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HIPPOCAMPAL DYNAMICS OF SYNAPTIC NF-KAPPA B DURING INHIBITORY AVOIDANCE LONG-TERM MEMORY CONSOLIDATION IN MICE

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Abstract—Since the discovery that long-term memory is dependent on protein synthesis, several transcription factors have been found to participate in the transcriptional activity needed for its consolidation. Among them, NF-kappa B is a constitutive transcription factor whose nuclear activity has proven to be necessary for the consolidation of inhibitory avoidance in mice. This transcription factor has a wide distribution in the nervous system, with a well-reported presence in dendrites and synaptic terminals. Here we report changes in synaptosomal NF-kappa B localization and activity, during long-term memory consolidation. Activity comparison of synaptosomal and nuclear NF-kappa B, indicates different dynamics for both localizations. In this study we identify two pools of synaptosomal NF-kappa B, one obtained with the synaptoplasm (free fraction) and the second bound to the synaptosomal membranes. During the early steps of consolidation the first pool is activated, as the membrane associated transcription factor fraction increases and concomitantly the free fraction decreases. These results suggest that the activation of synaptic NF-kappa B and its translocation to membranes are part of the consolidation of long-term memory in mice.

Key words: memory consolidation, synapse, NF-kappa B, I kappa B alpha.

INTRODUCTION

Long-term memory has proven to be dependent on gene expression for a variety of species (Agranoff et al., 1966; Davis and Squire, 1984), and this gene expression is driven by transcription factors such as CREB, NF-kappa B, AP-1, Zif268, C/EBP and others (Alberini, 2009). The most rapid induction is regulated by constitutive transcriptions factor like CREB and NF-kappa B, that exert their regulation in the nucleus directly upon activation (Kaltschmidt et al., 1994; Kaltschmidt and Kaltschmidt, 2009).

NF-kappa B, in neurons is in equilibrium between its inactive form, a dimer (typically p65/p50) bound to the inhibitor (IκB), and the free dimer that is capable to translocate to the nucleus and bind DNA (Kaltschmidt et al., 1994; Kaltschmidt and Kaltschmidt, 2000; Meffert and Baltimore, 2005). IκB blocks the nuclear localization signal (NLS) and DNA binding site. When IκB releases NF kappa B, the dimer is able to bind DNA and therefore may be considered as active (Baeuerle and Baltimore, 1988). The transcription factor is extensively expressed in the brain and has a strong presence in memory-related areas including the hippocampus (Kaltschmidt and Kaltschmidt, 2001).

The neuronal activation of the transcription factor NF-kappa B has been associated with synaptic plasticity and the consolidation of long-term memory (Freudenthal and Romano, 2000; Meffert et al., 2003; Romano et al., 2006; Kaltschmidt and Kaltschmidt, 2009). During long-term potentiation (LTP) of the perforant pathway, NF-kappa B is activated in the mice hippocampus (Freudenthal et al., 2004), the p50 knockout mice have impaired late-LTP (Oikawa et al., 2012) and NF-kappa B target genes are regulated after LTP induction in the perforant pathway of the rat (Ryan et al., 2012). NF-kappa B is activated during long-term memory consolidation and reconsolidation of crabs, rat and mice (Merlo et al., 2002; Freudenthal et al., 2005; Boccia et al., 2007; O'Sullivan et al., 2007) and the inhibition of the NF-kappa B pathway in the hippocampus, during consolidation and reconsolidation proves to be amnesic for the inhibitory avoidance memory task (Merlo et al., 2002; Freudenthal et al., 2005; Boccia et al., 2007). Also, NF-kappa B is directly involved in spine density control, its regulation throughout IKK activation

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Abbreviations: ANOVA, analysis of variance; AP-1, activator protein 1; C/EBP, CCAAT-enhancer-binding proteins; CREB, cAMP response element-binding protein; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ELK-1, ETS domain-containing protein Elk-1; EMSA, electrophoretic mobility shift assay; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; iS, immediate shock; LTP, long-term potentiation; NF-kappa B, nuclear factor kappa-light-chain-enhancer of activated B cells; NLS, nuclear localization signal; PFA, paraformaldehyde; PMSF, phenylmethylsulfonyl fluoride; PO, phosphate; ROD, relative optical density; SC, synaptosomal content; SDS, sodium dodecyl sulfate; SDSm, SDS-treated membranes; Stat3, Signal transducer and activator of transcription 3; TE, triton extract; WB, Western blots; Zif268, zinc finger protein 225.

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increases spine density, and inhibition decreases it (Russo et al., 2009; Boersma et al., 2011).

Several transcription factors have been observed in dendrites: NF-kappa B, CREB, Stat3 and ELK-1 (Kaltschmidt et al., 1993; Suzuki et al., 1997, 1998; Sgambato et al., 1998; Murata et al., 2000). NF-kappa B in particular, has been reported both in axons (Sulejczak and Skup, 2000; Mindorff et al., 2007) and dendrites (Kaltschmidt et al., 1993; Suzuki et al., 1997; Heckscher et al., 2007; Boersma et al., 2011). In this last localization p65 has been reported in close proximity to the post-synaptic densities (Suzuki et al., 1997; Boersma et al., 2011).

Synaptosomal activation of NF-kappa B has been observed during long-term memory consolidation in the crab *Neohelice granulata* (Freudenthal and Romano, 2000). Depolarization and/or glutamate, activates NF-kappa B and triggers its transport to the nucleus in cell culture experiments (Wellmann et al., 2001; Meffert et al., 2003). The main interpretation of this evidence has been that the transcription factor is part of the synapse-to-nucleus communication for trans-synaptic regulation of gene expression (Kaltschmidt et al., 1993; Wellmann et al., 2001; Meffert et al., 2003), and that the difference in signaling between the peri-somatic and synaptic transcription factor relays in post-translational modifications (Suzuki et al., 1998). Although a local role for the transcription factor has been identified in drosophila's neuromuscular junction (Heckscher et al., 2007), no previous studies have proposed a similar function in central mammalian synapses. Here, we describe a system in which NF-kappa B could have a local role during long-term memory consolidation.

For this study we chose the inhibitory avoidance in mice, for two main reasons, first: this model is able to induce long-term retention with one trial, allowing evaluation of the consolidation dynamics in a more time precise manner (Boccia et al., 2004); and second: the nuclear activation of this transcription factor and its requirement has been thoroughly reported by our group for this paradigm (Freudenthal et al., 2005).

We identify two different pools of NF-kappa B (p65 subunit) that can be obtained from synaptosomes, the first free in the cytoplasm and the second strongly bound to the membranes. During long-term memory consolidation the free fraction of transcription factor is activated, and the proportion of the membrane pool increases at expenses of the free pool. These results indicate the synaptic NF-kappa B activation and migration to membranes are early parts of the consolidation of the inhibitory avoidance long-term memory in mice.

EXPERIMENTAL PROCEDURES

Animals

The experiments were carried out following the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication No. 80–23/96) and local regulations. CF-1 male mice (*Mus musculus*) (Fundacal, Buenos Aires, Argentina) were used (age 60–70 days; weight 25–30 g). The outbred CF-1 mice where chosen keeping in mind that this genetic background will yield more general results than a strain and also that they show strong retention in the one-trial paradigm used for the present study. 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. All efforts were made to reduce the number of animals used and ameliorate animal suffering.

Apparatus and behavioral procedure

Inhibitory avoidance behavior was studied in a one-trial learning, step-through type situation (Boccia et al., 2004), which utilizes the natural preference of mice for dark environments. 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 center. The mice were not habituated to the dark compartment before the learning trial. All mice were trained between 8 a.m. and 10 a.m. 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 1.2 mA, 50 Hz, 1 s. Retention was evidenced by median delay scores of 300 s when entering the dark compartment during testing 48 h post training (Freudenthal et al., 2005). Two groups of eight mice were used for the experiments, Shocked (S) and Naïve (N). This number of animals is enough to evidence significant differences in biochemical and behavioral experiments. For some experiments a third group of animals was used denominated the immediate shock group (iS). The iS animals are placed directly inside of the dark compartment and immediately receive the electric shock after which they are returned to their home cage. The Naïve group of mice were housed in the same conditions as that of the experimental groups, this group was included in order to estimate basal levels.

Nuclear extracts

The mice were killed by cervical dislocation at different intervals after training (see Results), Naïve animals were sacrificed at the same time as S and iS animals for each experiment. The brains were rapidly removed, and the hippocampi were dissected according to the method of Glowinski and Iversen (1966). To obtain nuclear extracts, tissues were homogenized in 250 µl of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 g/ml pepstatin A, 10 g/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 10 g/ml aprotinin) with eight strokes in a Dounce homogenizer, type B pestle. The homogenate was centrifuged for 15 min at 1000*g* and the supernatant was discarded. The pellet was resuspended in 30 µl of buffer B (20 mM HEPES, pH 7.9, 800 mM KCl, 1.5 mM MgCl2, 0.4 mM EDTA, 0.5 mM DTT, 50% glycerol, 1 g/ml pepstatin A, 10 g/ml leupeptin, 0.5 mM PMSF, and 10 g/ml aprotinin) and incubated for 20 min on ice. A centrifugation for 15 min at 12,000*g* was then performed. The supernatant (nuclear extract) was stored at 80 °C until used. The entire extraction protocol was performed at

4 °C. The same protocol was used for cortex tissue but 500 μl were used of buffer A instead.

Synaptosomal extracts

The mice were killed and the hippocampus was removed as described previously in Nuclear extracts. To obtain synaptosomal crude extracts (P2), tissues were homogenized in 250 μl of buffer 1 (Tris–HCl 20 mM, sucrose 320 mM, EDTA 1 mM, pH 7.4, 1 μg/ml pepstatin A, 10 μg/ml leupeptin, 0.5 mM PMSF) with fifteen strokes in a Dounce homogenizer, type B pestle. The homogenate was centrifuged for 10 min at 1000*g*. The supernatant was separated in a new tube and the pellet was resuspended in 250 μl of buffer 1 and centrifuged again for 10 min at 1000*g*. The new supernatant was pooled with the previous one. This 500 μl of supernatant was centrifuged for 30 min at 13,000*g*. The last supernatant was discarded, and the pellet (synaptosomal crude extract, P2) was used for further extractions. Four volumes of hypotonic buffer (Tris–HCl 5 mM, $CaCl_2$ 50 μM, pH 8.1) were added to P2, the pellet was resuspended vigorously and it was centrifuged for 50 min at 15,800*g*. The supernatant (synaptosomal content, SC) was kept at −20 °C until used. The pellet was resuspended in hypotonic buffer with 1% triton detergent and centrifuged 50 min at 15,800*g*. The supernatant (Triton extract, TE) was kept at −20 °C until used. The pellet was resuspended in hypotonic buffer 1% sodium dodecyl sulfate (SDS) (SDS-treated membranes, SDSm) and kept at −20 °C until used.

Percoll purified synaptosomes were obtained following the procedure described by Nagy and Delgado-Escueta (1984) with modifications from Rodrigues et al. (2005). Briefly, the P2 is obtained and placed on a discontinuous 2 ml percoll gradient (3%, 10% and 23%) and centrifuged for 20 min at 15,000*g*. The synaptosomes are recovered with a Pasteur pipette from the interface formed. After pure synaptosomes were obtained, the same protocol as described previously was used to obtain SC, TE and SDSm from P2 extract.

Western blots

For Western blot assay, loading buffer was added to the samples that were incubated at 95 °C for 5 min and immediately placed on ice. Twenty micrograms of each protein sample was run on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) for 30 min at 80 V in the stacking phase and for about 90 min at 120 V in the resolving phase 10% acrylamide. The proteins were then electro transferred for 45 min at 100 V to a polyvinylidenedifluoride membrane for immunoblotting. Western blot assays were performed with anti-NFκB p65 (Santa Cruz Biotechnology, Dallas, Texas, U.S.A.; c-20 sc-372; at 1:1000 dilution), anti-IκB (Santa Cruz Biotechnology, Dallas, Texas, U.S.A.; sc-847; at 1:500 dilution) antibodies, synaptic markers anti-PSD95 (Cell Signalling, Danvers, MA, U.S.A.; #2507; at 1:1000 dilution) and antibody anti-NMDA NR1 (Sigma Aldrich, St. Louis, MO, U.S.A.; G8913; at 1:500 dilution for hippocampi; and custom antibody (EZ1) made by EZBiolabs (Carmel, IN, U.S.A.) against the peptide C-LQNQKDTVLPRRAIEREEGQLQLCSRHRES-NH2 the same immunogen as the used for the Chemicon 1516, at 1:100 dilution for cortex tissue) antibodies, and the nuclear marker anti-Lamin B1 (Santa Cruz Bio-technology, Dallas, Texas, U.S.A.; sc-13036; at 1:1000 dilution) antibodies following the manufacturer's protocol. Detection was made with the Advanced ECL kit (Amersham General Electric, Pittsburgh, PA, U.S.A.) as described by the manufacturer and the signals were digitalized by FUJIFILM-Intelligent Dark Box II apparatus with image reader LAS-1000 software. The relative optical density (ROD) was estimated using ImageJ 1.4 software. The protein size was estimated by its relative mobility using ImageJ 1.40 software and correlating these measurements with the molecular weights of pre-stained standard proteins (Full-Range Rainbow Molecular Weight Markers: 12–225 kDa, Amersham, Pittsburgh, PA, U.S.A.). Protein contents of the extracts were measured in duplicate with the BCA protein assay reagent kit (Thermo Scientific, Waltham, MA, U.S.A.); and each sample quantities were controlled with these measurements. Proteins were relativized to the Naïve group for each gel for control.

Determination of DNA-binding activity

κB-like DNA binding activity in nuclear and SC fractions was assessed using electrophoretic mobility shift assay (EMSA). An amount of 1.75 pmol of double-stranded oligonucleotide DNA containing the NF-κB binding site (5′-AGTTGAG**GGGACTTTCC**CAGGC-3′, binding site in bold) (Promega, Madison, WI, U.S.A.) was labeled with P^{32} at 37 °C for 10 min in 10 μl containing Tris–HCl, 70 mm; $MgCl_2$, 10 mm; DTT, 5 mm; (cP^{32})ATP (Nen/Dupont), 15 uCi; 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 labeled oligonucleotide DNA probe was added followed by incubation for a further 30 min at 0 °C. The reaction mixture was electrophoresed on a 6% non-denaturing polyacrylamide 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, Rochester, NY, U.S.A.). Using this probe, in previous works three specific retarded bands were found (Freudenthal et al., 2004). Extracts from this study were used to reproduce these results using the radioactive probe (yielding the three bands detailed before) and the same samples were used to compare with the blot technique (Fig. 1). The ROD of the first band corresponding to the p65–p50 hetero dimer was estimated using NIH Image J 1.40 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, Dallas, TX, U.S.A.). Protein contents of the extracts were measured in triplicate by the Bradford method (Sigma, St. Louis, MO, USA) and checked for

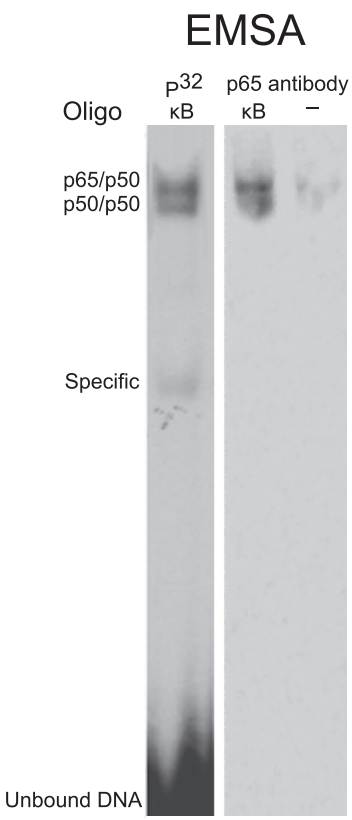

Fig. 1. Comparison of radioactive EMSA and EMSA blot. Hippocampal nuclear extracts were used for these EMSAs. The left panel shows a typical Radioactive EMSA image were the p65/p50 and p50/p50 dimmers are evidenced by the P^{32} labeled oligokB. The right panel shows a typical EMSA blot image were the p65/p50 is evidenced by the p65 antibody. This panel compares extract incubated with oligokB and without.

quality and quantity by comparing pattern intensities SDS–PAGE. EMSA Blot assays were preformed as described previously for radioactive EMSAs but with the following modifications: the oligonucleotides were not labeled and after the electrophoresis the proteins were then electro transferred for 45 min at 100 V to a polyvinylidenefluoride membrane for immunoblotting. NF-kappa B subunit p65 was detected with the anti-NFκB p65 (Santa Cruz Biotechnology, Dallas, Texas, U.S.A.; c-20 sc-372; at 1:1000 dilution) as described previously in the Western blot section. EMSA Blots were compared with Radioactive EMSA for validation (Fig. 1).

Immunofluorescence

Mice were anesthetized with ketamine/xilacine and perfused with PFA (paraformaldehyde) 4%. Brains were extracted and kept in 15 ml 4% PFA for 2 h. Then brains were transferred to 15 ml of 30% sucrose solution in phosphate buffer (PO) pH 7.4 and left overnight. Brains were frozen in isopentane kept at −80 °C. Sections were cut at 25 μm using a cryostat. Suitable sections were selected and were washed in PO buffer with increasing concentrations of triton detergent. Sections were kept 1 h in blocking solution (PO-triton 0.03%, BSA1%, Normal rabbit serum 2%) and then overnight with primary antibodies dilutions in PO-triton 0.03% with anti-NFκB p65 (Santa Cruz Biotechnology; c-20 sc-372; at 1:100 dilution), anti-IκB (Santa Cruz Biotechnology; sc-847; at 1:100 dilution) antibodies and anti-p65 NLS (Chemicon; Mab3026; at 1:50 dilution). Washes were made in PO buffer with increasing concentrations of triton detergent. The slices were then incubated with AlexaFluorlabeled488 goat anti-rabbit (Invitrogen, A11008, 1:500 dilution) for 1 h at room temperature. Washes were made in PO buffer with increasing concentrations of triton detergent and slices were mounted on slides with 20%PBS, 80% glycerol. Fluorescence in tissue samples was visualized using a confocal laser-scanning microscope (Olympus FV300, Japan) equipped with a 488-nm argon laser (543-nm HeNe Green), an UplanFl 10X objective (NA 0.3), an UplanFl 20X objective (NA 0.5), and a dichroic cube SDM 570 to split the acquisition channels. The specificity of the secondary antibody was evaluated by carrying out the immunofluorescence protocol without the primary antibody. Image processing was performed using ImageJ 1.40 software (NIH, USA http://rsb.info.nih.gov/ij/).

Data analysis

ROD values for each group were related to the mean ROD values of the N group. One-way analysis of variance (ANOVA) comparisons were used to estimate general significance. Differences between groups were assessed by post hoc Fisher Least Significant Difference (LSD) test using a free statistical software package.

RESULTS

NF-kappa B is present in the nucleus, perinuclear cytoplasm and dendrites

Initially, we performed immunofluorescence to detect NF-kappa B pathway components, p65 and IκBα, in mice hippocampus. Three different antibodies where used, one for IκBα and two for the p65 subunit of NF-kappa B, one of these for total p65 and the other for p65 with the NLS exposed (activated form). The subcellular activation levels of NF-kappa B are suggested by the presence of p65 and IκB at each site (as in some nuclei were p65 is abundant and IκB has low levels, therefore evidencing activity of NF-kappa B), and by the immunostaining of the activated form of p65, that is clearly visible in the dendrites of the stratum radiatum. P65 and IκB show a somatic and neuropilar distribution in the two main parts of the hippocampal formation, the dentate gyrus and Ammon's horn.

The subcellular distribution of NF-kappa B is evident in the compact cellular arrangement of the hippocampal CA1 pyramidal cell layer, and its adjacent neuropile, the *stratum radiatum* (Fig. 2A), equally thus in the Dentate Gyrus and CA3 (data not shown).

In CA1 the dimer component p65 is present in nucleus, perinuclear cytoplasm and dendrites, where it is detected with antibodies for the total protein and the form of p65 that has its NLS exposed. Most of the visualized p65 is in these three cellular compartments in

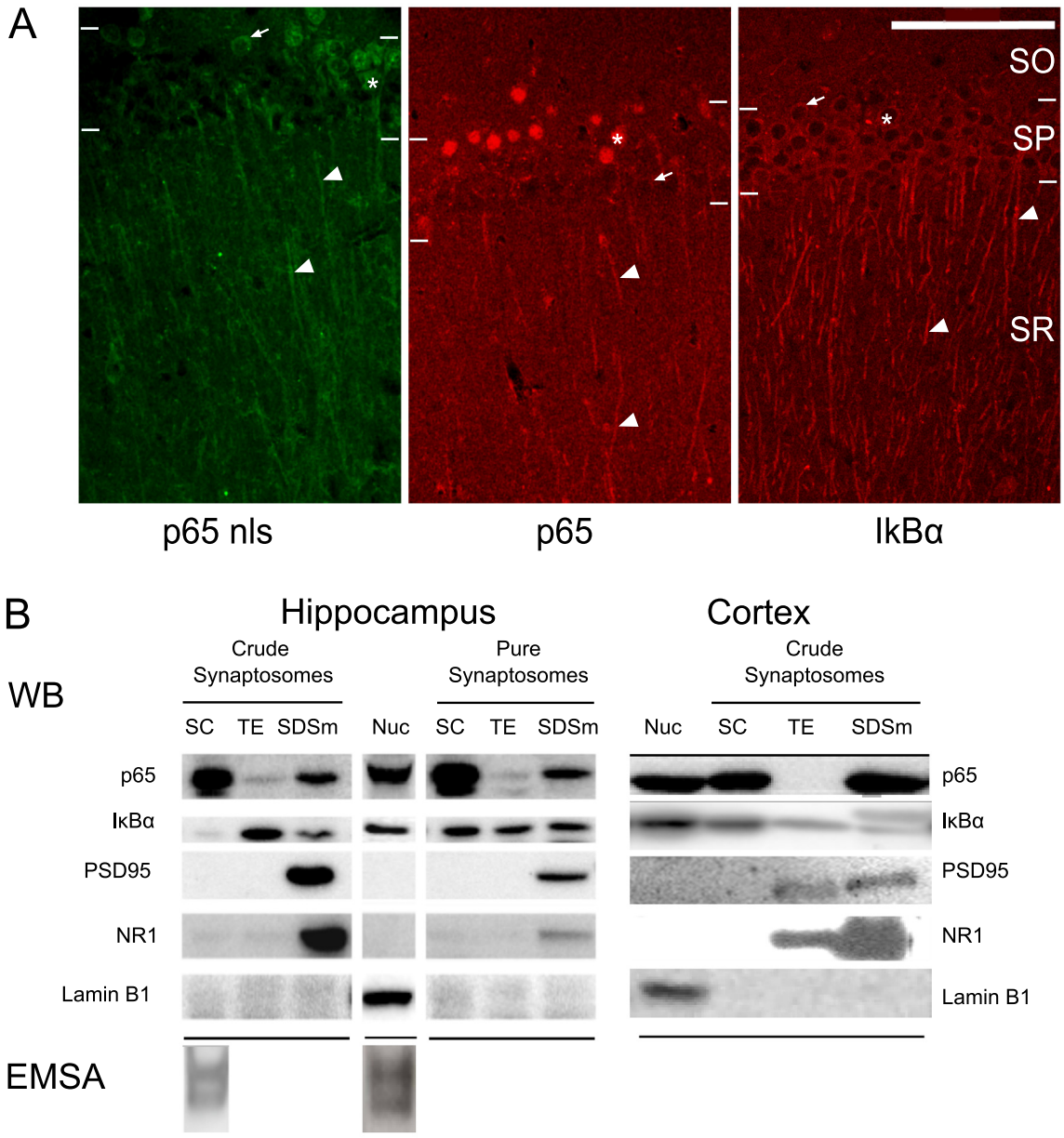

Fig. 2. NF-kappa B neuronal localization and characterization. (A) Immunofluorescence of coronal sections show CA1 area of the hippocampus labeled with the antibodies for p65 NLS, total p65 and IκBα (left, center and right accordingly). Arrows indicate perinuclear cytoplasm, arrowheads indicate location at dendrites and asterisk indicates nucleus. The different layers are marked for each panel, *stratum oriens* (SO), *stratum pyramidale* (SP), *stratum radiatum* (SR). (B) Western blots (WB) of hippocampus (left panel) and cortex (right panel) for Nuclear (Nuc), Synaptosomal content (SC), Triton extract (TE) and SDS-treated membranes (SDSm) extracts. These last three, both from pure (percoll gradient synaptosomes) and crude (P2) synaptosomal preparations. Antibodies used were NF-kappa B component (p65), NF-kappa B inhibitor (IκBα), Post synaptic density marker (PSD-95), Post synaptic density marker (NMDA Nr1) and Nuclear marker (Lamin B1). Underlined represents same tissue extraction. EMSA shows the presence of NF-kappa B DNA binding activity at nucleus and synaptosomal content.

variable proportions for each cell. IκBα is clearly seen in the dendrites of the stratum radiatum and in the perinuclear cytoplasm of the cells in the stratum pyramidale, its presence in the nucleus is almost undetectable in naïve animals (Fig. 2A).

NF-kappa B in synaptosomes is present in two different pools

The subcellular localization of NF kappa B seen in the immunofluorescence studies is corroborated by the results of Western blots (WB). The nuclear presence of p65 and IκBα is clearly detected, and extract origin is confirmed by Lamin B1 (Fig. 2B). Two main pools of p65 are clearly evidenced in synaptosomes; one pool of soluble p65 found in the synaptosomal content (SC) and another distinct pool strongly bound to membranes. This membrane p65 is found in the SDS-treated membrane extract (SDSm), which also show the presence of the synaptic membrane markers PSD-95 and the NMDA NR1 subunit. Almost no p65 is detected in membranes

treated with the mild detergent triton (TE), nonetheless IκB is clearly visible in this extract (Fig. 2B). This distribution pattern is found both in the hippocampus and whole-cortex extracts.

The percoll method is a further purification step in the synaptosome preparation protocol that yields purer synaptosomes than the crude synaptosomes (P2). The extracts obtained from the P2 and the ones from the percoll purified synaptosomes show a similar distribution for p65, IκBα and the synaptic membrane markers PSD-95 and NMDA receptor subunit 1 (NR1) (Fig. 2B).

In nucleus and synaptosomal content extracts, NF-kappa B DNA binding activity can be evidenced by EMSA (Fig. 2B). This supports the results seen in Fig. 2A by the p65 NLS antibody, suggesting the activated form of NF-kappa B at these same sites. Synaptosomal fractions (SC, TE, SDSm) are sequentially extracted from each synaptosome preparation (crude or percoll purified) and thus each series of fractions came from a different animal.

Two main points can be drawn from this section: first and foremost, NF-kappa B is found free in the synaptosomal content and strongly bound to synaptosomal membranes both in the hippocampus and cortex. Second, crude extracts show similar protein distribution than percoll purified synaptosomes, allowing us to use this faster protocol for the experiments aimed to evaluate the NF-kappa B dynamics during memory consolidation.

Synaptosomal NF-kappa B is activated during consolidation with a different time course than nuclear activation

In the inhibitory avoidance task, mice are placed on an illuminated platform with an entrance to a dark compartment; trained mice receive an electric shock once inside. Retention is evaluated as a measure of the latency to step through in following trials. The inhibitory avoidance long-term memory consolidation requires activated nuclear NF-kappa B 45 min post-training (Freudenthal et al., 2005). This experiment was designed to evaluate the time course of synaptic activation of NF-kappa B.

Two animal groups were evaluated: animals that received an electric shock when entered the dark compartment from the platform (shocked, S) and Naïve animals (N). Typically, S animals show a strong retention 48 h after training, evidencing long-term memory (Boccia et al., 2004). This protocol is known to affect DNA binding activity of nuclear NF-kappa B after training (Freudenthal et al., 2005). Hippocampal SC extracts were obtained 5, 15 and 45 min after training and the DNA binding activity was assayed by EMSA (Fig. 3A). Five minutes post-training there is a significant increment in NF-kappa B DNA binding activity in S mice ($S = 192.5\%$, $t = 2.821$, $p < 0.01$) when compared to N mice (Fig. 3B). No significant difference was observed 15 min after training within experimental groups (Fig. 3C). Though not statistically significant; there is a decreasing tendency for the DNA binding activity of NF-kappa B for S mice 45 min post training (Fig. 3D). Fig. 3E compares NF-kappa B DNA binding activity in nuclear and SC extracts. Synaptosomal activation of NF-kappa B occurs 5 min post training while nuclear activation of NF-kappa B occurs at 45 min post-training with a previous inhibition at 15 min. This indicates a clear time separation for the pathway activation between these sub-cellular localizations, one 5 min post-training at the synapse, seen in this study, and another 45 min post-training at the nucleus (data for nuclear activity at 15 and 45 min post-training from Freudenthal et al., 2005). As demonstrated previously, nuclear NF-kappa B activity after training is due to the association between context and foot-shock and not only due to the foot-shock (Boccia et al., 2007).

Synaptosomal NF-kappa B activation 5 min post-training is also not due to the effect of the foot-shock only but to the association of the aversive stimulus with the context. This has been evidenced using the iS group of animals. This immediate shock produces no context-fear learning (Landeira-Fernandez et al., 2006), and there is no activation of NF-kappa B 5 min after training in the synaptosomal content (One way ANOVA $p < 0.05$ $F = 197.76$; $S = 161.41\%$ $p < 0.05$, Si = 93.59%) (Fig. 4), supporting the idea that synaptosomal NF-kappa B is activated specifically during memory consolidation.

NF-kappa B pathway components translocate to synaptosomal membranes during consolidation

Changes in the distribution of NF-kappa B during consolidation were evaluated by normalized WB analysis of hippocampal synaptosomal extracts for the two groups. The amounts of p65 in SC are significantly lower 5 min after training for S mice compared to N animals ($S = 71.3\%$, $t = 3.637$, $p < 0.01$) (Fig. 5B). No differences were found for p65 in this extract 15 and 45 min post-training between groups. There is a significant increase of p65 in SDSm fraction 5 min post-training for S mice when compared to N group ($S = 197.6\%$, $t = 5.296$, $p < 0.01$) (Fig. 5A). No change was observed in the amount of p65 in SDSm 15 and 45 min post-training between groups (Fig. 5A). Membrane IκBα obtained in TE increased for S mice 5 min post-training ($S = 132\%$, $t = 2.677$, $p < 0.01$). The amount of IκBα decreased 15 min post-training in S mice compared to N ($S = 70.5\%$, $t = 3.656$, $p < 0.01$) and 45 min post-training there were no significant differences ($p = 0.205$) (Fig. 5C). These results suggest that the synaptic NF-kappa B is dynamic and changes during consolidation, moving from the synaptoplasm to the membranes.

The relation of membrane NF-kappa B pathway components change during consolidation

The amount of membrane p65 (p65 SDSm) in relation to the amount of soluble p65 (p65 SC) is higher for S animals when compared to N mice 5 min post-training ($S = 259\%$, $p < 0.01$) (Fig. 6A). This relation returns to naïve levels at 15 min post-training. The relation suggests that membrane p65 increases at the expense of soluble p65. While exploring the NF-kappa B pathway

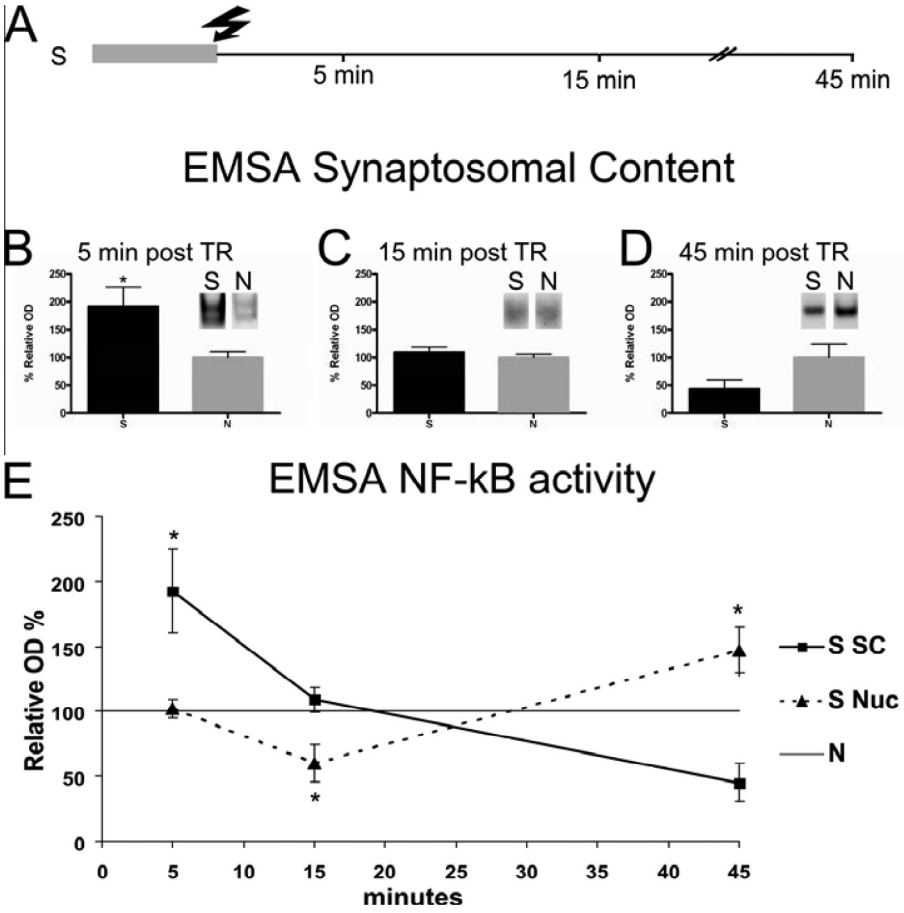

Fig. 3. NF-kappa B DNA binding activity during consolidation. (A) Training protocol for shocked (S) mice and tissue extraction times, 5, 15 and 45 min post training. (B–D) Bar graphs show NF-kappa B DNA binding activity in synaptosomal content (SC) relative to naïve (N) 5 (B), 15 (C) and 45 (D) minutes post training. Insets show representative EMSAs for each time and group. (E) NF-kappa B binding activity time course in synaptosomal content and nuclear extracts (Nuc), relative to N. $^{*}p < 0.05$.

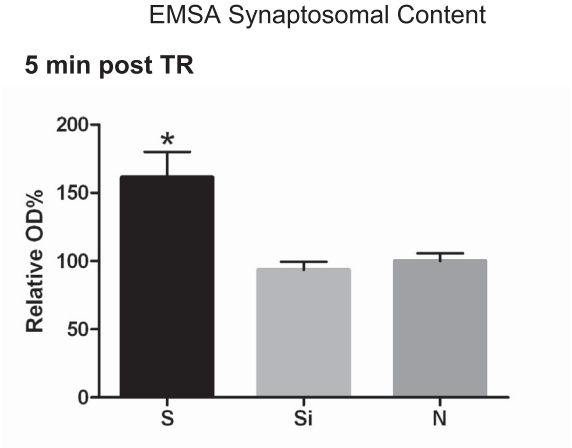

Fig. 4. NF-kappa B is activated during footshock-context association. Bar graph shows NF-kappa B DNA binding activity in synaptosomal content (SC) relative to naïve (N) for Shock (S) and immediate shock (iS) groups, 5 min post training. $^{*}p < 0.05$.

components in membranes is important to know if the transcription factor is activated or inhibited by IκB, in order to evaluate possible interactions that evidence the role at this site. As the membrane extracts have detergents that interfere with protein interactions, activity cannot be evaluated by EMSA, therefore we assess the ratio, p65SDSm/IκBαTE, to estimate the activation level of the transcription factor. The membrane relation between p65 and IκBα is higher 15 min post-training for S mice compared to N animals ($S = 182\%$, $p < 0.01$) (Fig. 6B). This relation is not significantly different 5 and 45 min post-training. This result suggests that membrane NF-kappa B activity (in terms of p65SDSm/IκBαTE relation) during consolidation has its own dynamics, different from nuclear and synaptosomal free fractions, with an increment in activity 15 min after training.

DISCUSSION

The presence of p65 and IκBα proteins is an indicator of an NF-kappa B functional pathway. In the hippocampus it is possible to localize these proteins both in the neuronal somas and the adjacent neuropiles of the hippocampus, i.e., pyramidal layer and the stratum radiatum of CA1 (Fig 2A). This localization suggests not only its classical dichotomous distribution between the nucleus and the peri-nuclear cytoplasm, but it shows as well a strong neuropilar presence (Fig 2). This subcellular localization

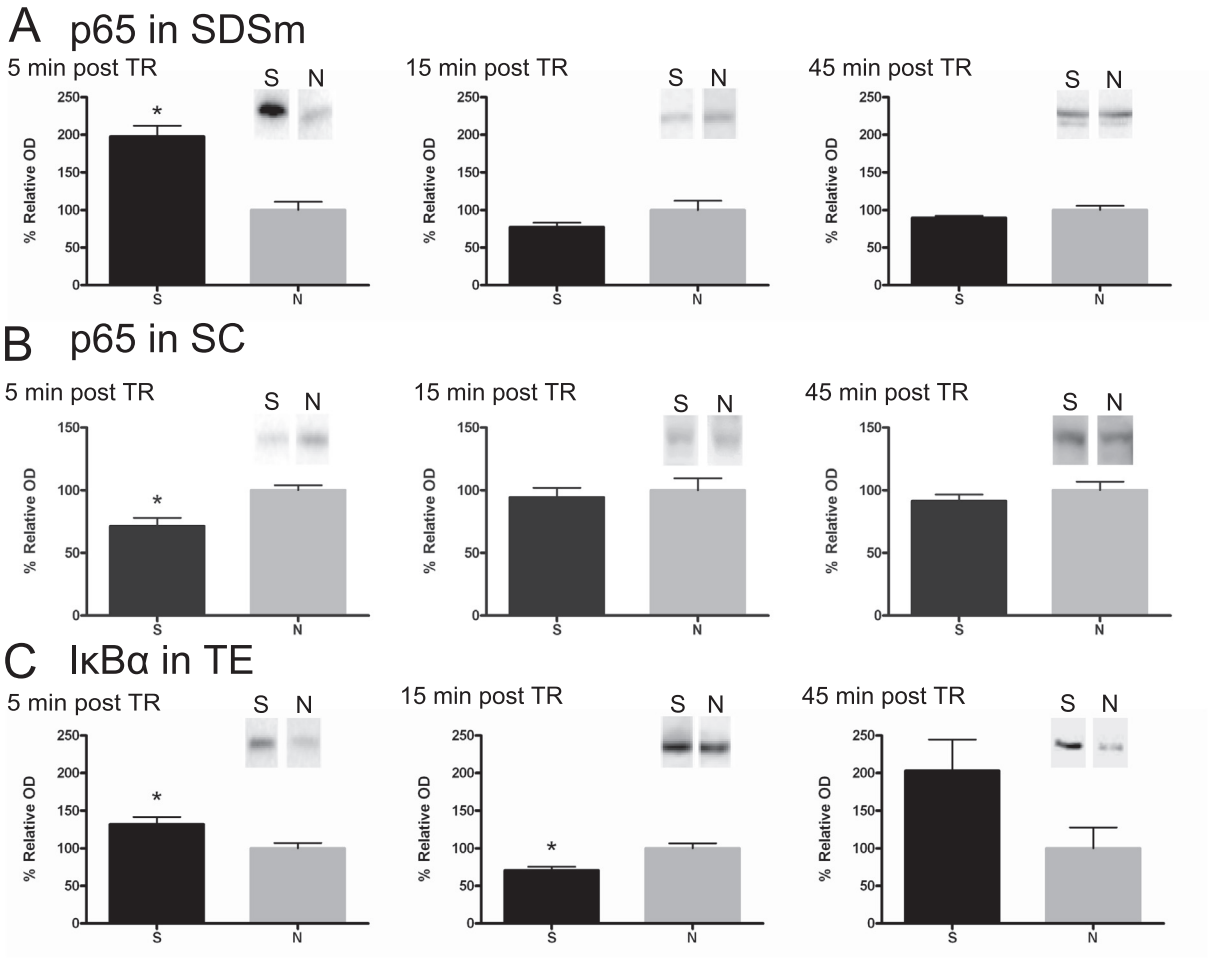

Fig. 5. Synaptosomal NF-kappa B pathway component dynamics during consolidation. Bars are mean ± SEM. Amount of p65 in (A) SDS-treated membranes (SDSm) and (B) Synaptosomal content (SC) and (C) IκBα in triton extracts (TE), for shocked (S) and naïve (N) animals, 5, 15 and 45 min post training (left, center and right accordingly). Protein quantities where controlled as described in 'Western blots' section. Insets show representative western blots for each extract. $^{*}p < 0.05$.

has been previously reported in the rat, mouse, crab and fruit fly. (Kaltschmidt et al., 1993; Meberg et al., 1996; Suzuki et al., 1998; Freudenthal and Romano, 2000; Heckscher et al., 2007; Boersma et al., 2011). In this work we found a similar distribution in the mouse hippocampus and, for the first time, we described the synaptic dynamics of the NF-kappa B pathway in the hippocampus during memory consolidation.

NF-kappa B pathway in the nervous system has been reported to have a basal level of activation in cultured neurons (Kaltschmidt et al., 1994). Here we report the *in vivo* soma and dendritic basal levels of activation. The localization of this pathway in the dendrite supports the idea that this transcription factor plays a local and specific role in plasticity and memory.

Pure and crude synaptosomal extracts proved to have a very similar localization pattern of synaptic proteins. This confirms that our observations are indeed dynamics of NF kappa B at the synapse.

We report two differentiable pools of NF-kappa B in synaptosomes; the first free in the synaptoplasm (SC extract) and the second anchored to membranes. Both pools have p65 and IκBα, but the anchored pool of p65 is only extracted with 1% SDS (strong anionic detergent) but not with 1% triton (mild non-ionic detergent). Some reports suggested this particular association for p65 and IκBα in neurons in culture (Suzuki et al., 1997; Heckscher et al., 2007; Schmeisser et al., 2012). This is the first time that these pools are described in the mouse hippocampus, and that the exchange dynamics between pools are described (Figs. 2, 5 and 6). P65 has the ability to interact with PDZ domains of the membrane protein X11L in neurons suggesting a possible membrane anchoring target (Tomita et al., 2000).

NF-kappa B is activated in the nucleus 45 min after training following a previous inhibition at 15 min post-training (Freudenthal et al., 2005). The activation at the synaptosomal content after a training session, takes 5 min (Fig. 3B). This activation is specific to the footshock-context association (Fig. 4).

Interestingly we found that the two pools of NF-kappa B (anchored in membrane and free in synaptoplasm) are not static but rather dynamic. The amount of NF-kappa B at these sites varies during consolidation (Fig. 5). Specifically, 5 min post-training there is an increase in

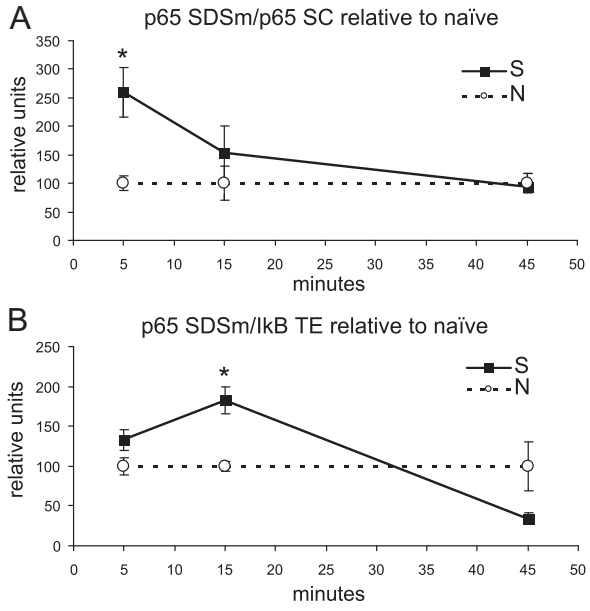

Fig. 6. Relation of anchored p65 with free p65 and with TE IκBα during consolidation. (A) Relation of p65 in SDS-treated membranes (SDSm) to p65 in synaptosomal content (SC) for shocked (S) animals, relative to naïve (N), 5, 15 and 45 min post training. (B) Relation of p65 in SDS-treated membranes (SDSm) to IκB in triton extract (TE) for shocked (S) animals, relative to naïve (N), 5, 15 and 45 min post training. Protein quantities where controlled as described in 'Western blots' section. $^{*}p < 0.05$.

the amount of membrane associated NF-kappa B at the expense of the free NF-kappa B. This is clearly evidenced in Fig. 6A where the p65 SDSm/p65 SC relation shows a remarkably high increase relative to N animals. The p65 SDSm/p65 SC relation remains unchanged between groups at 15 and 45 min post training, implicating a small temporal window where NF-kappa B changes its localization. This short window of close interaction with synaptic membranes postulates the possibility of a local signaling role for further changes during consolidation.

On the other hand, the presence of IκBα at the membranes of synaptosomes is noteworthy. Not all IκBα is as strongly bound to membranes as p65, given that a fraction can be removed with triton present in the TE buffer (Fig. 2). Nevertheless its role at the synapse may be central in regulating the activity of NF-kappa B. The amount of IκBα at the membranes increases together with p65, suggesting that at this time the transcription factor associates to the membranes with its inhibitor (Fig. 5C).

It is only at 15 min post-training that the amount of IκBα decreases in the membranes (TE) and in relation to the amount of p65 SDSm (Figs. 5C and 6B). This suggests that NF-kappa B translocates to the membranes after the synapse is activated through the training-related synaptic activity, but in association with its inhibitor. At the same time, the pool of synaptic NF-kappa B that does not translocate to the membrane is freed from IκBα and may be involved in the activation or recruiting of other proteins *in situ*, or may be itself be prepared for transport to the nucleus. Later, 15 min post training at the membranes, the p65/IκBα relation augments relative to N animals, suggesting a membrane NF-kappa B activation (Fig. 6B). The consequences of the dynamics of this transcription factor probably involve labeling and preparing the synapse for remodeling and synaptic plasticity changes, acting in the memory consolidation process.

We hypothesize that during this anchoring period the equilibrium of posttranslational modifications of synaptic proteins is altered. In the nucleus, one way in which NF-kappa B is capable of inducing post-translational modifications of proteins is by recruiting acetyltransferases (Zhong et al., 1998). The acetyltransferase ARD1/NAA10 is present in dendrites, regulating its development (Ohkawa et al., 2008). ARD1 is able to associate with p65 (Xu et al., 2012). Such an interaction is one possible way in which NF-kappa B could achieve its local role at the synapse during memory consolidation.

Regarding a local role of the transcription factor, it has been reported that the synaptic plasticity changes that required NF-kappa B did not occur when the transactivation and DNA binding domain were mutated (Boersma et al., 2011), suggesting that the role of the transcription factor in synaptic changes always requires a nuclear function. An alternative interpretation is that NF-kappa B local function relays in its capacity to interact with other molecules other than DNA, and that the modifications of the protein affect its interaction capacity with synaptic substrates (Huxford et al., 1998; Malek et al., 1998).

CONCLUSIONS

Strong evidences indicate that the role of NF-kappa B in neuronal plasticity and memory is crucial. The importance of this transcription factor in the expressions of proteins involved in memory storage is widely reported and represents a conserved mechanism in vertebrates and invertebrates (Romano et al., 2006; Kaltschmidt and Kaltschmidt, 2009). The results presented here suggest that NF-kappa B may have a new local synaptic role during consolidation. We also show the first evidence of a novel localization for NF-kappa B bound to membranes of hippocampal synaptosomes. This is also the first report showing NF-kappa B synaptic dynamics during consolidation of the inhibitory avoidance learning paradigm in mice.

CONFLICT OF INTEREST

Authors have no conflict of interest to declare.

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REFERENCES

Agranoff BW, Davis RE, Brink JJ (1966) Chemical studies on memory fixation in goldfish. Brain Res 1:303–309.

Alberini CM (2009) Transcription factors in long-term memory and synaptic plasticity. Physiol Rev 89:1.

Baeuerle PA, Baltimore D (1988) Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-kappa B transcription factor. Cell 53:211–217.

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. http://dx.doi.org/10.1523/JNEUROSCI.4430-07.2007.

Boccia MM, Acosta GB, Blake MG, Baratti CM (2004) Memory consolidation and reconsolidation of an inhibitory avoidance response in mice: effects of i.c.v. injections of hemicholinium-3. Neuroscience 124:735–741. http://dx.doi.org/10.1016/j.neuroscience.2004.01.001.

Boersma MCH, Dresselhaus EC, De Biase LM, Mihalas AB, Bergles DE, Meffert MK (2011) A requirement for nuclear factor-kappaB in developmental and plasticity-associated synaptogenesis. J Neurosci 31:5414–5425. http://dx.doi.org/10.1523/JNEUROSCI.2456-10.2011.

Davis HP, Squire LR (1984) Protein synthesis and memory: a review. Psychol Bull 96:518–559.

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. http://dx.doi.org/10.1111/j.1460-9568.2005.04126.x.

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, Romano A, Routtenberg A (2004) Transcription factor NF-kappaB activation after in vivo perforant path LTP in mouse hippocampus. Hippocampus 14:677–683. http://dx.doi.org/10.1002/hipo.20020.

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.

Heckscher ES, Fetter RD, Marek KW, Albin SD, Davis GW (2007) NF-kappaB, IkappaB, and IRAK control glutamate receptor density at the Drosophila NMJ. Neuron 55:859–873. http://dx.doi.org/10.1016/j.neuron.2007.08.005.

Huxford T, Huang DB, Malek S, Ghosh G (1998) The crystal structure of the IkappaBalpha/NF-kappaB complex reveals mechanisms of NF-kappaB inactivation. Cell 95(6):759–770.

Kaltschmidt B, Kaltschmidt C (2000) Constitutive NF-kappa B activity is modulated via neuron-astroglia interaction. Exp Brain Res 130:100–104.

Kaltschmidt B, Kaltschmidt C (2001) DNA array analysis of the developing rat cerebellum: transforming growth factor-beta2 inhibits constitutively activated NF-kappaB in granule neurons. Mech Dev 101:11–19.

Kaltschmidt B, Kaltschmidt C (2009) NF-kappaB in the nervous system. Cold Spring Harb Perspect Biol 1:a001271. http://dx.doi.org/10.1101/cshperspect.a001271.

Kaltschmidt C, Kaltschmidt B, Baeuerle PA (1993) Brain synapses contain inducible forms of the transcription factor NF-kappa B. Mech Dev 43:135–147.

Kaltschmidt C, Kaltschmidt B, Neumann H, Wekerle H, Baeuerle PA (1994) Constitutive NF-kappa B activity in neurons. Mol Cell Biol 14:3981–3992.

Landeira-Fernandez J, DeCola JP, Kim JJ, Fanselow MS (2006) Immediate shock deficit in fear conditioning: effects of shock manipulations. Behav Neurosci 120:873–879. http://dx.doi.org/10.1037/0735-7044.120.4.873.

Malek S, Huxford T, Ghosh G (1998) Ikappa B alpha functions through direct contacts with the nuclear localization signals and the DNA binding sequences of NF-kappaB. J Biol Chem 273(39):25427–25435.

Meberg PJ, Kinney WR, Valcourt EG, Routtenberg A (1996) Gene expression of the transcription factor NF-kappa B in hippocampus: regulation by synaptic activity. Brain Res Mol Brain Res 38:179–190.

Meffert MK, Baltimore D (2005) Physiological functions for brain NF-kappaB. Trends Neurosci 28:37–43. http://dx.doi.org/10.1016/j.tins.2004.11.002.

Meffert MK, Chang JM, Wiltgen BJ, Fanselow MS, Baltimore D (2003) NF-kappa B functions in synaptic signaling and behavior. Nat Neurosci 6:1072–1078. http://dx.doi.org/10.1038/nn1110.

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

Mindorff EN, O'Keefe DD, LabbÈ A, Yang JP, Ou Y, Yoshikawa S, van Meyel DJ (2007) A gain-of-function screen for genes that influence axon guidance identifies the NF-kappaB protein dorsal and reveals a requirement for the kinase Pelle in Drosophila photoreceptor axon targeting. Genetics 176:2247–2263. http://dx.doi.org/10.1534/genetics.107.072819.

Murata S, Usuda N, Okano A, Kobayashi S, Suzuki T (2000) Occurrence of a transcription factor, signal transducer and activators of transcription 3 (Stat3), in the postsynaptic density of the rat brain. Brain Res Mol Brain Res 78:80–90.

Nagy A, Delgado-Escueta AV (1984) Rapid preparation of synaptosomes from mammalian brain using nontoxic isoosmotic gradient material (Percoll). J Neurochem 43:1114–1123.

O'Sullivan NC, McGettigan PA, Sheridan GK, Pickering M, Conboy L, O'Connor JJ, Moynagh PN, Higgins DG, Regan CM, Murphy KJ (2007) Temporal change in gene expression in the rat dentate gyrus following passive avoidance learning. J Neurochem 101:1085–1098. http://dx.doi.org/10.1111/j.1471-4159.2006.04418.x.

Ohkawa N, Sugisaki S, Tokunaga E, Fujitani K, Hayasaka T, Setou M, Inokuchi K (2008) N-acetyltransferase ARD1-NAT1 regulates neuronal dendritic development. Genes Cells Devoted Mol Cell Mech 13:1171–1183. http://dx.doi.org/10.1111/j.1365-2443.2008.01235.x.

Oikawa K, Odero GL, Platt E, Neuendorff M, Hatherell A, Bernstein MJ, Albensi BC (2012) NF-κB p50 subunit knockout impairs late LTP and alters long term memory in the mouse hippocampus. BMC Neurosci 13:45. http://dx.doi.org/10.1186/1471-2202-13-45.

Rodrigues RJ, Alfaro TM, Rebola N, Oliveira CR, Cunha RA (2005) Co-localization and functional interaction between adenosine A(2A) and metabotropic group 5 receptors in glutamatergic nerve terminals of the rat striatum. J Neurochem 92:433–441. http://dx.doi.org/10.1111/j.1471-4159.2004.02887.x.

Romano A, Freudenthal R, Merlo E, Routtenberg A (2006) Evolutionarily-conserved role of the NF-kappaB transcription factor in neural plasticity and memory. Eur J Neurosci 24:1507–1516. http://dx.doi.org/10.1111/j.1460-9568.2006.05022.x.

Russo SJ, Wilkinson MB, Mazei-Robison MS, Dietz DM, Maze I, Krishnan V, Renthal W, Graham A, Birnbaum SG, Green TA, Robison B, Lesselyong A, Perrotti LI, BolaÒos CA, Kumar A, Clark MS, Neumaier JF, Neve RL, Bhakar AL, Barker PA, Nestler EJ (2009) Nuclear factor kappa B signaling regulates neuronal morphology and cocaine reward. J Neurosci 29:3529–3537. http://dx.doi.org/10.1523/JNEUROSCI.6173-08.2009.

Ryan MM, Ryan B, Kyrke-Smith M, Logan B, Tate WP, Abraham WC, Williams JM (2012) Temporal profiling of gene networks associated with the late phase of long-term potentiation in vivo. PloS One 7:e40538. http://dx.doi.org/10.1371/journal.pone.0040538.

Schmeisser MJ, Baumann B, Johannsen S, Vindedal GF, Jensen V, Hvalby OC, Sprengel R, Seither J, Maqbool A, Magnutzki A, Lattke M, Oswald F, Boeckers TM, Wirth T (2012) IκB kinase/nuclear factor κB-dependent insulin-like growth factor 2 (Igf2) expression regulates synapse formation and spine maturation via Igf2 receptor signaling. J Neurosci 32:5688–5703. http://dx.doi.org/10.1523/JNEUROSCI.0111-12.2012.

Sgambato V, Vanhoutte P, PagËs C, Rogard M, Hipskind R, Besson MJ, Caboche J (1998) In vivo expression and regulation of Elk-1,

a target of the extracellular-regulated kinase signaling pathway, in the adult rat brain. J Neurosci 18:214–226.

Sulejczak D, Skup M (2000) Axoplasmic localisation of the NF kappa B p65 subunit in the rat brain. Acta Neurobiol Exp (Warsz) 60:217.

Suzuki T, Mitake S, Okumura-Noji K, Yang JP, Fujii T, Okamoto T (1997) Presence of NF-kappaB-like and IkappaB-like immunoreactivities in postsynaptic densities. Neuroreport 8:2931–2935.

Suzuki T, Usuda N, Ishiguro H, Mitake S, Nagatsu T, Okumura-Noji K (1998) Occurrence of a transcription factor, cAMP response element-binding protein (CREB), in the postsynaptic sites of the brain. Mol Brain Res 61:69–77. http://dx.doi.org/10.1016/S0169-328X(98)00199-5.

Tomita S, Fujita T, Kirino Y, Suzuki T (2000) PDZ domain-dependent suppression of NF-kappaB/p65-induced Abeta42 production by a neuron-specific X11-like protein. J Biol Chem 275:13056–13060.

Wellmann H, Kaltschmidt B, Kaltschmidt C (2001) Retrograde transport of transcription factor NF-kappa B in living neurons. J Biol Chem 276:11821–11829. http://dx.doi.org/10.1074/jbc.M009253200.

Xu H, Jiang B, Meng L, Ren T, Zeng Y, Wu J, Qu L, Shou C (2012) N-α-acetyltransferase 10 protein inhibits apoptosis through RelA/p65-regulated MCL1 expression. Carcinogenesis 33:1193–1202. http://dx.doi.org/10.1093/carcin/bgs144.

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.

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