Methods of detection of the transcription factor NF-κB in rat hippocampal slices

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Abstract

The hippocampus is one of the most studied sites for understanding the cellular and molecular mechanisms underlying long-term potentiation (LTP) and long-term depression (LTD), mechanisms believed to underlie the formation and storage of memories. The early-phases of LTP and LTD have been most intensively studied and have been shown to involve the activation of several kinases and phosphatases, respectively. The factors involved in the later stages have largely yet to be elucidated. We have focused our attention on the transcription factor NF-κB as a possible factor involved in such late-phase processes, and have developed both immunocytochemistry and electrophoretic mobility shift assay (EMSA) to measure the activated forms of this factor. This is important as many of the studies in this area are performed in vitro and to our knowledge quantitative assessment has not previously being deemed feasible in slice work. The pro-inflammatory cytokines TNF-α and IL-1β both led to pronounced nuclear activation of NF-κB in the dentate granule cells as demonstrated by immunostaining and EMSA, respectively. Electrophysiological measurements taken from slices treated with TNF-α showed that it inhibited LTP (field excitatory post-synaptic potentials (fEPSP) $116 \pm 10\%$, $n = 9$, 60 min post-tetanus compared to control fEPSP $185 \pm 9\%$, $n = 6$; $P < 0.001$). The neurotransmitter L-glutamate also led to activation of NF-κB and electrophysiology recordings showed a small but sustained increase in synaptic transmission (fEPSP $106 \pm 12\%$, 30 min post-drug). These methods provide valuable tools to forward our understanding of the role of NF-κB in plasticity as well as in many neurological disorders being mimicked by in vitro studies. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

The transcription factor nuclear factor κB (NF-κB) is now being recognised as a functionally important component in mechanisms inherent to neuronal plasticity (Kaltschmidt et al., 1993; Guerrini et al., 1995; Perez-Otano et al., 1996; Terai et al., 1996; Albensi and Mattson, 2000; Freudenthal and Romano, 2000). NF-κB has been shown to be present in both inducible and constitutive forms in cerebellar granule cells and in hippocampal pyramidal neurons (Guerrini et al., 1995; Kaltschmidt et al., 1995). In its inactive form, NF-κB consists of a three-subunit complex consisting of two subunits of the Rel transcription factor family (e.g. 50 kDa (p50) and 65 kDa (p65)) and an inhibitory subunit, called IκB. Activation of NF-κB depends on the dissociation of IκB from the heterotrimeric complex via serine phosphorylation and subsequent proteosomal proteolysis of the IκB subunit. This then allows the active NF-κB heterodimer to translocate to the nucleus where it can activate its downstream target genes. NF-κB is activated in response to various intercellular signals, including the cytokines interleukin-1 and tumour necrosis factor-α, neurotrophic factors, and neurotransmitters such as glutamate (Mattson et al., 2000).

Mechanisms underlying long-term potentiation (LTP), in conjunction with the mechanisms of long-term depression (LTD) are thought to be responsible for information storage by the hippocampus (Madison et al., 1991; Bliss and Collingridge, 1993; Malenka, 1994). Both processes can be induced by electrical or chemical means, high frequency stimulation (HFS) causes LTP and low frequency LTD. LTP results in a long-lasting increase in synaptic transmission, whereas LTD results

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in a persistent functional decrease in synaptic activity. Emerging data is implicating NF-κB as a key component in LTD (Albensi and Mattson, 2000) and previous work has shown a role for NF-κB in LTP (Meberg et al., 1996; Freudenthal et al., 1998).

The present work describes two experimental approaches that may be used to monitor the activation status of NF-κB in the brain. The first employs immunocytochemistry, which facilitates the localisation of both latent and active NF-κB in the brain and the second exploits the electrophoretic mobility shift assay (EMSA), a technique which may be used to quantify the levels of activated NF-κB in the brain.

2. Methodology

2.1. Stimulation of hippocampal slices

Transverse hippocampal slices (350 μm) were prepared by standard methods from adult male Wistar rats (50–80 g) as previously described (O'Connor et al., 1994). Slices were equilibrated in oxygenated artificial cerebrospinal fluid (aCSF) for a 60-min period before transfer to a perfusion chamber held at 30 °C for experimentation. Extracellular recordings of field excitatory post-synaptic potentials (fEPSP) were elicited by stimulation of the medial perforant path of the dentate gyrus by a monopolar glass electrode at a frequency of 0.05 Hz. Responses were recorded by a glass electrode placed in the middle third of the molecular layer in the presence of the GABA$_A$ receptor antagonist picrotoxin (100 μM; Sigma) and stimulus strength was adjusted to give a response 35% of maximal. Stable baseline recordings were made before application of drugs. LTP was induced by HFS consisting of eight pulses at 200 Hz at 2 s intervals at a stimulus strength corresponding to 75% of maximal. Recordings were analysed off-line using the Strathclyde electrophysiology software (J. Dempster, Edinburgh, UK). All results are expressed as mean ± S.E.M. Data were analysed statistically using the Student's *t*-test.

2.2. Drugs used in analysis

Recombinant rat TNF-α (R&D systems) and recombinant human interleukin-1β (IL-1β) (R&D systems) were prepared in bovine serum albumin (BSA; Sigma). Final BSA concentration was 10^{-5} w/v. L-glutamate (Sigma) was prepared in double distilled H$_2$O. Stable baseline recordings (20 min) were obtained before application of drugs. Both TNF-α (4 ng/ml) and IL-1β (10 ng/ml) were bath-applied for 20 min pre-tetanus, L-glutamate (100 μM) was perfused for 6 min to the hippocampal slice followed by a 5 min washout. For immunostaining/EMSA electrophysiological responses were followed for 30 min post-tetanus/post-drug wash-out before removal of the slice from the perfusion chamber for processing for activated NF-κB. For TNF-α treated slices, responses were followed for 60 min post-tetanus to determine the effect of drug on synaptic transmission, HFS was given to L-glutamate treated slices at 30 min post-drug, and the response followed for 30 min.

2.3. Immunohistochemistry analysis of tissue sections

Following electrophysiology, the hippocampus was dissected out and immersed in 10% formol-saline for overnight fixation. The sections were dehydrated through a series of graded alcohols, cleared in chloroform for 30 min and then impregnated with paraplast plus by immersion in a molten wax bath overnight. Sections of 8 μm were cut on a microtome and mounted onto silanized coated slides. Active NF-κB was probed by using an anti-p65 antibody (Mouse IgG 3 subclass, 10 μg/ml; Chemicon International Ltd., UK), which specifically recognises an epitope normally masked by IκB. Thus immunoreactivity provides an index of NF-κB activation. The binding of the antibody to p65 was visualised using the Vectastain Elite ABC kit (Vector Labs, CA, USA) according to the manufacturer's recommendations with the exception of an additional step. Microwave treatment of tissue sections in citrate buffer (pH 6.0) for 10 min was necessary for detection of NF-κB in paraffin sections. The chromagen diaminobenzidine tetrahydrochloride (DAB, Sigma) was used which produces a brownish precipitate, the counterstain used was Haematoxylin, which stains nuclei blue. For Control, a mouse IgG antibody (Chemicon International Ltd., UK) was used as the primary antibody, in such slides no brown staining (corresponding to NF-κB) was seen, only that of haematoxylin. Slides were analysed on a microscope coupled with axiovision software.

2.4. Preparation of subcellular fractions from rat hippocampal slices

Subcellular fractions were prepared with modifications to previously described methods (Osborn et al., 1989; Freudenthal and Romano, 2000). The dentate gyrus region of the rat hippocampus was dissected out following electrophysiology and frozen immediately at −70 °C in 50 μl of phosphate buffered saline. Frozen hippocampal slices (× 3) were pooled and then re-suspended in 500 μl of hypotonic buffer A (10 mM Hepes, pH 7.9, containing 1.5 mM of MgCl$_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, Pepstatin A, 1 μg/ml and Leupeptin, 10 μg/ml). Cells were pelleted by centrifugation at 6000 × *g* for 3 min, the supernatant removed and re-suspended in 250 μl of buffer A

containing 0.1% (v/v) of Nonidet P-40. Cell lysis was facilitated by homogenizing the tissue with a pestle on ice for 15 min. Lysates were then centrifuged at 11 000 × *g* for 15 min. The pellets were re-suspended in 25 µl of buffer B (20 mM Hepes, pH 7.9, 0.5 M KCl, 1.5 mM MgCl₂, 0.4 mM EDTA, 0.5 mM DTT, 50% (w/v) of glycerol, 0.5 mM PMSF, Pepstatin A, 1 µg/ml and Leupeptin, 10 µg/ml), and incubated on ice for 15 min. Finally, a centrifugation step of 15 min at 14 000 × *g* was performed, and the supernatants which represented the nuclear extracts were removed and stored at −70 °C.

2.5. Electrophoretic mobility shift assay

The nuclear extracts were first diluted in buffer to give the appropriate DNA-protein binding conditions (20 mM Hepes, pH 7.9, 0.1 M KCl, 0.4 mM EDTA, 0.5 mM DTT, 20% glycerol). Protein concentrations were then determined by the Bradford assay (Bradford, 1976). Nuclear extracts (5–10 µg) were incubated with 50 000 cpm of a 22-bp oligonucleotide containing the NF-κB consensus sequence (5′-AGTTGAGGGGACTTTCC-CAGGC-3′) (Promega, Madison, WI) that had been previously labeled with $[\gamma\text{-}^{32}P]$ ATP (10 mCi/mmol) by T4 polynucleotide kinase. Incubations were performed for 30 min at room temperature in the presence of 2 µg of poly (dI-dC) and 10 mM Tris–HCl, pH 7.5 containing 0.1 M NaCl, 1 mM EDTA, 5 mM DTT, 4% (w/v) of glycerol, and 0.1 mg/ml nuclease free BSA. Samples were run on native 4% (w/v) polyacrylamide gels that were subsequently dried and autoradiographed.

3. Results and discussion

The transcription factor NF-κB is a potent transcriptional activator that resides in a latent form in the cytoplasm of cerebellar granule cells and neuronal cells of the hippocampus, and is activated in response to ionotropic glutamate receptor activation through both pharmacological and electrical means (Guerrini et al., 1995; Meberg et al., 1996). Indeed, accumulating evidence suggests that NF-κB may be involved in the memory paradigms of both LTP (Meberg et al., 1996) and LTD (Albensi and Mattson, 2000) since increased levels have been detected following tetanically-induced LTP and inhibitors of NF-κB have been shown to inhibit the induction of LTD in the hippocampus. These findings emphasize the importance of developing methods to detect activated forms of this transcription factor.

Immunocytochemistry provided a qualitative approach to detect activated levels of NF-κB in hippocampal slices. In order to detect NF-κB in paraffin-sections we found that microwave pre-treatment was essential. This procedure has previously been shown to enhance immunoreactivity in paraffin sections for a wide variety of antibodies (Shi et al., 1991). It remains unclear how this procedure improves antigen detection. In addition to the chemical reaction with reagents such as citrate, it may be the major effect of the microwave pre-treatment is primarily due to the heat generated (Suurmeijer and Boon, 1993).

The result of this procedure meant that the antibody no longer bound specifically to the activated form of NF-κB but also detected the cytosolic-located IκB-NF-κB bound complex (Fig. 1A and C). The images clearly show that NF-κB is present at high levels in the cytoplasm of the dentate granule cells of the hippocampus. Immunostaining at the single cell level was used to detect activated NF-κB in hippocampal slices that had been treated with L-glutamate (Fig. 1B), the major excitatory neurotransmitter in the brain and the pro-inflammatory cytokine TNF-α (Fig. 1D and F). As can be seen from the image L-glutamate led to activation of NF-κB in the dentate granule cells of the hippocampus (indicated by double arrows) when compared to control slice (Fig. 1A). Similar observations have been made in cerebellar granule cells, using the glutamate receptor agonists L-glutamate (Guerrini et al., 1995), kainate and N-methyl-D-asparate (NMDA), as well as with membrane depolarization by KCl (Kaltschmidt et al., 1995).

Electrophysiological measurements were also made from hippocampal slices perfused with L-glutamate (Fig. 2). A small but sustained increase in the magnitude of the fEPSP was seen (fEPSP $106 \pm 12\%$ 30 min post-drug, $n = 4$; not significant). This is in contrast to a study by Collins and Davies (1994) in which a large and sustained increase in the magnitude of the fEPSP was seen following bath application of L-glutamate. However these differences could be attributed to the fact that field recordings were taken from the CA1 as opposed to the dentate gyrus region in the present study. HFS induced LTP in the glutamate treated slices to values similar to control.

We demonstrate by immunocytochemistry that treatment of hippocampal slices with TNF-α leads to pronounced nuclear staining of NF-κB in the dentate granule cells (Fig. 1D and F) and the pyramidal CA1 neurons (data not shown) of the rat hippocampus. Levels of TNF-α are found elevated in many neurological conditions in the brain including Alzheimer's and Parkinson's disease, stroke and ischemia, conditions known to be associated with memory deficits (Fiore et al., 1996; Mattson et al., 1997; Zaremba and Losy, 2001). Electrophysiological measurements taken from slices treated with TNF-α prior to HFS showed that it significantly inhibited LTP in the dentate gyrus (fEPSP $116 \pm 10\%$ 60 min post-tetanus, $n = 9$ compared to controls fEPSP $185 \pm 9\%$, $n = 6$; $P < 0.001$). Similar findings of TNF-α inhibition of LTP have previously been reported (Tancredi et al., 1992; Cunningham et al.,
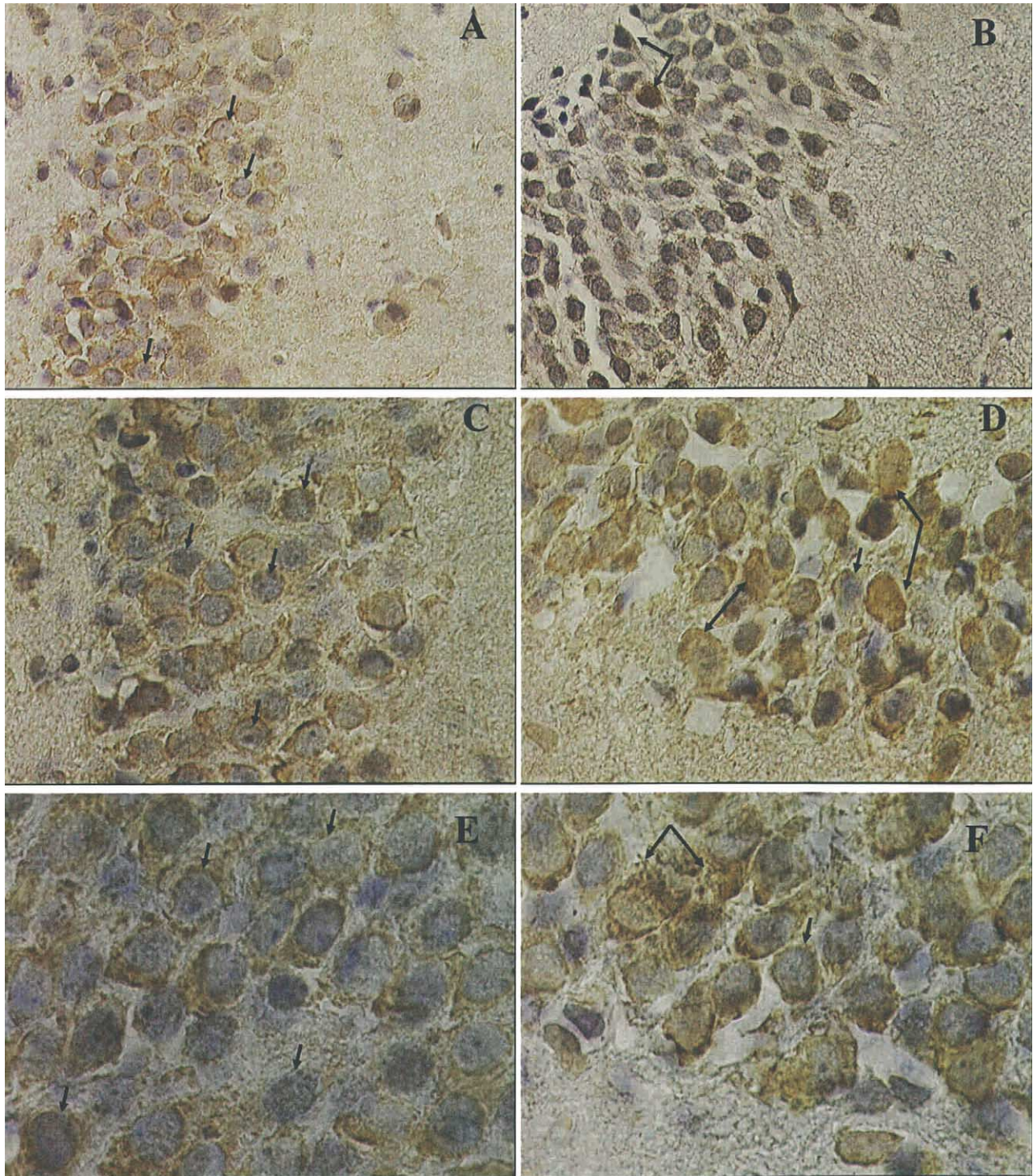
Fig. 1. Immunostaining for the transcription factor NF-κB in the dentate gyrus region of the rat hippocampus. The brown staining given by diaminobenzidine represents positive immunostaining for NF-κB, the blue is that of haematoxylin, a counterstain which stains the nuclear chromatin blue in colour. (A) Represents an untreated slice, no obvious nuclear staining is apparent. Single arrows denote granule cells with no apparent activated NF-κB. Cytosolic staining for NF-κB is clearly seen, this is the result of the microwave treatment used in the procedure ($\times 40$ magnification). (B) L-glutamate (100 μM) was perfused through the slice for 6 min and the slice was removed at 30 min post-drug washout. Nuclear immunostaining is indicated by double arrows (D). TNF-α (4 ng/ml) was perfused through the slice for 20 min pre-tetanus and the slice removed at 30 min post-tetanus. Pronounced nuclear staining for activated NF-κB is seen (double arrows, $\times 60$ magnification) compared to control slice (C). (F) TNF-α treated slice at higher magnification ($\times 100$) shows increased nuclear staining compared to control slice (E). Immunostaining for each condition was repeated ($n = 3$).
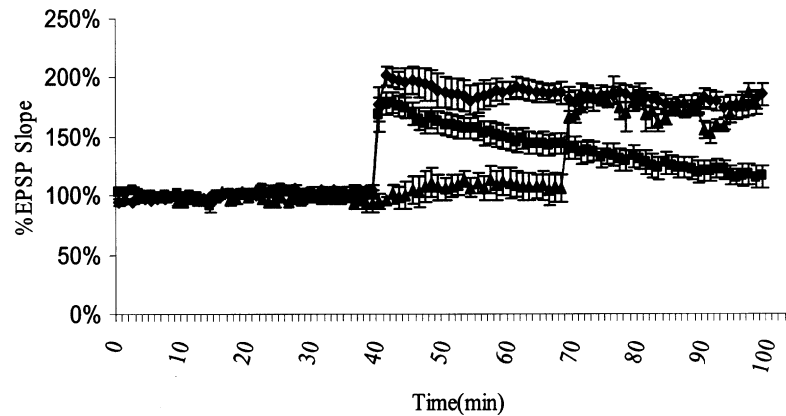
Fig. 2. TNF-α leads to an inhibition of LTP. Slices were pre-incubated with TNF-α (4 ng/ml) prior to HFS and it was shown to significantly inhibit LTP (fEPSP 116±10%, ■; $n = 9$, at 60 min post-tetanus compared to controls 185±9%, ▶; $n = 6$; $P < 0.001$). L-glutamate (100 μM) was perfused for 6 min and led to a small but sustained increase in synaptic transmission (fEPSP 106±12%, ▲; $n = 4$; not significant). At 30 min post-drug HFS was given and the potentiated responses were similar to controls.

1996). The mechanism underlying this process of inhibition has not to date been addressed, but in the present study we have illustrated that TNF-α promotes strong activation of NF-κB in the dentate granule cells which may suggest it has some regulatory role in this process.

The EMSA technique provides for a quantitative assessment of nuclear NF-κB levels and although developed for cell lines it had not been modified to allow for NF-κB detection from hippocampal slices. Slight modifications to previously published methodologies enabled detection of NF-κB from hippocampal slices (Osborn et al., 1989; Freudenthal and Romano, 2000). A low-salt buffer with detergent followed by a high-salt nuclear extraction protocol was employed. Nuclear NF-κB was detectable from untreated hippocampal slices (Fig. 3) and this represents constitutive NF-κB. In agreement with Stephenson et al. (2000) the EMSA was found to provide for a more sensitive index of nuclear NF-κB activity when compared to immunocytochemistry. Constitutive NF-κB has previously been detected in the hippocampus through immunostaining using indirect immunofluorescence (Kaltschmidt et al., 1994), which is more sensitive than light microscopy. Application of IL-1β at pathophysiological concentrations (10 ng/ml) to hippocampal slices led to increased levels of activated NF-κB when compared to control slices (Fig. 3). This pro-inflammatory cytokine has previously been shown to cause an inhibition of LTP in the dentate gyrus region (Cunningham et al., 1996; Coogan et al., 1999) and CA1 region (Bellinger et al., 1993) of the rat hippocampus. To our knowledge, this is the first documentation of the use of the EMSA technique to detect activated nuclear NF-κB from hippocampal slices derived from in vitro work. This technique may be used to quantify levels of activated NF-κB in slice work through densitometric analysis of

the NF-κB-complexed retarded bands seen from the autoradiographs.

Albensi and Mattson (2000) have suggested that NF-κB may be involved in LTD, to further investigate its possible physiological role in LTD these techniques will be most useful in the context of chemically induced metabotropic glutamate receptor-LTD (chem-LTD), using as agonists 1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD) in the dentate gyrus and (RS)-3,5-dihydroxyphenylglycine in the CA1 region of the hippocampus. The role of metabotropic glutamate receptors (mGluR) in hippocampal LTD has been a recent focus of attention. Previously it was thought that LTD in the CA1 region of the adult-rat hippocampus
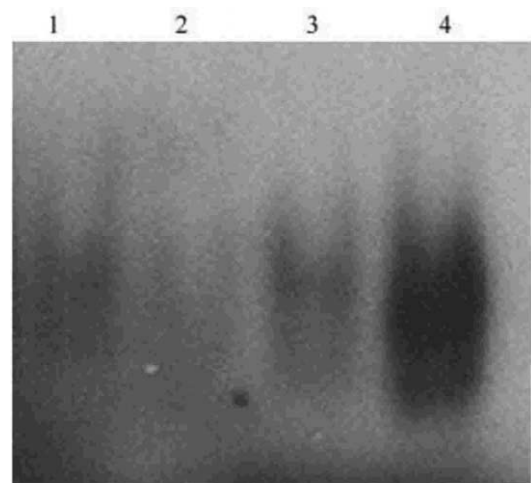

Fig. 3. EMSA analysis of activated NF-κB from hippocampal slices. Lanes 1–3 inclusive represent pooled nuclear extracts derived from 3 hippocampal slices. Nuclear extracts from IL-1β (10 ng/ml) treated hippocampal slices (lane 1, 5 μg protein), untreated hippocampal slices (lane 2, 5 μg protein), IL-1β (10 ng/ml) treated hippocampal slices (lane 3, 8 μg protein) and 132N1 human astrocytoma cell line (lane 4, 8 μg protein and positive control for detection system) were generated and subjected to EMSA analysis. The bands represent the retarded NF-κB-DNA complexes. EMSA analysis was repeated and reproducible findings were observed ($n = 3$).

was solely NMDA-dependent. However, an-mGluR dependent form of LTD is expressed in the CA1 region of neonates and in the dentate gyrus of the adult-rat hippocampus (Bolshakov and Siegelbaum, 1994; O'Mara et al., 1995). The biochemical cascades involved in mGluR-dependent LTD, unlike NMDA-dependent LTD and LTP, have been less extensively studied. Induction of chem-LTD is achieved by perfusing the agonist throughout the hippocampal slice and as such a larger population of neurons are activated as opposed to tetanic-LTD, in which biochemical analysis is hampered by the fact that purification methods yield low-levels of stimulated cells. Evaluation of the contribution of NF-κB to the inhibitory effects of TNF on LTP will also be facilitated by the application of these techniques. In summary, these two methods will be of great value in increasing our understanding of the physiological and pathological roles of NF-κB in brain.

Acknowledgements

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