NF-κB pathway of gene expression regulation (Lopez-Salon et al., 2001; Merlo and Romano, 2007). This applies not only for memory formation, but also for memory reprocessing after retrieval. Labilization-reconsolidation has previously been associated with memory reinforcement in different species and tasks (Frenkel et al., 2005; Inda et al., 2011; Forcato et al., 2011, 2013), though both processes have been shown to occur when the reactivating stimuli fulfill certain boundary conditions. We propose that the detection of a mismatch (Pedreira et al., 2004) and the triggering of labilization-reconsolidation, involve an active inhibition of negative constraints like CaN that will, in turn, allow memory processes like transcription factor activation to take place.

The elucidation of the identity and dynamics of mechanisms involved in memory formation and reprocessing after retrieval is important not only for its theoretical value but also for the development of therapeutic strategies for pathologies related to memory dysfunctions (Lee et al., 2006; Fiorenza et al., 2011). In particular, the study of the cellular and molecular mechanisms involved in associative fear will be enlightening to contribute to the development of treatments for human phobias and post-traumatic stress disorder.

Acknowledgments

The authors thank M. E. Pedreira and L. Orelli for helpful comments on the manuscript, and J. E. Kamienkowski for helpful advice on statistics.

REFERENCES

Ahn HJ, Hernandez CM, Levenson JM, Lubin FD, Liou HC, Sweatt JD. 2008. c-Rel, an NF-kappaB family transcription factor, is required for hippocampal long-term synaptic plasticity and memory formation. Learn Mem 15:539–549.

Alberini CM. 2005. Mechanisms of memory stabilization: are consolidation and reconsolidation similar or distinct processes? Trends Neurosci 28:51–56.

Alberini CM. 2009. Transcription factors in long-term memory and synaptic plasticity. Physiol Rev 89:121–145.

Alexanian AR, Bamburg JR. 1999. Neuronal survival activity of s100betabeta is enhanced by calcineurin inhibitors and requires activation of NF-kappaB. FASEB J Off Publ Fed Am Soc Exp Biol 13:1611–1620.

Anokhin KV, Tiunova AA, Rose SPR. 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.

Baumgartel K, Genoux D, Welzl H, Tweedie-Cullen RY, Koshibu K, Livingstone-Zatchej M, Mamie C, Mansuy IM. 2008. Control of the establishment of aversive memory by calcineurin and Zif268. Nat Neurosci 11:572–578.

Beattie EC, Carroll RC, Yu X, Morishita W, Yasuda H, von Zastrow M, Malenka RC. 2000. Regulation of AMPA receptor endocytosis by a signaling mechanism shared with LTD. Nat Neurosci 3:1291–1300.

Biswas G, Anandatheerthavarada HK, Zaidi M, Avadhani NG. 2003. Mitochondria to nucleus stress signaling: a distinctive mechanism of NFkappaB/Rel activation through calcineurin-mediated inactivation of IkappaBbeta. J Cell Biol 161:507–519.

Bito H, Deisseroth K, Tsien RW. 1996. CREB phosphorylation and dephosphorylation: A Ca(2+)- and stimulus duration-dependent switch for hippocampal gene expression. Cell 87:1203–1214.

Blanchard RJ, Blanchard DC. 1969. Crouching as an index of fear. J Comp Physiol Psychol 67:370–375.

Blumenthal DK, Takio K, Hansen RS, Krebs EG. 1986. Dephosphorylation of cAMP-dependent protein kinase regulatory subunit (type II) by calmodulin-dependent protein phosphatase. Determinants of substrate specificity. J Biol Chem 261:8140–8145.

Boccia M, Freudenthal R, Blake M, de la Fuente V, Acosta G, Baratti C, Romano A. 2007. Activation of hippocampal nuclear factor-kappa B by retrieval is required for memory reconsolidation. J Neurosci 27:13436–13445.

Carayol N, Chen J, Yang F, Jin T, Jin L, States D, Wang CY. 2006. A dominant function of IKK/NF-kappaB signaling in global lipopolysaccharide-induced gene expression. J Biol Chem 281: 31142–31151.

de la Fuente V, Freudenthal R, Romano A. 2011. Reconsolidation or extinction: transcription factor switch in the determination of memory course after retrieval. J Neurosci 31: 5562–5573.

Donella-Deana A, Krinks MH, Ruzzene M, Klee C, Pinna LA. 1994. Dephosphorylation of phosphopeptides by calcineurin (protein phosphatase 2B). Eur J Biochem FEBS 219:109–117.

Dong J, Jimi E, Zhong H, Hayden MS, Ghosh S. 2008. Repression of gene expression by unphosphorylated NF-kappaB p65 through epigenetic mechanisms. Genes Dev 22:1159–1173.

Ehrlich I, Humeau Y, Grenier F, Ciocchi S, Herry C, Lüthi A. 2009. Amygdala inhibitory circuits and the control of fear memory. Neuron 62:757–771.

El-Armouche A, Bednorz A, Pamminger T, Ditz D, Didié M, Dobrev D, Eschenhagen T. 2006. Role of calcineurin and protein phosphatase-2A in the regulation of phosphatase inhibitor-1 in cardiac myocytes. Biochem Biophys Res Commun 346:700–706.

Federman N, de la Fuente V, Zalcman G, Corbi N, Onori A, Passananti C, Romano A. 2013. Nuclear factor κB-dependent histone acetylation is specifically involved in persistent forms of memory. J Neurosci Off J Soc Neurosci 33:7603–7614.

Fernandez AM, Fernandez S, Carrero P, Garcia-Garcia M, Torres-Aleman I. 2007. Calcineurin in reactive astrocytes plays a key role in the interplay between proinflammatory and anti-inflammatory signals. J Neurosci 27:8745–8756.

Fiorenza NG, Sartor D, Myskiw JC, Izquierdo I. 2011. Treatment of fear memories: Interactions between extinction and reconsolidation. An Acad Bras Ciênc 83:1363–1372.

Forcato C, Rodriguez ML, Pedreira ME. 2011. Repeated labilization-reconsolidation processes strengthen declarative memory in humans. PLoS One 6:e23305.

Forcato C, Fernandez RS, Pedreira ME. 2013. The role and dynamic of strengthening in the reconsolidation process in a human declarative memory: What decides the fate of recent and older memories? PloS One 8:e61688.

Franklin KBJ, Paxinos G. 2001. The mouse brain in stereotaxic coordinates. London: Academic.

Frantz B, Nordby EC, Bren G, Steffan N, Paya CV, Kincaid RL, Tocci MJ, O'Keefe SJ, O'Neill EA. 1994. Calcineurin acts in synergy with PMA to inactivate I kappa B/MAD3, an inhibitor of NF-kappa B. EMBO J 13:861–870.

Frenkel L, Maldonado H, Delorenzi A. 2005. Memory strengthening by a real-life episode during reconsolidation: An outcome of water deprivation via brain angiotensin II. Eur J Neurosci 22:1757–1766.

Freudenthal R, Romano A. 2000. Participation of Rel/NF-kappaB transcription factors in long-term memory in the crab Chasmagnathus. Brain Res 855: 274–281.

Freudenthal R, Boccia MM, Acosta GB, Blake MG, Merlo E, Baratti CM, Romano A. 2005. NF-kappaB transcription factor is required for inhibitory avoidance long-term memory in mice. Eur J Neurosci 21:2845–2852.

Genoux D, Haditsch U, Knobloch M, Michalon A, Storm D, Mansuy IM. 2002. Protein phosphatase 1 is a molecular constraint on learning and memory. Nature 418:970–975.

Gerdjikov TV, Beninger RJ. 2005. Differential effects of calcineurin inhibition and protein kinase A activation on nucleus accumbens amphetamine-produced conditioned place preference in rats. Eur J Neurosci 22:697–705.

Glowinski J, Iversen L. 1966. Regional studies of catecholamines in the rat brain. 3. Subcellullar distribution of endogenous and exogenous catecholamines in various brain regions. Biochem Pharmacol 15:977–987.

Havekes R, Nijholt IM, Luiten PGM, Van der Zee EA. 2006. Differential involvement of hippocampal calcineurin during learning and reversal learning in a Y-maze task. Learn Mem Cold Spring Harb N 13:753–759.

Havekes R, Nijholt IM, Visser AK, Eisel UL, Van der Zee EA. 2008. Transgenic inhibition of neuronal calcineurin activity in the forebrain facilitates fear conditioning, but inhibits the extinction of contextual fear memories. Neurobiol Learn Mem 89:595–598.

Ikegami S, Inokuchi K. 2000. Antisense DNA against calcineurin facilitates memory in contextual fear conditioning by lowering the threshold for hippocampal long-term potentiation induction. Neuroscience 98:637–646.

Inda MC, Muravieva EV, Alberini CM. 2011. Memory retrieval and the passage of time: From reconsolidation and strengthening to extinction. J Neurosci 31:1635–1643.

Kaltschmidt B, Widera D, Kaltschmidt C. 2005. Signaling via NF-kappaB in the nervous system. Biochim Biophys Acta 1745:287–299.

Kandel ER. 2001. The molecular biology of memory storage: a dialogue between genes and synapses. Science 294:1030–1038.

Kim Y, Moon JS, Lee KS, Park SY, Cheong J, Kang HS, Lee HY, Kim HD. 2004. Ca2+/calmodulin-dependent protein phosphatase calcineurin mediates the expression of iNOS through IKK and NF-kappaB activity in LPS-stimulated mouse peritoneal macrophages and RAW 264.7 cells. Biochem Biophys Res Commun 314:695–703.

Klee CB, Ren H, Wang X. 1998. Regulation of the calmodulin-stimulated protein phosphatase, calcineurin. J Biol Chem 273: 13367–13370.

Kuno T, Mukai H, Ito A, Chang CD, Kishima K, Saito N, Tanaka C. 1992. Distinct cellular expression of calcineurin A alpha and A beta in rat brain. J Neurochem 58:1643–1651.

Lee JL. 2009. Reconsolidation: maintaining memory relevance. Trends Neurosci 32:413–420.

Lee JL, Everitt BJ, Thomas KL. 2004. Independent cellular processes for hippocampal memory consolidation and reconsolidation. Science 304:839–843.

Lee JL, Milton AL, Everitt BJ. 2006. Reconsolidation and extinction of conditioned fear: Inhibition and potentiation. J Neurosci 26: 10051–10056.

Lee JLC, Hynds RE. 2013. Divergent cellular pathways of hippocampal memory consolidation and reconsolidation. Hippocampus 23: 233–244.

Letzkus JJ, Wolff SBE, Meyer EMM, Tovote P, Courtin J, Herry C, Lüthi A. 2011. A disinhibitory microcircuit for associative fear learning in the auditory cortex. Nature 480:331–335.

Levenson JM, Choi S, Lee SY, Cao YA, Ahn HJ, Worley KC, Pizzi M, Liou HC, Sweatt JD. 2004. A bioinformatics analysis of memory consolidation reveals involvement of the transcription factor c-rel. J Neurosci 24:3933–3943.

Lin CH, Lee CC, Gean PW. 2003a. Involvement of a calcineurin cascade in amygdala depotentiation and quenching of fear memory. Mol Pharmacol 63:44–52.

Lin CH, Yeh SH, Leu TH, Chang WC, Wang ST, Gean PW. 2003b. Identification of calcineurin as a key signal in the extinction of fear memory. J Neurosci 23:1574–1579.

Lin CH, Yeh SH, Lu HY, Gean PW. 2003c. The similarities and diversities of signal pathways leading to consolidation of conditioning and consolidation of extinction of fear memory. J Neurosci 23: 8310–8317.

Liu JO, Nacev BA, Xu J, Bhat S. 2009. It takes two binding sites for calcineurin and NFAT to tango. Mol Cell 33:676–678.

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

Lubin FD, Sweatt JD. 2007. The IkappaB kinase regulates chromatin structure during reconsolidation of conditioned fear memories. Neuron 55:942–957.

Malleret G, Haditsch U, Genoux D, Jones MW, Bliss TV, Vanhoose AM, Weitlauf C, Kandel ER, Winder DG, Mansuy IM. 2001. Inducible and reversible enhancement of learning, memory, and long-term potentiation by genetic inhibition of calcineurin. Cell 104:675–686.

Martin SJ, Grimwood PD, Morris RG. 2000. Synaptic plasticity and memory: an evaluation of the hypothesis. Annu Rev Neurosci 23: 649–711.

Meffert MK, Baltimore D. 2005. Physiological functions for brain NF-kappaB. Trends Neurosci 28:37–43.

Merlo E, Romano A. 2007. Long-term memory consolidation depends on proteasome activity in the crab Chasmagnathus. Neuroscience 147:46–52.

Merlo E, Freudenthal R, Romano A. 2002. The IkappaB kinase inhibitor sulfasalazine impairs long-term memory in the crab Chasmagnathus. Neuroscience 112:161–172.

Merlo E, Freudenthal R, Maldonado H, Romano A. 2005. Activation of the transcription factor NF-kappaB by retrieval is required for long-term memory reconsolidation. Learn Mem 12:23–29.

Mulkey RM, Endo S, Shenolikar S, Malenka RC. 1994. Involvement of a calcineurin/inhibitor-1 phosphatase cascade in hippocampal long-term depression. Nature 369:486–488.

Myers KM, Davis M. 2007. Mechanisms of fear extinction. Mol Psychiatry 12:120–150.

Nader K. 2003. Memory traces unbound. Trends Neurosci 26:65–72.

Nader K, Schafe GE, Le Doux JE. 2000. Fear memories require protein synthesis in the amygdala for reconsolidation after retrieval. Nature 406:722–726.

Paterson JM, Smith SM, Harmar AJ, Antoni FA. 1995. Control of a novel adenylyl cyclase by calcineurin. Biochem Biophys Res Commun 214:1000–1008.

Pedreira ME, Maldonado H. 2003. Protein synthesis subserves reconsolidation or extinction depending on reminder duration. Neuron 38:863–869.

Pedreira ME, Perez-Cuesta LM, Maldonado H. 2004. Mismatch between what is expected and what actually occurs triggers memory reconsolidation or extinction. Learn Mem 11:579–585.

Pons S, Torres-Aleman I. 2000. Insulin-like growth factor-I stimulates dephosphorylation of ikappa B through the serine phosphatase calcineurin (protein phosphatase 2B). J Biol Chem 275:38620–38625.

Price CJ, Kim P, Raymond LA. 1999. D1 dopamine receptor-induced cyclic AMP-dependent protein kinase phosphorylation and potentiation of striatal glutamate receptors. J Neurochem 73:2441–2446.

Raman IM, Tong G, Jahr CE. 1996. Beta-adrenergic regulation of synaptic NMDA receptors by cAMP-dependent protein kinase. Neuron 16:415–421.

Schwaninger M, Blume R, Krüger M, Lux G, Oetjen E, Knepel W. 1995. Involvement of the Ca(2+)-dependent phosphatase calcineurin in gene transcription that is stimulated by cAMP through cAMP response elements. J Biol Chem 270:8860–8866.

Sheridan CM, Heist EK, Beals CR, Crabtree GR, Gardner P. 2002. Protein kinase A negatively modulates the nuclear accumulation of NF-ATc1 by priming for subsequent phosphorylation by glycogen synthase kinase-3. J Biol Chem 277:48664–48676.

Si J, Yang J, Xue L, Yang C, Luo Y, Shi H, Lu L. 2012. Activation of NF-κB in basolateral amygdala is required for memory reconsolidation in auditory fear conditioning. PloS One 7:e43973.

Silva AJ, Josselyn SA. 2002. The molecules of forgetfulness. Nature 418:929–930.

Sweatt JD. 2003. Mechanisms of memory. San Diego, California: Academic Press. http://www.loc.gov/catdir/toc/els051/2003107470.html http://www.loc.gov/catdir/description/els051/2003107470.html.

Tong G, Shepherd D, Jahr CE. 1995. Synaptic desensitization of NMDA receptors by calcineurin. Science 267:1510–1512.

Traynelis SF, Wahl P. 1997. Control of rat GluR6 glutamate receptor open probability by protein kinase A and calcineurin. J Physiol 503 (Pt 3):513–531.

Von Hertzen LSJ, Giese KP. 2005. Memory reconsolidation engages only a subset of immediate-early genes induced during consolidation. J Neurosci Off J Soc Neurosci 25:1935–1942.

Wahl C, Liptay S, Adler G, Schmid RM. 1998. Sulfasalazine: a potent and specific inhibitor of nuclear factor kappa B. J Clin Invest 101: 1163–1174.

Wang JH, Kelly PT. 1996. The balance between postsynaptic Ca(2+)-dependent protein kinase and phosphatase activities controlling synaptic strength. Learn Mem 3:170–181.

Weber CK, Liptay S, Wirth T, Adler G, Schmid RM. 2000. Suppression of NF-kappaB activity by sulfasalazine is mediated by direct inhibition of IkappaB kinases alpha and beta. Gastroenterology 119:1209–1218.

Winder DG, Mansuy IM, Osman M, Moallem TM, Kandel ER. 1998. Genetic and pharmacological evidence for a novel, intermediate phase of long-term potentiation suppressed by calcineurin. Cell 92:25–37.

Yang J, Yu J, Jia X, Zhu W, Zhao L, Li S, Xu C, Yang C, Wu P, Lu L. 2011. Inhibition of nuclear factor-κB impairs reconsolidation of morphine reward memory in rats. Behav Brain Res 216:592–596.

Yeh SH, Lin CH, Lee CF, Gean PW. 2002. A requirement of nuclear factor-kappaB activation in fear-potentiated startle. J Biol Chem 277:46720–46729.

Zhong H, SuYang H, Erdjument-Bromage H, Tempst P, Ghosh S. 1997. The transcriptional activity of NF-kappaB is regulated by the IkappaB-associated PKAc subunit through a cyclic AMP-independent mechanism. Cell 89:413–424.

Zhong H, Voll RE, Ghosh S. 1998. Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. Mol Cell 1:661–671.

Zhong H, May MJ, Jimi E, Ghosh S. 2002. The phosphorylation status of nuclear NF-kappa B determines its association with CBP/p300 or HDAC-1. Mol Cell 9:625–636.