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Nuclear factor kappa B-dependent Zif268 expression in hippocampus is required for recognition memory in mice

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ABSTRACT

Long-term memory formation requires gene expression after acquisition of new information. The first step in the regulation of gene expression is the participation of transcription factors (TFs) such as nuclear factor kappa B (NF-κB), which are present before the neuronal activity induced by training. It was proposed that the activation of these types of TFs allows a second step in gene regulation by induction of immediate-early genes (IEGs) whose protein products are, in turn, TFs. Between these IEGs, *zif268* has been found to play a critical role in long-term memory formation and reprocessing after retrieval. Here we found in mice hippocampus that, on one hand, NF-κB was activated 45 min after training in a novel object recognition (NOR) task and that inhibiting NF-κB immediately after training by intrahippocampal administration of NF-κB Decoy DNA impaired NOR memory consolidation. On the other hand, Zif268 protein expression was induced 45 min after NOR training and the administration of DNA antisense to its mRNA post-training impaired recognition memory. Finally, we found that the inhibition of NF-κB by NF-κB Decoy DNA reduced significantly the training-induced Zif268 increment, indicating that NF-κB is involved in the regulation of Zif268 expression. Thus, the present results support the involvement of NF-κB activity-dependent Zif268 expression in the hippocampus during recognition memory consolidation.

1. Introduction

After learning, memory stabilization requires synapse-to-nucleus communication to induce gene expression and production of new proteins. Synaptic signaling induces a fast activation of transcription factors (TFs) which are present before the neuronal activity induced by training. The resulting changes in gene expression during discrete time periods after learning are key processes in consolidation (Alberini, 2009). Thus, a first wave of gene expression regulation is achieved by these proteins. Nuclear factor kappa B (NF-κB) is a TF that shows relatively high basal expression. Such characteristic warrants the presence of the proteins before the neuronal activity that occurs during information acquisition. This fact implies that NF-κB can be promptly activated by synaptic activity and extracellular signals. Such activation takes place by protein–protein interaction or by covalent modifications, usually phosphorylations. In this way, the presence of this TF in regulatory regions of different genes and the induction of transcription can be rapidly achieved. NF-κB is bound to IκB, a regulatory protein that inhibits and retains NF-κB in the neuronal soma. After synaptic activation, the IκB kinase (IKK) complex phosphorylates IκB and then releases the TF for nuclear translocation (Małek, Borowicz, Jargiełło, & Czuczwar, 2007). The participation of NF-κB in memory consolidation and reconsolidation processes constitutes an evolutionary conserved feature, since it was shown that its activation is a necessary mechanism in crabs and rodents (reviewed in Romano, 2012, chap. 6; Snow, Stoesz, Kelly, & Albensi, 2014). All these evidence locate NF-κB as a key player among the molecular mechanisms involved in long term memory (LTM) formation.

Experimental data accumulated in recent years provide evidence that this first wave of gene expression regulation after training involves the induction of immediate-early genes (IEGs), which are characterized for its fast inducible expression. Some IEGs protein products act in turn as TFs. Between them, Zif268 (also known as Egr1, Krox-24, NGF1-A, TIS8, and Zenk), a member of the early growth response (Egr) family of proteins, is involved in neural plasticity and memory processes (Bozon, Davis, & Laroche, 2002; Soulé et al., 2008).

The induction of long-term potentiation (LTP) in dentate gyrus of the hippocampus is associated with rapid and robust transcrip-

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tion of *zif268* gene in the activated granule cells (Cole, Saffen, Baraban, & Worley, 1989; Wisden et al., 1990). The study of LTP in *zif268* mutant mice support a crucial role of this IEG in the maintenance of late phases of potentiation for more than 24 h (Jones et al., 2001). Besides this role in neural plasticity models, *zif268* expression was found to be induced in many learning and memory paradigms and in different brain areas (Guzowski, Setlow, Wagner, & McGaugh, 2001; Maddox, Monsey, & Schafe, 2011; Malkani & Rosen, 2000; Soulé et al., 2008). As well, a large variety of types of memories are affected by impaired Zif268 protein expression, suggesting that Zif268 function in neurons are part of the basic mechanisms implicated in memory consolidation and reconsolidation (reviewed in Veyrac, Besnard, Caboche, Davis, & Laroche, 2014).

Recently, the binding of NF-κB to the promoter region of *zif268* has been identified mediating UV-irradiating cell death (Thyss et al., 2005), suggesting that NF-κB is involved *zif268* expression. Furthermore, after fear conditioning reconsolidation, the phosphorylation and acetylation of histones was increased in *zif268* promoter and these epigenetic mechanisms were dependent on the NF-κB pathway activity (Lubin & Sweatt, 2007).

In the present work we studied in the hippocampus the involvement of NF-κB and Zif268 TFs in novel object recognition (NOR) task, and we evaluated if the regulation of Zif268 expression is dependent on NF-κB during memory consolidation.

2. Materials and methods

2.1. Animals

CF1 male mice, 6–8 weeks old, provided by the animal facility from the School of Veterinary Sciences of the University of Buenos Aires (FVET-UBA) in Argentina were used for all the experiments. Animals were housed in groups of four individuals with the exception of cannulated mice that were housed individually. Water and food was provided ad libitum and mice were kept under a 12 h light/dark cycle (lights on at 8:00 A.M.) at a temperature of 21–22 °C. Experiments were conducted during the light phase. Experiments were performed in accordance with local regulations and the National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals* (NIH publication 80-23/96). All procedures were performed with the approval of the Institutional Committee for the Use and Care of Laboratory Animals (CICUAL, protocol number approval #29) from the University of Buenos Aires. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Behavioral procedures for NOR task

Animals were handled for 2 days (3 min/day) followed by 3 days of habituation that involved the placement of each animal in the experimental box with no object presentation (5 min/day) (see Federman et al., 2013 for a full description of the apparatus used). The following day, the training session took place. Each animal of the trained group was placed in the experimental chamber for 15 min with two identical objects, while each mouse of the habituated group was placed in the chamber for 15 min without objects. The objects used were 100 ml transparent beakers or blue blocks (Rasti® toys), both of similar size. The objects were scrubbed with a tissue soaked in 96% alcohol (Sanicol) and then rinsed with bi-distilled water to ensure that no olfactory cues were present.

For behavioral experiments, memory retention was tested 24 h after training. Testing session was the same for trained and habituated groups and it involved introducing the animals to the experimental chamber and allowing them to explore two different objects (one beaker and one block) for 5 min. One of the objects was identical to those explored during the training session (familiar object) and the other was a different object (novel object). The objects were exposed in the same locations of the chamber as they were in the training session. The location of the novel object was exchanged between the left and the right for different animals to avoid place preference during the evaluation session. The training and testing sessions were filmed with a web camera as described before (Federman et al., 2013).

For molecular studies, animals were killed at different time points after training session and tissue was collected as described in Section 2.4.

2.3. NOR behavioral data analysis

Time spent exploring an object was established as previously described (Federman et al., 2013). Briefly, during training, total time of exploration and percentage of time that the animal spent exploring the left and right objects were determined. During the testing session, the total exploration time for each object was determined and the relative time of novel object exploration was calculated as the discrimination index (DI%) calculated as follows:

$$\mathrm{DI}\% = (t\ \text{novel} - t\ \text{familiar})/(t\ \text{novel} + t\ \text{familiar}) * 100\%.$$

The mean DI% value was calculated for the different groups of animals.

Animals showing low exploration times during training or testing session were excluded from the experiments (below 2 standard deviation of the mean time of exploration). Furthermore, for each experiment we verified that during the training session total time of exploration and the percentage of time spent exploring the objects during training did not differ significantly between groups. Similarly, total time of exploration in the testing session was compared in all of the behavioral experiments and it was verified that there were no differences between groups in this parameter.

2.4. Nuclear-enriched protein extraction

Mice were killed by cervical dislocation at different time points after training (see Results in Section 3). Brains were rapidly removed, and the hippocampus was dissected according to the method described by Glowinski and Iversen (1966). After dissection, the hippocampus was immediately placed in 250 µl of buffer A (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DDT, 0.5 ng/ml pepstatin A, 10 ng/ml leupeptin, 0.5 mM PMSF, and 50 ng/ml aprotinin). Each hippocampus was then homogenized in buffer A with 7 strokes of a tight dounce. The homogenate was centrifuged at 1000*g* for 15 min. The pellet was completely resuspended in 30 µl of buffer B (20 mM Hepes (pH 7.9), 1.2 M KCl, 1.5 mM $MgCl_2$, 0.4 mM EDTA, 0.5 mM DTT, 50% glycerol) and incubated on ice for 20 min. Samples were then centrifuged at 10,000*g* for another 15 min. The supernatant was transferred to a new tube and stored at −80 °C until used for western blot or electrophoresis mobility shift assay (EMSA) techniques. The entire extraction protocol was performed at 4 °C.

2.5. Determination of DNA-binding activity by EMSA

DNA binding activity of NF-κB in nuclear fractions was assessed using the EMSA-Blot technique (de la Fuente, Freudenthal, & Romano, 2011; Freudenthal et al., 2005). Briefly, 10 µg of nuclear-enriched protein extracts were incubated for 40 min on ice with an aliquot of 1.75 pmol of double-stranded DNA oligonucleotide containing the NF-κB binding site (5-AGTTGAG**GGGACTTTCC**CAGGC-3; binding site in bold) (Promega). The reaction mixture was then electrophoresed on a 6% non-denatur-

ing polyacrylamide gel in 0.25% TBE and transferred to a PVDF membrane as described by Salles et al. (Salles A. and Freudenthal R., personal communication). Protein levels in homogenate samples were determined using the BCA Protein Assay Kit (Pierce).

2.6. SDS–PAGE

For assessment of Zif268 protein levels, nuclear or cytosolic enriched protein extracts were separated by SDS–PAGE (20 μg, 10% resolving gel, 4% stacking gel, 90 min 110 V) and then transferred onto a PVDF membrane (1 h, 100 V). Protein concentration of the extracts was measured in triplicate by the Bradford method (Bradford, 1976).

2.7. Immunoblot assays

For the incubation protocols, SDS–PAGE and EMSA PVDF membranes were activated with methanol for 1 min, rinsed in water and then blocked for 1 h. Blocking buffer (BB) consisted on T-TBS (Tris-buffered saline, 0.1% Tween-20) and 4% non fat dry milk. Membranes were then incubated with primary antibody O.N. at 4 °C. After incubation, membranes were washed three times on T-TBS (5–10 min each time) and incubated for 1 h with secondary antibody dissolved in BB (anti-rabbit, 1:5000, sc-2030, Santa Cruz Biotechnology) at room temperature. Finally it was washed twice with T-TBS, once with TBS and protein detection was performed with Luminol chemiluminescence kit (Santa Cruz Biotechnology) as described by the manufacturer. Signals were digitalized by FUJIFILM-Intelligent Dark Box II apparatus with image reader LAS-1000 software. Relative optical density (R.O.D.) was estimated using NIH ImageJ 1.43u software. Primary antibodies used were anti-Zif268 antibody dissolved in BB (1:1000, sc-110, Santa Cruz Biotechnology), anti-NF-κB p65 (1:1000 T-TBS, sc-372, Santa Cruz Biotechnology) and anti-c-Fos (1:1000 T-TBS, sc-7202, Santa Cruz Biotechnology). For nuclear housekeeping measurements the primary antibody used was anti-Lamin B dissolved in BB antibody (1:500, sc56143, Santa Cruz Biotechnology) and as the secondary antibody, anti-mouse antibody dissolved in BB (1:5000, sc-2005, Santa Cruz Biotechnology). For cytosolic housekeeping measurements, the primary antibody used was anti-Actin dissolved in BB (1:5000, sc-1616-R, Santa Cruz Biotechnology) and anti-rabbit as the secondary antibody, dissolved in T-TBS (anti-rabbit, 1:5000, sc-2030, Santa Cruz Biotechnology).

2.8. Surgery and drug infusion

Mice were implanted under deep anesthesia (80 μl of ketamine 5% w/v and 10 μl of xylazine at 20 mg/ml) with 23-gauge guide cannulae in the dorsal region of the hippocampus as described before (de la Fuente et al., 2011). In all cases, infusions were bilateral, had a volume of 1 μl and were performed immediately after training session. After behavioral procedures histological examination of cannulae placements was performed. For this purpose, animals were decapitated, brains were placed in 4% paraformaldehyde for 1 d followed by 30% sucrose for an additional 24 h and after this treatment brains were sliced using a vibratome. Finally, cannulae placement was verified with a magnifying glass. Only data from animals with cannulae located in the dorsal hippocampus (DH) were included in the analysis.

2.9. Drugs

NF-κB Decoy (double-stranded DNA oligonucleotide 5′-GAG**GGGACTTTC**CCA-3′; consensus sequence in bold) and mDecoy (5′-GAG**GCGACTTTC**CCA-3′; base changed underlined) (Albensi & Mattson, 2000) were dissolved in STE solution. In previous reports, we have included in the experiments an STE group (vehicle group) showing no differences on behavior in comparison with the double stranded DNA group (mDecoy) (Freudenthal et al., 2005). Decoy or mDecoy were used at a concentration of 0.47 μg/μl and delivered 0.26 pmol per side (Boccia et al., 2007; Freudenthal et al., 2005).

Zif268 antisense oligonucleotides (ASO) and scrambled oligonucleotides (MSO) (ODN; Genbiotech S.R.L.) were designed as in previous studies (Katche, Goldin, Gonzalez, Bekinschtein, & Medina, 2012). The Zif268 ASO ODN encodes an antisense sequence for a part of Zif268 mRNA near the translation start site (GenBank accession number NM 007913.5, bases 362–379). The scrambled ODN served as a control and did not show significant homology to sequences in the GenBank database. Both ODNs contained phosphorothioate linkages on the three terminal bases of both the 5′ and 3′ ends and phosphodiester internal bonds, as this nucleotide design is reportedly more stable than unmodified phosphodiester ODNs in vivo and less toxic than fully phosphorothioate ODNs (Guzowski, 2002). The following sequences were used, ASO: 5′-GGT AGT TGT CCA TGG TGG-3′ and MSO: 5′-GTG TTC GGT AGG GTG TCA-3′. Both ODNs were resuspended in sterile saline to a concentration of 1 nmol/μl. For the behavioral Zif268 knockdown experiment, a control group infused with ODNs vehicle, sterile saline, was used (1 μl per side) to rule out the possibility of an unspecific effect of oligonucleotide injection on the animal behavior.

2.10. Data analysis

In the behavioral experiments statistical analysis was performed by unpaired two-tailed *Student's t test* or one-way ANOVA followed by Unequal N HSD post hoc comparison test, comparing mean discrimination index percentages (DI%) between the different groups. Immunoblot and EMSA data were analyzed by unpaired two-tailed *Student's t test*. All data are presented as mean ± SEM.

3. Results

3.1. Hippocampal NF-κB is involved in novel object recognition memory

As a first step in our experiments we tested if a training session of 15 min of object exploration is sufficient to render good object discrimination in CF1 animals in a testing session 24 h after training. Previously, this training condition induced a strong memory in another mouse strain, C57BL6 (Federman et al., 2013). A group of animals (TR) was trained with two similar objects and another group (HAB) was exposed to the chamber without objects. Twenty-four h after training mice received a testing session consisting in the exposure to two different objects, one similar to that utilized during training and another one different. The discrimination index was estimated, observing a good retention of about 40% (Fig. 1A) ($t = 4.556$, $p = 0.0002$), similar to that observed in the other strain.

Next, we studied the participation of NF-κB in NOR memory consolidation in hippocampus. Previous results from our group showed that NF-κB activation peaked at 45 min during consolidation in two memory tasks in mice, inhibitory avoidance (Freudenthal et al., 2005) and contextual fear conditioning (de la Fuente, Federman, Fustiñana, Zalcman, & Romano, 2014), therefore, we decided to quantify NF-κB activation 45 min after training. Two groups of animals were used as in the previous experiment, TR and HAB, and 45 min after training animals were killed, the hippocampus was dissected and nuclear extracts were obtained. To estimate NF-κB activity, electromobility shift-assays (EMSAs) were performed with these nuclear protein extracts incubated with

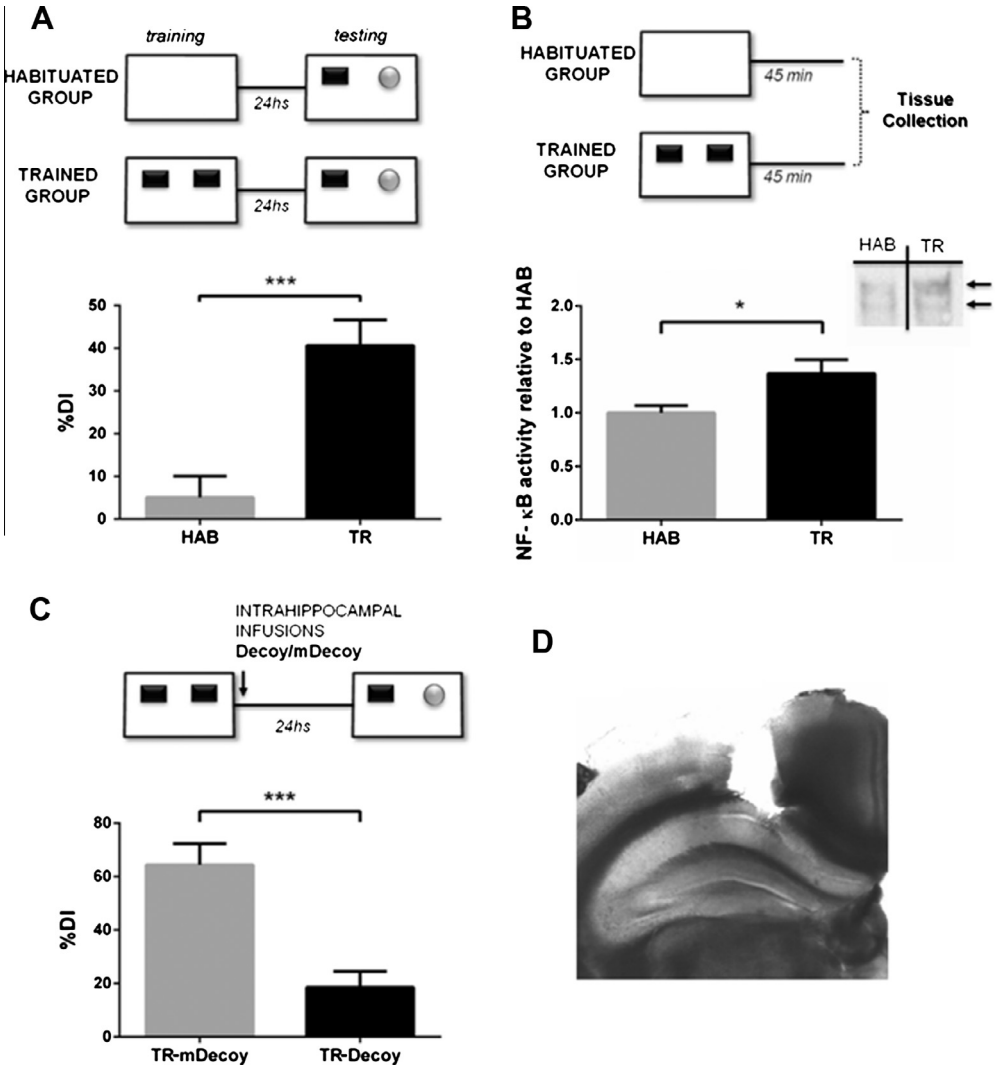

Fig. 1. NF-κB activation in the hippocampus is necessary for NOR long-term memory formation. (A) The diagram shows a representation of NOR training and testing session. Trained group showed preferential exploration for the novel object compared to the habituated group (n_{HAB} = 11, n_{TR} = 11). Student's *t* test, $^{***}p < 0.001$. (B) Tissue collected 45 min after the training session was subjected to an EMSA-Blot to determine NF-κB activity in the nucleus of hippocampal neurons. Arrows stands for p65 specific containing bands (n_{HAB} = 9, n_{TR} = 10). Student's *t* test, $^{*}p < 0.05$. (C) Intrahippocampal injections of an oligodeoxynucleotide that inhibits NF-κB activity (Decoy) results in NOR memory impairment as compared with a group injected with a mutant oligodeoxynucleotide (mDecoy) that has no effect on NF-κB activity ($n_{TR\text{-}mDecoy}$ = 9, $n_{TR\text{-}Decoy}$ = 9). Student's *t* test, $^{***}p < 0.001$. Data is presented as mean ± SEM in all cases. (D) Image showing an example of cannula position in the DH.

NF-κB DNA oligonucleotide probe. The NF-κB component p65 in the DNA–protein complexes was detected using a specific antibody and the obtained bands were quantified. NF-κB nuclear activity was significantly higher in TR group compared to the control HAB group (Fig. 1B) (t = 2.419, p = 0.027), indicating that NF-κB is activated 45 min after NOR training in hippocampus.

To evaluate if the NF-κB activation observed in the previous experiment is required for NOR memory consolidation we performed the following experiment. Two groups of cannulated mice were trained and immediately after training were bilaterally injected into the dorsal hippocampus (DH) with the specific inhibitor of NF-κB, NF-κB Decoy DNA oligonucleotide (TR-Decoy) which contains the κB consensus sequence to titrate off NF-κB from its normal binding sites, or a mutated DNA oligonucleotide mDecoy (TR-mDecoy) that has one base mutation which impedes the transcription factor recognition of the consensus sequence and therefore does not inhibit NF-κB (Boccia et al., 2007; de la Fuente et al., 2011; Freudenthal et al., 2005). In previous reports from our lab we found that NF-κB Decoy DNA oligonucleotide enters the cells of the DH and induces NF-κB inhibition 15 min and 2 h after intrahippocampal injection (Boccia et al., 2007; Freudenthal et al., 2005). Twenty-four h after training animals were tested and TR-Decoy group showed a lower discrimination index (DI%) (Fig. 1C) (t = 4.632, p = 0.0003). The result of this experiment indicates that inhibiting NF-κB activation in the DH impairs NOR memory.

3.2. Zif268 expression after NOR training

In the following experiment we evaluated whether Zif268 is expressed in the hippocampus after NOR training. For this end, a time course of Zif268 protein levels was performed, estimating the amount of protein in TR group relative to the HAB group at 45 min, 90 min and 3 h after training. At these time points after training, animals were killed, the hippocampus was dissected and nuclear protein extracts were obtained. Western blots were performed with a specific Zif268 antibody and the levels of the protein relative to the levels of Lamin B housekeeping were estimated by densitometry of the specific band obtained. A statistically significant increment in the levels of Zif268 protein was found 45 min after training, returning to the level of HAB group after 90 min (Fig. 2) (45 min: t = 2.529, p = 0.0205; 90 min: t = 0.3832,

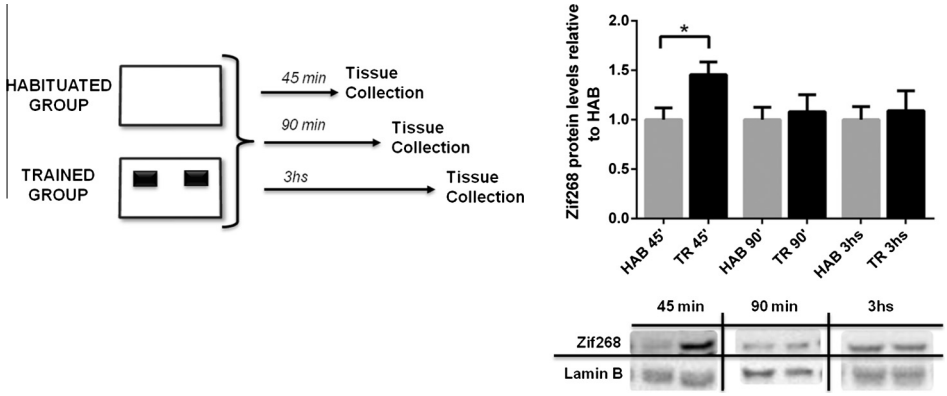

Fig. 2. Zif268 protein levels have a transient increase 45 min after NOR training. Hippocampal tissue was collected at different time points after NOR training session, and Zif268 protein levels in the nucleus of hippocampal neurons were estimated by western blot ($n_{HAB45} = 9$, $n_{TR45} = 12$; $n_{HAB90} = 9$, $n_{TR90} = 8$; $n_{HAB3hs} = 9$, $n_{TR3hs} = 7$). Data is presented as mean ± SEM of Zif268/Lamin *b* ratio. Student's *t* test, $^{*}p < 0.05$.

$p = 0.7069$; 3 h: $t = 0.388$, $p = 0.7047$). These results indicate that Zif268 expression has a transient increase after training and that such increment coincides with the period in which NF-κB is activated (see Fig. 1B).

3.3. Hippocampal Zif268 is required for NOR memory consolidation

To evaluate if Zif268 expression observed in the previous experiment is required for NOR memory consolidation we performed the following experiment. Three groups of cannulated mice were trained and immediately after training were bilaterally injected into its DH with a single stranded DNA oligonucleotide antisense to part of Zif268 mRNA (TR-ASO group), a scrambled oligonucleotide (TR-MSO group), or saline vehicle (TR-Saline group), respectively. Twenty-four h after training the three groups were tested. Fig. 3 A shows the results of testing session. Normal DI% for TR-Saline and TR-MSO groups, but a significantly lower DI% for TR-ASO group was found ($F = 6.103$, $p = 0.006$). These results support memory impairment induced by ASO administration.

To corroborate that ASO ODN infused in the hippocampus induces a reduction in the levels of Zif268 compared to the infusion of MSO ODN, two groups of animals were trained and injected with ASO or MSO. Forty-five min after training animals were killed, the hippocampus was dissected and nuclear extracts were obtained. The determination of Zif268 protein levels by western blot revealed a significant reduction induced by ASO (Fig. 3B) ($t = 2.402$, $p = 0.0227$). These results support that ASO infusion induced a knock down in Zif268 and that this effect induced memory impairment, indicating that Zif268 hippocampal expression is necessary for NOR memory consolidation.

3.4. Zif268 expression is dependent on NF-κB

NF-κB consensus sequences were found in the promoter regions of *zif268* (Ahn et al., 2008), and promoter NF-κB binding was identified in UVB-irradiated HaCaT cells (Thyss et al., 2005). To obtain evidence that NF-κB is necessary for the protein expression of Zif268 during NOR memory consolidation in the hippocampus, we performed the following experiment. Two groups of mice were trained and immediately after they were injected bilaterally in the DH with Decoy (TR-Decoy group) or mDecoy (TR-mDecoy group). Forty-five min after training animals were killed, the hippocampus was dissected and nuclear extracts were obtained (Fig. 4A). Western blots were performed with Zif268 antibody and protein levels relative to Lamin B housekeeping protein were estimated by densitometry of the specific band obtained. A statistically significant reduction in the protein level of TR-Decoy regarding TR-mDecoy was found (Fig. 4B) ($t = 2.258$, $p = 0.0397$), indicating that NF-κB

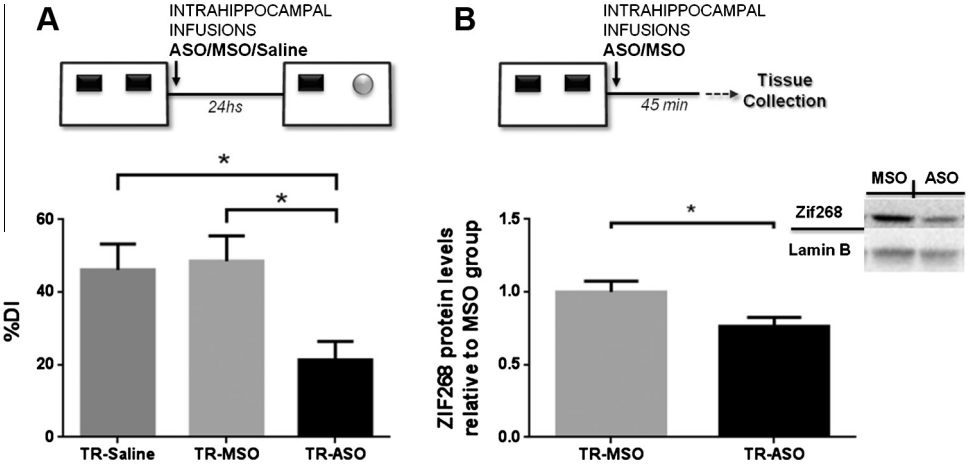

Fig. 3. Zif268 protein expression in the hippocampus after training is necessary for NOR long-term memory consolidation. (A) Zif268 expression was inhibited immediately after training by intrahippocampal infusion of an oligonucleotide antisense to Zif268 mRNA (TR-ASO group) and NOR performance was assessed 24 h later. Control groups were injected with a missense oligonucleotide (TR-MSO) or the drug vehicle (TR-Saline) respectively ($n_{TR\text{-}Saline} = 12$, $n_{TR\text{-}MSO} = 12$, $n_{TR\text{-}ASO} = 9$). One-way ANOVA ($F = 6.103$), followed by Unequal N HSD post-hoc test, $^{*}p < 0.05$. B. Zif268 protein expression 45 min after NOR training is significantly reduced in animals injected with ASO as compared with the MSO group ($n_{TR\text{-}MSO} = 18$, $n_{TR\text{-}ASO} = 14$). Student's *t* test, $^{*}p < 0.05$. Data is expressed as mean ± SEM in all cases.

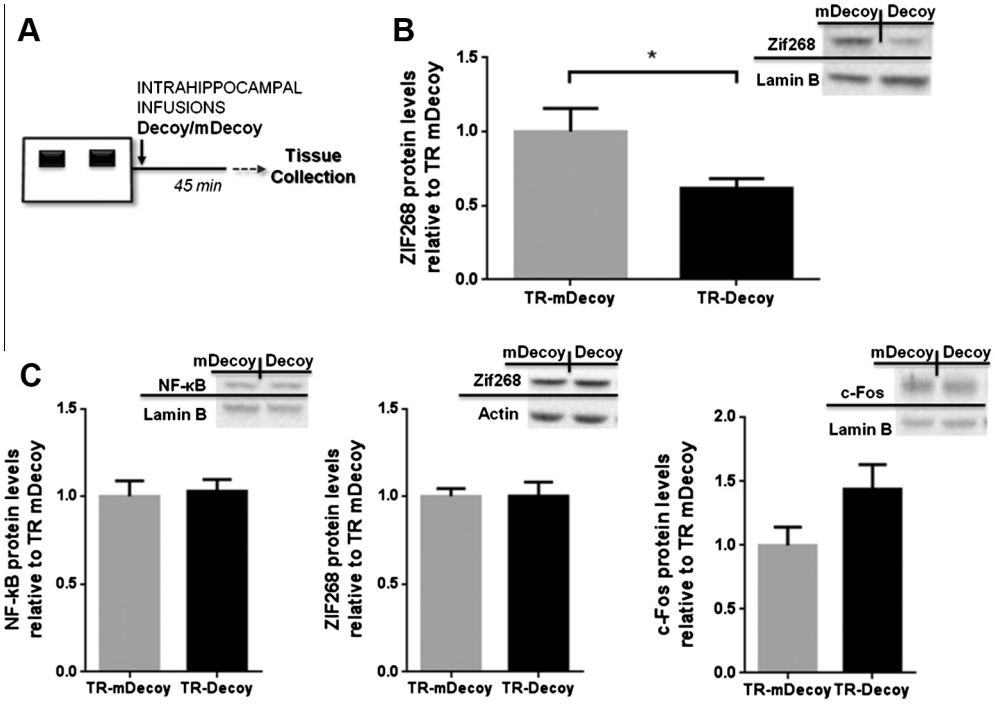

Fig. 4. NF-κB inhibition after NOR training reduces Zif268 protein expression in the hippocampus. (A) Diagram outlining the experimental design. (B) Zif268 protein levels in hippocampal nuclear extracts measured 45 min after training and drug infusions ($n_{TR\text{-}mDecoy}$ = 12, $n_{TR\text{-}Decoy}$ = 13). Data is expressed as mean ± SEM of Zif268/Lamin *b* ratio. Student's *t* test, $^{*}p < 0.05$. (C) Decoy injection does not affect NF-κB nor Zif268 nuclear translocation (left and middle graph, respectively) and it does not affect c-Fos expression (right graph). (NF-κB nuclear protein levels: $n_{TR\text{-}mDecoy}$ = 12, $n_{TR\text{-}Decoy}$ = 13, Zif268 cytoplasmic protein levels: $n_{TR\text{-}mDecoy}$ = 14, $n_{TR\text{-}Decoy}$ = 11, c-Fos nuclear protein levels: $n_{TR\text{-}mDecoy}$ = 14, $n_{TR\text{-}Decoy}$ = 11).

inhibition induced an impairment in Zif268 expression. To rule out the possibility that NF-κB effect over Zif268 expression could be the result of a decrease in NF-κB nuclear translocation instead of a decrease in its nuclear activity, we measured NF-κB nuclear protein levels. We did not find significant differences in NF-κB nuclear protein levels between TR-mDecoy and TR-Decoy groups (Fig. 4C, left graph) ($t = 0.2628$, $p = 0.80$). We also analyzed the cytosolic fraction of the hippocampus homogenate to rule out the possibility that the inhibition of NF-κB may be affecting Zif268 translocation to the nucleus instead of its protein expression. If translocation of Zif268 to the nucleus was affected, we would expect increased levels of cytoplasmic Zif268 in the TR-Decoy group in comparison to TR-mDecoy group. The results show no significant differences in the cytoplasmic fraction between both groups (Fig. 4C, middle graph) ($t = 0.011$, $p = 0.99$). Finally, to exclude the possibility that the inhibition of Zif268 expression could be a consequence of the consolidation process being interrupted after NF-κB inhibition, we decided to study the effect of Decoy injection over the expression of c-Fos, a protein that has also been involved in memory formation (He, Yamada, & Nabeshima, 2002; Katche et al., 2010; Lamprecht & Dudai, 1996; Tischmeyer & Grimm, 1999). *c-fos*, as well as *zif268*, is an immediate-early gene that codes for a transcription factor and whose expression is induced upon neuronal activation. *c-fos* and other immediate-early genes suchs as *Arc*, and *c-jun*, have *bona fide* sites for NF-κB in its promoter, but they have not been shown to be functional. For this experiment, we trained cannulated animals to the NOR task, we injected Decoy or mDecoy immediately after training and 45 min later we sacrificed mice and hippocampus was isolated. We decided to analyze the nuclear protein levels at this time-point as we expected *c-fos* expression to have a similar timeline to that of *zif268*, as both are IEGs and code for transcription factors. Nuclear protein levels for c-Fos show no significant differences between TR-Decoy and TR-mDecoy groups (Fig. 4C, right graph) ($t = 1.879$, $p = 0.073$). Altogether, these experiments, indicate that the effect of NF-κB inhibition over Zif268 expression is not due to a general blockade of the consolidation process nor an unspecific effect on protein translocation. Our results support an inhibition on its protein expression.

4. Discussion

In the present work we obtained evidence on the participation of NF-κB and Zif268 in the hippocampus of mice during NOR memory consolidation. NF-κB is activated in the nucleus of neurons 45 min after training and the inhibition of this TF in DH impaired memory consolidation. Similarly, Zif268 expression is increased 45 min after training and the knock-down of the protein by a specific ASO injected in DH impaired NOR memory. Furthermore, the last experiment in this work supports that Zif268 protein expression induced during learning is dependent on NF-κB activity. Thus, not only we proved that both TFs as well as the hippocampus are necessary for NOR memory formation, but also we demonstrated that NF-κB regulates, either on a direct or an indirect manner, Zif268 protein expression in the hippocampus during this process.

NF-κB is involved in memory consolidation in different learning tasks in rodents (de la Fuente et al., 2014; Freudenthal et al., 2005; Meffert, Chang, Wiltgen, Fanselow, & Baltimore, 2003; Yeh, Lin, Lee, & Gean, 2002). We previously found that NF-κB activation peaked at 45 min during consolidation in other two memory tasks in mice, inhibitory avoidance (Freudenthal et al., 2005) and contextual fear conditioning (de la Fuente et al., 2014). These two tasks are of aversive nature due to the use of foot shock as unconditioned stimulus. Conversely, NOR task is not aversive and the fact that NF-κB is involved in this task, and that the activation time point is coincident with the other tasks, stress the importance and the generality of this TF in memory processes in the hippocampus. Hence, the study of gene expression induced by NF-κB activation is important to reveal the molecular characteristics of memory formation. Among the different genes that could be regulated by NF-κB,

zif268 stands out for its known involvement on learning and memory processes.

Our results show that Zif268 nuclear protein levels are transiently increased 45 min after NOR training. There is a large amount of literature that suggests a rapid increase in *zif268* expression after plasticity and learning events in different species (Bozon et al., 2002 for a review). Previous data show that in the dentate gyrus, Zif268 mRNA is transiently upregulated between 10 min and 2 h following LTP-inducing stimulation (Jones et al., 2001), and that Zif268 mRNA in rats is upregulated in the hippocampus 30 min after spatial learning in a navigation task (Guzowski et al., 2001) and after exposure to a novel stimulus (Hall, Thomas, & Everitt, 2000). In particular, recent studies have shown that spatial exploration of objects in mice triggers phosphorylation, acetylation, and methylation of histones at the *zif268* promoter in the hippocampus (Gräff, Woldemichael, Berchtold, Dewarrat, & Mansuy, 2012) as well as increased Zif268 mRNA 30 min after this type of experience (Poplawski et al., 2014). Altogether, these evidence and our results indicate that there is gene and protein expression of Zif268 after object exploration. In this work we also show that this increase is transient. This quick turnover of Zif268 in the nucleus suggests that this TF is involved in a tight temporal second wave of regulation of gene expression.

Regarding the functional role of Zif268 in object recognition memory formation, a study using knock-out mice has shown that homozygous mice have NOR memory impairment (Bozon et al., 2002). In agreement with these findings, our results are the first evidence that knocking down Zif268 specifically in the hippocampus of mice is sufficient to impair object recognition memory, indicating that this TF has a fundamental role in this structure during NOR memory consolidation. How Zif268 may be involved in this type of memory still remains elusive. Recent experiments suggest that Zif268 regulates the transcription of proteasome-related genes and hence modulates the activity of the proteasome (James, Conway, & Morris, 2006). It is possible that its role in memory formation is to keep the correct balance between new and old proteins involved in the mnemonic trace, as it has previously been shown that proteasome activity is involved in different forms of plasticity (Juo & Kaplan, 2004; Obin et al., 1999; Speese, Trotta, Rodesch, Aravamudan, & Broadie, 2003) and memory (Fustiñana et al., 2014; Jarome, Werner, Kwapis, & Helmstetter, 2011; Kaang & Choi, 2012; Merlo & Romano, 2007).

Finally, in the present work we found that the DNA binding inhibition of NF-κB by the administration of NF-κB Decoy in DH significantly reduced the expression of Zif268 induced by NOR training. Taking together all of the results in this work and the literature available, we suggest that NOR training triggers NF-κB activation in the nucleus of hippocampal neurons, that this event is followed by the expression of IEGs such as *zif268* and that NF-κB activation is involved in this last process by regulating Zif268 expression. Later, there is another wave of gene transcription regulated, at least in part, by Zif268 which is necessary for proper NOR memory consolidation. Whether NF-κB is regulating Zif268 expression on a direct manner by binding to its promoter is still an open question. We found a tight temporal correlation between NF-κB activation and Zif268 expression. Previous data also report there are many NF-κB binding sequences on *zif268* promoter (Ahn et al., 2008) and that NF-κB can bind to *zif268* promoter (Deckmann, Rörsch, Geisslinger, & Grösch, 2012; Thyss et al., 2005) suggesting that this interaction is probably taking place on a direct manner. Ongoing experiments aim to assess whether the observed effect is mediated by a direct action of NF-κB in *zif268* promoter and to study the presence of both TFs *in situ* in the hippocampus after NOR training. This analysis will shed further light about the direct action of NF-κB on Zif268 expression and about the identification of neurons involved in NOR consolidation.

5. Conclusions

In the present work, we evaluated the role of a TF of constitutive expression, NF-κB, and the role of an IEG, *zif268*, in NOR memory consolidation in hippocampus. Our results not only prove that both TF are necessary for proper NOR memory consolidation, but they also demonstrate that the hippocampus is a key structure for this process. Finally, we obtained evidence that support the regulation of the IEG expression by NF-κB, as a fast transducer of synaptic signals in the nucleus.

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