European Journal of Neuroscience, Vol. 21, pp. 2845–2852, 2005 

# NF-κB transcription factor is required for inhibitory avoidance long-term memory in mice

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*Keywords*: gel shift, hippocampus, κB decoy, sulfasalazine

## Abstract

Although it is generally accepted that memory consolidation requires regulation of gene expression, only a few transcription factors (TFs) have been clearly demonstrated to be specifically involved in this process. Increasing research data point to the participation of the Rel/nuclear factor-κB (NF-κB) family of TFs in memory and neural plasticity. Here we found that two independent inhibitors of NF-κB induced memory impairment in the one-trial step-through inhibitory avoidance paradigm in mice: post-training administration of the drug sulfasalazine and 2 h pretraining administration of a double-stranded DNA oligonucleotide containing the NF-κB consensus sequence (κB decoy). Conversely, one base mutation of the κB decoy (mut-κB decoy) injection did not affect long-term memory. Accordingly, the κB decoy inhibited NF-κB in hippocampus 2 h after injection but no inhibition was found with mut-κB decoy administration. A temporal course of hippocampal NF-κB activity after training was determined. Unexpectedly, an inhibition of NF-κB was found 15 min after training in shocked and unshocked groups when compared with the naïve group. Hippocampal NF-κB was activated 45 min after training in both shocked and unshocked groups, decreasing 1 h after training and returning to basal levels 2 and 4 h after training. On the basis of the latter results, we propose that activation of NF-κB in hippocampus is part of the molecular mechanism involved in the storage of contextual features that constitute the conditioned stimulus representation. The results presented here provide the first evidence to support NF-κB activity being regulated in hippocampus during consolidation, stressing the role of this TF as a conserved molecular mechanism for memory storage.

## Introduction

Increasing experimental data links nuclear factor-κB (NF-κB) with processes that require synaptic plasticity. Reports with different approaches support the participation of this transcription factor (TF) in long-term potentiation in mouse hippocampus (Meberg *et al*., 1996; Albensi & Mattson, 2000; Freudenthal *et al*., 2004). The first evidence indicating that NF-κB is activated during memory consolidation was found in the context-signal memory paradigm of the crab *Chasmagnathus*. In this model, a marked correlation between memory formation and NF-κB activation was found (Freudenthal *et al*., 1998; Freudenthal & Romano, 2000). Furthermore, the inhibition of this TF by a specific inhibitor κB (IκB) kinase (IKK) inhibitor, sulfasalazine, induced amnesia (Merlo *et al*., 2002). It has been proposed that this TF can play a dual role, acting as a signalling molecule after its activation at the synapse and as a transcription initiator upon reaching the nucleus (Kaltschmidt *et al*., 1993). Activated NF-κB was found in synaptic terminals after long-term memory (LTM) induction (Freudenthal & Romano, 2000) and evidence for its retrograde transport to the nucleus has been reported in primary neural cultures (Wellmann *et al*., 2001; Meffert *et al*., 2003), giving physiological support for the synapse-to-nucleus signalling role of this TF. Recent studies in rodents give further support for the role of NF-κB in memory. This TF is required in the rat amygdala for fear conditioning (Yeh *et al*., 2002; Yeh *et al*., 2004) and double-knockout mice for p65 (Rel A) and tumour necrosis factor receptor showed a deficit in spatial memory (Meffert *et al*., 2003). Thus, NF-κB-dependent gene expression seems to be a conserved mechanism required for memory storage.

Rel–NF-κB is a conserved family of TFs present in several cell types and involved in different processes such as the immune response, development and apoptosis. In response to specific stimuli, NF-κB translocates to the nucleus as a consequence of the proteolytic degradation of its inhibitor protein, IκB. The signal for degradation is IκB phosphorylation by a specific kinase, IKK (DiDonato *et al*., 1997; Mercurio *et al*., 1997). In the nervous system it is activated by signals involved in neural plasticity such as glutamate, depolarization, increase in intracellular $Ca^{2+}$ (reviewed in O'Neill & Kaltschmidt, 1997), neuropetides such as angiotensin II (Frenkel *et al*., 2002), neural cell adhesion molecule (N-CAM) and cytokines (tumour necrosis factor and interleukin-1) (Krushel *et al*., 1999; Choi *et al*., 2001). Several genes related to neural plasticity contain NF-κB

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*Received 1 December 2004, revised 25 February 2005, accepted 21 March 2005*

doi:10.1111/j.1460-9568.2005.04126.x

recognition sites, such as those whose protein products are inducible nitric oxide synthase, N-CAM, angiotensinogen, cytokines and opiod peptides.

Here we study the participation of NF-κB in memory using the inhibitory avoidance task, which is in part dependent on hippocampal function. For this purpose we analysed (i) the effect of NF-κB inhibition on memory using two independent strategies: the administration of the NF-κB pathway inhibitor sulfasalazine, which acts by direct inhibition of IKK, and the use of a double-stranded DNA oligonucleotide containing the NF-κB consensus sequence (κB decoy) for direct inhibition of the TF, and (ii) the temporal course of NF-κB activity in hippocampus during memory consolidation.

## Materials and methods

### *Animals*

CF-1 male mice (Fundacal, Buenos Aires, Argentina) were used (age 60–70 days; weight 25–30 g). They were individually caged and remained singly housed throughout the experimental procedures. Mice were kept in a lodging room maintained at 21–23 °C on a 12-h light–dark cycle (lights on at 06.00 h), with *ad libitum* access to dry food and tap water. Experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication no. 80–23/96), and local regulations. All efforts were made to reduce the number of animals used.

### *Apparatus and behavioural procedure*

Inhibitory avoidance behaviour was studied in a one-trial learning, step-through type situation (Boccia *et al*., 2004), which utilizes the natural preference of mice for a dark environment. The apparatus consists of a dark compartment (20 × 20 × 15 cm) with a stainless-steel grid floor and a small (5 × 5 cm) illuminated and elevated platform attached to its front centre. The mice were not habituated to the dark compartment before the learning trial. During training, each mouse was placed on the platform and received a footshock as it stepped into the dark compartment. The footshock-training conditions were 0.8 mA, 50 Hz, 1 s (Boccia & Baratti, 2000); these yield median retention scores of ≈150 s. For pharmacological experiments, a retention test was performed 48 h after training. Each mouse was placed on the platform and the step-through latency was recorded. The retention test was finished either when the mouse stepped into the dark compartment or failed to cross within 300 s. In the latter case the mouse was immediately removed from the platform and assigned a score of 300 s. In the testing session the footshock was omitted.

### *I.c.v. injections*

Mice were prepared (Boccia *et al*., 2004), 48 h before training, for the i.c.v. injection of saline or drug solution so that a minimum of time was necessary for injection, which was administered under light ether anaesthesia in a stereotaxic instrument. Preliminary operations were also performed under ether anaesthesia and consisted of deflecting the scalp and drilling a hole through the skull. The skull was covered with bone wax and the mouse returned to its home cage. Vehicle or drug solution was injected unilaterally at random into either the left or right lateral ventricle at a volume of 1 or 1.7 μL, depending of the drug injected. The injection coordinates were −0.34 mm with respect to bregma, 1.00 mm to the right or left of the centre and 2.10 mm in depth (Franklin & Paxinos, 1997). Injections were performed over 90 s through a 30-gauge blunt stainless steel needle attached to a 10-μL Hamilton syringe with PE-10 tubing and driven by hand. Accuracy of the i.c.v. injection was >90% as determined by Methylene Blue injections performed regularly.

### *Drugs*

2–4 hydroxy((4-((2-pyridinilamino)sulphonyl)phenyl)azo) benzoic acid (sulfasalazine; Sigma, USA) was freshly dissolved in saline solution with 10 mM HEPES, pH 7.6, plus 20% DMSO, final pH 7.6. 1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indole-3-acetic acid (indomethacin) was dissolved at a concentration of 1 mM or less with saline with 10 mM HEPES pH 7.6 plus 20% DMSO. κB Decoy (5′-GAG**GGGACTTTCC**CA-3′, consensus sequence in bold) and mut κB Decoy (5′-GAG**GCGACTTTCC**CA-3′, base changed underlined) were dissolved in saline at 36.6 μg/μL.

The drugs sulfasalazine and indomethacin were administered i.c.v. immediately post-training at a volume of 1 μL and κB decoy and one base mutation of the κB decoy (mut-κB decoy) were administered i.c.v. 2 h before training at a volume of 1.7 μL.

### *Experimental groups*

For the first and third pharmacological experiments, different groups of 10 mice each were trained (shocked groups; SH) and immediately after training received an i.c.v. injection of 1 μL of either vehicle or different doses of drug (five doses for sulfasalazine and two doses for indomethacine). In the second experiment, together with the two SH groups, other two groups of 10 mice were included in the design, trained without footshock (unshocked groups; USH) and receiving saline or drug injection. The four groups were tested for retention 48 h after training. In the third experiment six groups of 10 mice each were injected with vehicle or sulfasalazine (5 mM, i.c.v.) immediately, 3 or 24 h after training, and the retention test was performed 48 h afterwards. For the fourth pharmacological experiment, 1.7 μL of κB decoy DNA oligonucleotide, or mut-κB decoy containing one C instead of G base, were injected 2 h before training in SH and USH groups. For the experiments designed to estimate the NF-κB activity in hippocampus, SH and USH mice were trained and killed by cervical dislocation at different times after training, obtaining nuclear extracts from hippocampus. In each case, an untreated naïve (NV) group of mice housed in the same conditions as that of the experimental groups was included in order to estimate basal activity.

### *Nuclear extracts and determination of DNA-binding activity*

Mice were killed by cervical dislocation at different times after training as indicated in Results. Brains were rapidly removed and both hippocampi dissected according to the method of Glowinski & Iversen (1966). To obtain nuclear extracts, tissues were homogenized in 250 μL of buffer A (HEPES, 10 mM, pH 7.9; $MgCl_2$, 1.5 mM; KCl, 10 mM; DTT, 1 mM; pepstatin A, 1 μg/mL; leupeptin, 10 μg/mL; PMSF, 0.5 mM; and aprotinin, 10 μg/mL) with eight strokes in a Dounce homogenizer, type B pestle. The homogenate was centrifuged for 15 min at 1000 ***g***, the pellet was resuspended in 30 μL of buffer B (Hepes, 20 mM, pH 7.9; KCl, 1.2 M; $MgCl_2$, 1.5 mM; EDTA, 0.4 mM; DTT, 0.5 mM; glycerol, 50%; pepstatin A, 1 μg/mL; leupeptin, 10 μg/mL; PMSF, 0.5 mM; and aprotinin, 10 μg/mL) and incubated for 15 min in ice. A centrifugation for 15 min at 12 000 ***g*** was then performed. The supernatant was kept at −70 °C until used. All the extraction protocol was performed at 4 °C. κ-B-like

DNA binding activity in nuclear fractions was assessed using electrophoretic mobility shift assay (EMSA). An amount of 1.75 pmoles of double-stranded oligonucleotide DNA containing the NF-κB binding site (5′-AGTTGAG**GGGACTTTCC**CAGGC-3′, binding site in bold) (Promega) was labelled at 37 °C for 10 min in 10 μL containing Tris-HCl, 70 mM; $MgCl_2$, 10 mM; DTT, 5 mM; (γP32)ATP (Nen/Dupont), 15 μCi; and T4 polynucleotide kinase (Promega), 10 U. DNA-protein binding was carried out in 20 μL containing Hepes, 20 mM, pH 7.9; KCl, 120 mM; EDTA, 0.4 mM; DTT, 0.5 mM; glycerol, 25%; and 10 μg of protein extract. Samples were incubated for 30 min at 0 °C and 1 μg of labelled oligonucleotide DNA probe was added followed by incubation for a further 30 min at 0 °C. The reaction mixture was electrophoresed on a 6% nondenaturing polyacrilamide gel in 0.25 × TBE (in mM: Tris, 22.3; boric acid, 22.3; and EDTA 0.5) for 2 h at 150 V. The gel was vacuum-dried and exposed overnight to XAR-5 film (Kodak). Using this probe, in previous work three specific retarded bands were found (Freudenthal *et al*., 2004). The relative optical density (ROD) of the first band corresponding to the p65–p50 heterodimer was estimated using NIH Image J 1.29x software. All measures were made with exposures within the linear range of the film. Images were digitized by means of a transmissive scanner (Umax PowerLook III). Protein contents of the extracts were measured in triplicate by the Bradford method (Sigma, St. Louis, MO, USA) and checked for quality and quantity by comparing pattern intensities in sodium dodecyl sulphate–polyacrilamide gel electrophoresis (SDS-PAGE). Competition assay was performed adding to the binding reaction molar excess of cold κB consensus sequence and nonrelated consensus sequences (CRE and AP-1) DNA oligonucleotides and supershift assay was performed, adding to the reaction 1 μL of p65 nuclear localization signal (NLS; Chemicon MAB 3026) or 2 μL of p65 C20 (Santa Cruz Biotechnology).

### *Data analysis*

Behavioural data are expressed as median latencies and interquartiles ranges to step-through during the retention test, and were analysed with Kruskal–Wallis nonparametric analysis of variance. Differences between groups were estimated by individual Mann–Whitney *U*-tests (two tailed; Siegel, 1956), considering *P*-values $< 0.05$ as significant. For the experiments designed to estimate NF-κB activity in hippocampus, ROD values for each group were related to the mean ROD values of the NV group. Dunnett *a priori* planned comparisons were used to estimate differences between NV and the other groups. Due to the lack of variance homogeneity and normality, these data were transformed to log $(x+1)$ before the statistical analysis.

## Results

### *The NF-κB inhibitor sulfasalazine impairs LTM*

Sulfasalazine is an NF-κB inhibitor (Wahl *et al*., 1998) acting by direct inhibition of IKKα and IKKβ by interference with the ATP binding site (Weber *et al*., 2000). Previous work performed in the crab *Chasmagnathus* model of LTM showed that sulfasalazine induced amnesia. In this work, two doses of sulfasalazine, 1 and 5 mM, were tested. Only the 5 mM solution effectively impaired crab LTM when administered immediately pretraining or 5 h after training, but not when injected immediately or 24 h after training, in coincidence with the periods during consolidation in which NF-κB is in the course of activation (Merlo *et al*., 2002). Furthermore, considering a potential effect of sulfasalazine on cyclooxygenase (COX), the effect of indomethacin, a drug that inhibits the prostaglandin but not NF-κB pathway (Yin *et al*., 1998), was evaluated in memory consolidation. This drug did not show amnestic effect on LTM and thus the disruption of memory formation found with sulfasalazine was attributed to IKK inhibition (Merlo *et al*., 2002).

In order to test the purported amnesic effect of sulfasalazine in mice, in the first experiment animals were shocked after their entrance into the dark compartment of the experimental device and then injected with vehicle (VEH) or with a 1-, 3-, 5-, 7- or 10-mM concentration of sulfasalazine. Forty-eight hours later animals were tested, determining latencies to step-through. As shown in Fig. 1A, the 5-mM and 7-mM doses presented significantly lower latencies than the VEH group ($U = 11.50$, $P < 0.01$ and $U = 14.5$, $P < 0.05$), suggesting an amnestic effect of these doses. No differences were found in 1 and 3 mM sulfasalazine with respect to the VEH group, indicating that these doses are too low to induce amnesia. Similarly, a 10-mM dose did not cause significantly different latencies from the VEH control group. Taken together, these results suggest a 'U'-type dose–response curve in which only the intermediate doses are effective. However, although not significant, higher latencies were observed with the 10-mM dose than with 1 mM, which can be interpreted as a nonspecific effect that caused a reduction in general activity.

To further evaluate the amnestic effect of the dose that showed the more clear amnestic effect (5 mM), a complete experiment design was used in the second experiment, in which animals were either shocked (SH group) or remained unshocked (USH group) during the training session. Half of the animals of each group were injected after training with either 5 mM sulfasalazine (SSZ) or vehicle (VEH). Thus, four groups were formed: SH–SSZ, SH–VEH, USH–SSZ and USH–VEH. Figure 1B shows the results of the testing session. SH–SSZ group showed a significantly lower level of latencies than SH–VEH group ($U = 13.50$, $P < 0.01$), indicating an amnesic effect. USH–SSZ group showed very low latencies, similar to USH–VEH, indicating that sulfasalazine did not cause nonspecific effects on animal responses.

In order to rule out possible nonspecific effects, induced by the drug, that would not be evaluated in the unshocked controls due to a floor effect, we performed a preliminary time course study of the effect of sulfasalazine using delayed injections at 3 and 24 h post-training. The rationale for this experiment was that if the drug effect is specific to memory formation its action should be restricted to the consolidation period, whereas no time windows would be expected for a nonspecific effect. For this purpose, six groups of 10 animals each were shocked after their entrance into the dark compartment of the experimental device and then injected with vehicle (VEH) or with 5 mM sulfasalazine (SSZ) at 0, 3 and 24 h after training. As expected for a specific effect of the drug in memory consolidation, only the group injected with sulfasalazine immediately after training showed significantly lower latencies than the VEH group ($U = 4.5$ $P < 0.01$) (Fig. 1C). Furthermore, the latencies of the SSZ groups injected at 3 and 24 h were significantly higher than those of the SSZ group injected immediately after training ($U = 7.0$ $P < 0.01$ and $U = 14.5$ $P < 0.01$, respectively).

Results obtained in this section thus indicate that sulfasalazine impaired retention performance when administered immediately post-training, suggesting that LTM is impeded in a dose and time-dependent manner.

### *The cyclooxygenase inhibitor indomethacin did not induce amnesia*

The following experiment was aimed at evaluating whether the amnesic effect of sulfasalazine could be explained as a potential effect

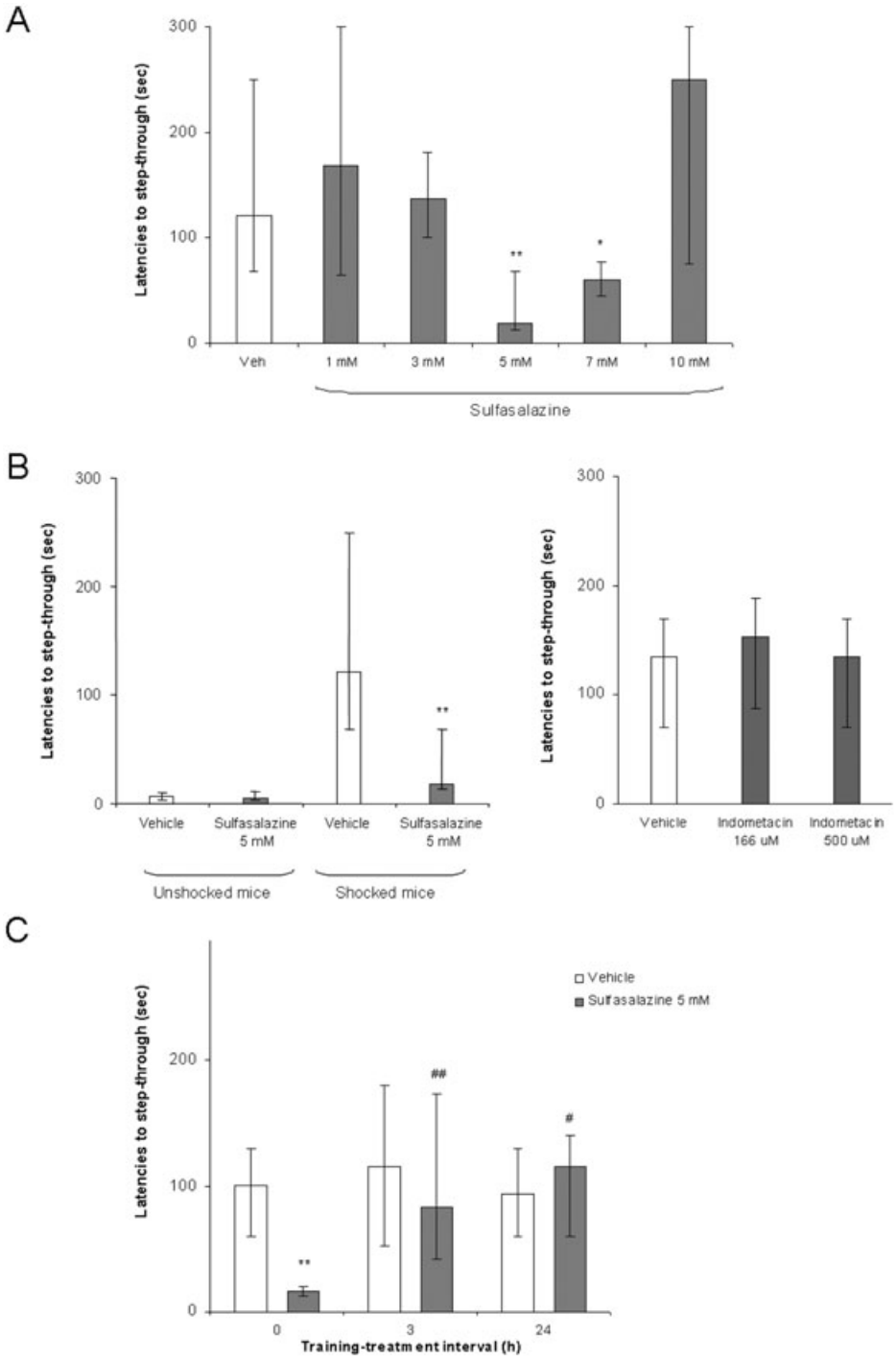


FIG. 1. Effect of sulfasalazine i.c.v. administration on long-term retention. (A) Test of performance of mice shocked during training session, having received either vehicle or 1-, 3-, 5-, 7- or 10-mM doses of sulfasalazine. (B, left histogram) Performance of mice shocked or unshocked during training injected with vehicle or 5 mM sulfasalazine immediately after training. (B, right histogram) Performance of mice shocked during training and injected either with vehicle or with 166 or 500 mM indomethacin immediately after training. (C) Performance of shocked mice during training injected with 5 mM sulfasalazine either immediately after training or 3 or 24 h after training. Values are median ± interquartile ranges of latencies to step-through; $n = 10$ per group; $^{*}P < 0.05$, $^{**}P < 0.01$ with respect to vehicle control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ with respect to immediate post-training administration.

on cyclooxygenase activity. For this purpose we used indomethacin, a cyclooxygenase and prostaglandin inhibitor that does not interfere with the NF-κB pathway (Yin *et al.*, 1998). Three groups were formed; one was injected post-training with vehicle (VEH), the second with 166 μM indomethacin (IND166) and the third group with 500 μM indomethacin (IND500). Higher doses were not used in order to avoid nonspecific effects on behaviour (Merlo *et al.*, 2002). All the animals received footshock during training. The results of the testing session are shown in Fig. 1C. All groups showed similarly high levels of latencies and no significant differences were found between groups,

indicating that indomethacin did not induce retention impairment in the examined doses.

### *The inhibition of NF-κB by κB decoy-impaired LTM*

An independent strategy to inhibit NF-κB activity was employed to further evaluate whether this pathway is required for memory consolidation. Instead of the upstream step inhibition employed in the first two experiments, we performed a direct inhibition of NF-κB using double-strand DNA containing the κB consensus sequence (κB decoy) to titrate off NF-κB from its normal binding sites. Initially, we tested whether NF-κB is actually inhibited by κB decoy in hippocampus, a brain area involved in this task. In accordance with previous studies (Freudenthal *et al*., 2004), EMSA performed with nuclear extracts obtained from hippocampus rendered two specific retarded bands, as characterized by competition assays (Fig. 2A). These bands were competed out by an excess of nonlabelled κB DNA oligonucleotide, while a 100-fold excess of unrelated sequences (CRE and AP-1) did not compete. The highest complex corresponds to the heterodimer p65–p50 (Krushel *et al*., 1999) as shown by supershift assay with a p65 antibody that recognizes the NLS, p65 NLS Ab. As expected, this antibody suppressed the p65–p50 complex instead of inducing a supershift band, as p65 NLS Ab recognizes the NLS sequence and then interferes with binding to DNA (Fig. 2A). This upper band was used for all densitometric analyses in this study. Sixty microgrammes of κB decoy was administered i.c.v. to eight naïve animals (Decoy group) and two control groups were established, one injected i.c.v. with saline solution (VEH group, $n = 8$) and the other with 60 μg of one base mutation of the κB sequence (mut-κB decoy; m-Decoy group; $n = 8$). Two hours after injection mice were killed and the hippocampus was dissected out. The densitometric analysis of EMSAs revealed that the Decoy group, but not the m-Decoy group, showed significantly lower levels of ROD values than VEH, indicating that the κB decoy inhibited NF-κB activity and that such

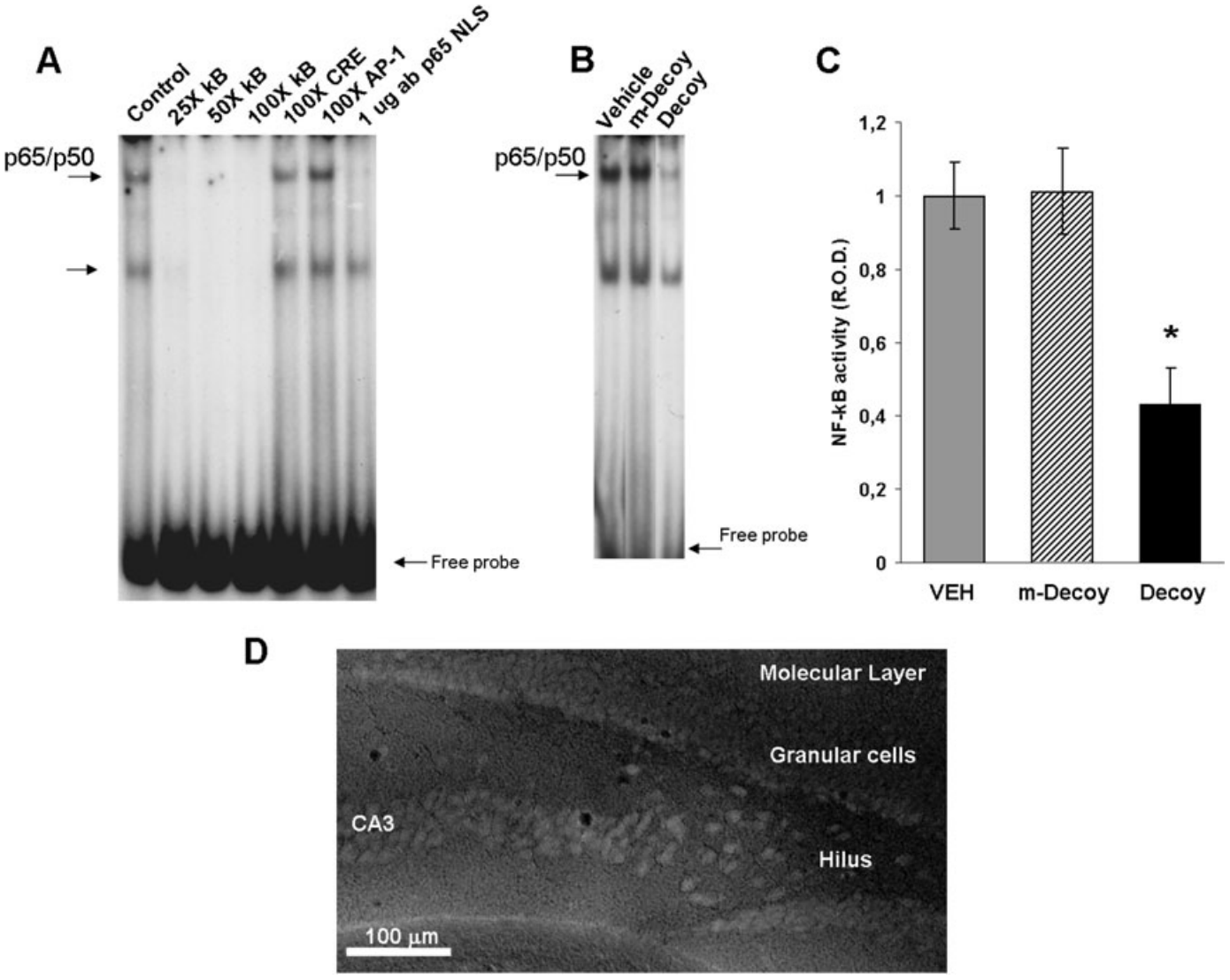


FIG. 2. Effect of κB decoy i.c.v. administration on hippocampal NF-κB activity. (A) Representative EMSA, competition assay with κB and with nonrelated consensus sequences (CRE and AP-1) and supershift assay with p65 NLS antibody. (B and C) κB decoy inhibition of NF-κB in hippocampus 2 h after administration. (B) Representative EMSA showing the p65–p50 complex from hippocampal nuclear extracts of animals injected with vehicle (VEH), mut-kB decoy (m-Decoy) or κB decoy (Decoy). (C) Mean ± SEM of ROD values obtained by densitometry; $n = 8$ per group, $^*P < 0.05$. (D) Micrograph showing κB decoy DNA-associated fluorescence in dentate girus and CA3 cells of the hippocampus 2 h after intraventricular administration of fluorescein-labelled κB decoy.

inhibition was sequence-specific (Fig. 2B and C). Sixty microgrammes of 5′ fluoresceine-labelled κB decoy was injected i.c.v. into a naïve mouse and 2 h later the animal was killed and brain sections were analysed, showing fluorescence in several areas of the brain. In Fig. 2D a micrograph shows κB decoy-associated fluorescence in CA3 and dentate gyrus hippocampal cells.

In the following experiment we evaluated whether κB decoy administration was able to induce amnesia. Animals were injected 2 h before training with κB decoy and divided into two groups. One of them received footshock during training (SH–Decoy) and the other remained unshocked (USH–Decoy). Mut-kB decoy and vehicle were also administered to shocked and unshocked groups (USH–m-Decoy, SH–m-Decoy, USH–VEH and SH–VEH), as a control for DNA and saline solution administration. Group performance during training was evaluated (Fig. 3A), showing no differences between groups. This fact suggests that DNA administration did not alter normal mouse performance during training. Figure 3B shows the results of the testing session performed 48 h after training. SH–Decoy group showed significantly lower latencies than the other shocked groups ($U = 4.00$, $P < 0.05$), indicating that the κB decoy induced amnesia and that mut-kB decoy administration did not affect mouse LTM and general activity at testing. The unshocked groups showed significant differences compared to SH–VEH and SH–m-Decoy, indicating retention. Furthermore, no differences were found between unshocked groups, demonstrating that DNA-injected animals performed normally at testing.

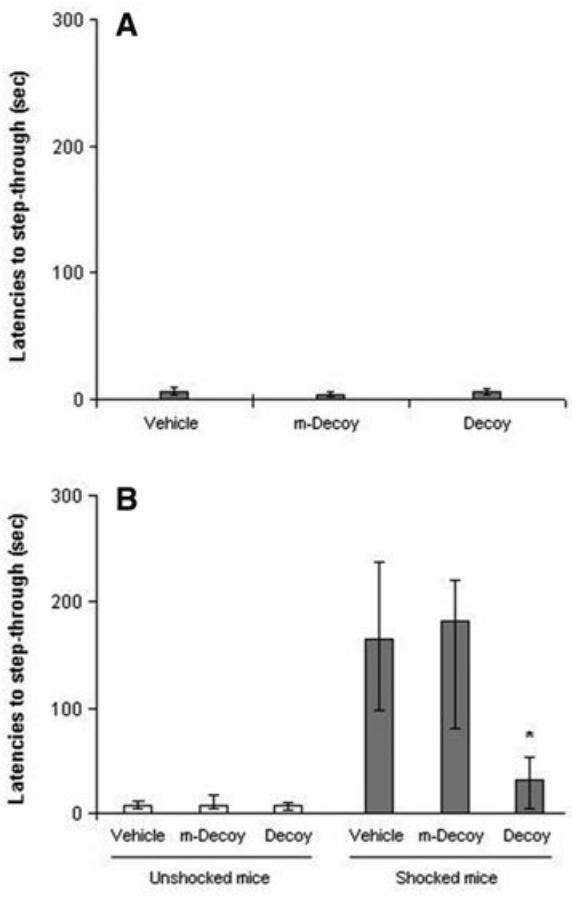


FIG. 3. Effect of κB decoy administration on long-term retention. Median ± interquartile ranges of latencies to step-through for animals that were injected with κB decoy (Decoy) or mut-kB decoy (m-Decoy) and shocked or unshocked during training. (A) Training performance. (B) Testing performance; *$P < 0.05$.

Therefore, the experiments of this section revealed that specific NF-κB inhibition by means of the administration of its DNA consensus sequence (κB decoy) impeded long-term retention, while one base mutation (mut-kB decoy) in this sequence yielded no amnestic effect.

### *Time course of hippocampal NF-κB activity during memory consolidation*

The hippocampal formation is a key structure implicated in the storage of some types of memories, such as that involved in contextual information processing. Inhibitory avoidance paradigms are known to be dependent on hippocampal function (e.g. Izquierdo *et al*., 1997). Results obtained in the previous section indicated that NF-κB activity is required for memory consolidation in this task. Therefore, in this section we evaluated whether NF-κB was actually activated in the hippocampus during consolidation. For this purpose we estimated NF-κB activity by EMSA at 15 and 45 min and at 1, 2 and 4 h after training, establishing three groups for each time point: animals treated with footshock during training (SH group), animals that did not received footshock during training (USH) and untreated naïve animals (NV). At the corresponding time after training, mice were killed and nuclear extracts from the hippocampus of each mouse were obtained. Figure 4A shows a representative EMSA for all time points and Fig. 4B shows the densitometric analysis of all the data. Surprisingly, we found a significantly lower level of ROD values 15 min after training in SH and USH groups than in the NV group ($P < 0.05$ for both comparisons in Dunnett's test), indicating an inhibition of NF-κB activity. Conversely, 45 min after training NF-κB ROD values were significantly higher in SH and USH ($P < 0.05$ for both comparisons in Dunnett's test) groups than in the NV group, indicating an increased TF activity in both groups at this time point. The activity in SH and USH decreased at 1 h and returned to NV levels at 2 and 4 h after training.

## Discussion

In the present study we report evidence supporting the participation of NF-κB in the consolidation of passive avoidance LTM in mice. Two independent strategies for the inhibition of NF-κB activity during memory consolidation were employed. Taken together, these two strategies led to the conclusion that NF-κB is required for memory formation, based on the fact that in both cases retention was impaired. In the first series of experiments, the administration of sulfasalazine, an IKK and thus NF-κB inhibitor was able to induce amnesia in a dose- and time-dependent manner. Post-training administration of drugs influence memory only if given soon after learning occurs (McGaugh, 1973), and this time-dependent nature of drug effects on memory is generally interpreted as evidence that they affect memory storage (McGaugh, 1989). Our results agree with this hypothesis and suggest an effect of sulfasalazine on memory storage-modulating mechanisms. Additionally, using the specific COX inhibitor indomethacin we did not find amnesia, suggesting that sulfasalazine exerts its amnestic effect specifically through inhibition of IKK. Similar results, at similar doses, were previously obtained in the context-signal memory paradigm in the crab *Chasmagnathus* (Merlo *et al*., 2002). In a second series of experiments we used κB decoy DNA to produce a direct inhibition of NF-κB. Two hours after brain administration of κB decoy, we found a high level of inhibition of NF-κB activity. Coincidentally, κB decoy was able to induce amnesia when

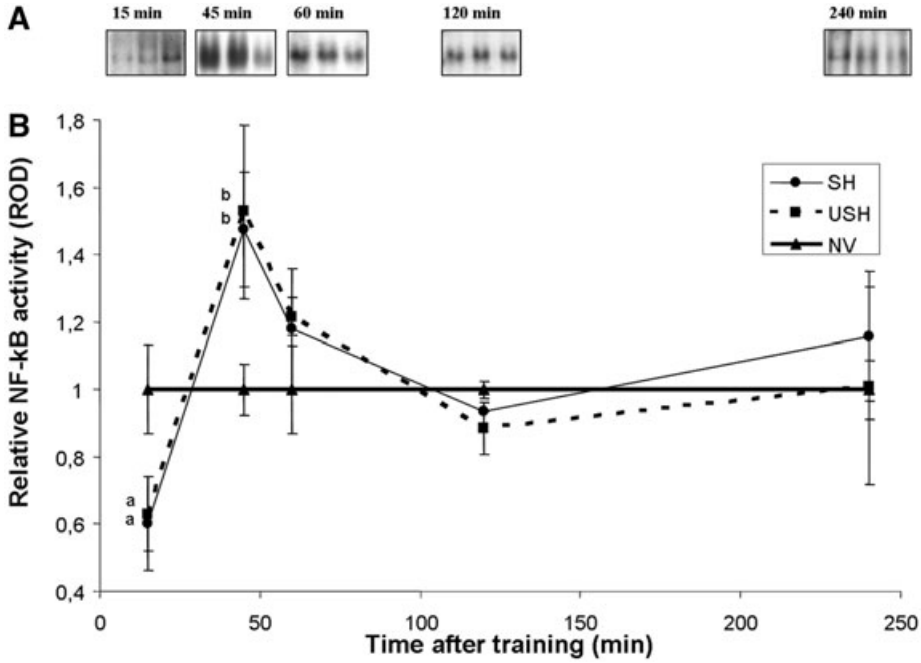


FIG. 4. Temporal course of NF-κB activity in hippocampus after training. (A) Representative EMSA for 15 and 45 min and 1, 2 and 4 h after training. (B) Mean ± SEM of ROD values for the p65–p50 complex at different time points after training. Number of animals per group: 15 min, $n = 8$; 45 min, $n = 12$; 1 h, $n = 6$; 2 h, $n = 2$ and 4 h, $n = 4$. a and b, $P < 0.05$, compared with the naive group for each time point.

administered 2 h before training. Considering that NF-κB is already activated in hippocampus 45 min after training (Fig. 3), κB decoy would be expected to block this TF activation during consolidation. The specificity of the κB decoy action on NF-κB was evaluated using a one-base mutation of its sequence. The lack of amnesic effect in this control rules out possible nonspecific effects of DNA administration.

The study of the temporal course of NF-κB activity in the hippocampal formation revealed that the NF-κB activation found 45 min after training occurred both in SH and USH groups. On the one hand, the similar level of activation observed in the two groups make hardly tenable the interpretation that attributes the observed activation to a nonspecific effect of footshock administration. On the other hand, these results suggest that this activation is not specific to the processing of the association between conditioned stimulus (CS) and unconditioned stimulus (US) but only to the codification of the CS. The fact that NF-κB activation in hippocampus is found not only in shocked animals but also in the unshocked group suggests that this process is involved in a nonassociative event and that the memory effects observed after NF-κB function inhibition is due, at least in part, to a disruption of this process in hippocampus. It has been proposed that the hippocampus is involved in the processing of contextual and spatial information implicated in contextual conditioning but it is not involved in footshock representation (Sanders *et al*., 2003). We propose that NF-κB activation is part of the molecular mechanisms required for the storage of contextual and spatial traits that constitute the CS codification, which will then be linked to US (footshock) information being processed in other brain areas such as amygdala and neocortex (Fanselow & Gale, 2003).

The temporal course study also revealed an initial inhibition found at 15 min after training in both SH and USH groups. Recent studies analysing the expression of many genes using microarray techniques call attention to the relevance of gene expression inhibition during consolidation, taking into account the important number of genes that are down-regulated during this process (Cavallaro *et al*., 2001; Cavallaro *et al*., 2002). Generally, the studies of the role of TFs involved in memory are focused on the positive regulation. The use of methods such as phospho-specific antibodies to estimate the level of TF activation does not allow the evaluation of inhibitions of the TF function, which may be an important mechanism in some neurons or neuronal circuits that participate in memory storage. In the present study, the use of EMSAs to estimate TF activity allowed us to detect this fast and transient inhibition of NF-κB during memory formation. Ongoing experiments are aimed at further characterizing the role of both the NF-κB inhibition and the NF-κB activation found in the present study in hippocampus during memory consolidation.

The role of NF-κB in neuronal plasticity and memory is becoming increasingly clear, representing a conserved mechanism involved in these processes from crustacea to mammals (Meberg *et al*., 1996; Freudenthal & Romano, 2000; Meffert *et al*., 2003; Freudenthal *et al*., 2004). It is proposed that Rel–NF-κB plays a pivotal role in the regulation of gene expression communicated from activity-dependent processes at the synapse. The results presented here provide the first evidence supporting the idea that NF-κB activity is regulated in hippocampus during consolidation, stressing the role of this TF as part of the molecular mechanism for memory storage.

## Acknowledgements

We are indebted to Dr Liliana Orelli for language correction. This work was supported by FONCYT PICT 06041 and Fundación Antorchas grants to A.R. and Grant B035 from the University of Buenos Aires to C.M.B.

## Abbreviations

COX, cyclooxygenase; EMSA, electrophoretic mobility shift assay; IκB, inhibitor κB; IKK, IκB kinase; IND, indomethacin-injected group of mice; κB decoy, a double-stranded DNA oligonucleotide containing the NF-κB consensus sequence; LTM, long-term memory; mut-κB decoy, one base mutation of the κB decoy; N-CAM, neural cell adhesion molecule; NF-κB, nuclear factor-κB; NV, untreated naïve group of mice; ROD, relative optical density; SH, shocked group; SSZ, sulfasalazine-injected group of mice; TF, transcription factor; USH, unshocked group; VEH, vehicle-injected group of mice.

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