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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.

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Abbreviations: ANOVA, analysis of variance; CREB, cAMP response element-binding protein; EDTA, ethylenediaminetetraacetic acid; EMSA, electrophoretic mobility shift assay; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; iS, immediate shock; LTP, long-term potentiation; NLS, nuclear localization signal; PFA, paraformaldehyde; PO, phosphate; ROD, relative optical density; SC, synaptosomal content; SDS, sodium dodecyl sulfate; SDSm, SDS-treated membranes; TE, triton extract; WB, Western blots.

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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 cAMP response element-binding protein (CREB), NF-kappa B, AP1, 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 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 DTT, 1 g/ml pepstatin A, 10 g/ml leupeptin, 0.5 mM 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

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