Retrograde Transport of Transcription Factor NF-κB in Living Neurons*

 Henning Wellmann†, Barbara Kaltschmidt‡, and Christian Kaltschmidt§

From the Institut für Neurobiochemie, Universität Witten/Herdecke, Stockumer Straße 10, D-58448 Witten, Germany

The mechanism by which signals such as those produced by glutamate are transferred to the nucleus may involve direct transport of an activated transcription factor to trigger long-term transcriptional changes. Ionotropic glutamate receptor activation or depolarization activates transcription factor NF-κB and leads to translocation of NF-κB from the cytoplasm to the nucleus. We investigated the dynamics of NF-κB translocation in living neurons by tracing the NF-κB subunit RelA (p65) with jellyfish green fluorescent protein. We found that green fluorescent protein-RelA was located in either the nucleus or cytoplasm and neurites, depending on the coexpression of the cognate inhibitor of NF-κB, IκB-α. Stimulation with glutamate, kainate, or potassium chloride resulted in a redistribution of NF-κB from neurites to the nucleus. This transport depended on an intact nuclear localization signal on RelA. Thus, in addition to its role as a transcription factor, NF-κB may be a signal transducer, transmitting transient glutamatergic signals from distant sites to the nucleus.

For short-term signals (e.g. synaptic activity) to trigger long-term changes, differential gene expression is required (1–4). This raises the issue of determining the signaling systems that translate short-term signals to changes in gene expression. Two possible mechanisms are a signal transducer that is retrograde-transported and that subsequently transmits information to a transcription factor, or a transcription factor that independently can fulfill both functions. There is evidence for each mechanism in different systems. With regard to the former mechanism, the nerve growth factor TrkA receptor functions in complex with nerve growth factor as a retrograde signal transducer, connecting extracellular signals at distant sites with nuclear gene expression via phosphorylation of the transcription factor CREB (5). NF-κB is a transcription factor that may act via the latter mechanism. Recently, it was reported that potentiated synapses are marked with a molecular tag that may sequester relevant proteins necessary for changes in gene expression (3). NF-κB is present in synaptic compartments (6–10) and rapidly activated independent of protein synthesis (11), making this factor a likely candidate as a synaptic tag (3). NF-κB is present in many neuronal cell types (for review, see Ref. 12) and, in neurons, can be constitutively active (13–15) or activated by a variety of stimuli such as glutamate (7, 16, 17) and amyloid β-peptide (18, 19). A physiological role was defined for NF-κB in neuroprotection against amyloid β-peptide (19) and oxidative stress and glutamate (20, 21). Depending on the context, NF-κB might also be involved in neurodegeneration (22).

To date, five mammalian NF-κB DNA-binding subunits are known: p50, p52, RelA (p65), c-Rel, and RelB (23, 24). The important role of the transactivating RelA subunit is apparent in re/α knockout mice, for which there is a high rate of embryonic mortality. Inhibitory subunits are IκB-α, IκB-β, IκB-γ (p105), IκB-δ (p100), and IκB-ε (25). Within the nervous system, heteromeric NF-κB is most frequently composed of two DNA-binding subunits (e.g. p50 or RelA) that either are constitutively active or form a complex with the inhibitory subunit IκB-α (6, 7, 13, 14, 16, 18, 26). Interactive ankyrin repeats of IκB-α can physically block the nuclear localization signal (NLS) on the RelA subunit (27, 28), preventing transport of the complex into the nucleus. Activation of NF-κB results in the degradation of IκB-α, which in turn exposes the NLS, allowing NF-κB to be transported into the nucleus (29). Thus, the specific post-translational regulation of NF-κB and its synaptic distribution support the idea that NF-κB functions both as a transcription factor in the nucleus, where it can function as a molecular switch for turning on gene expression, and as an immediate retrograde signal transducer, which unifies signal perception at distant sites (dendrites, axons, and synapses) (6, 12, 30). It is not known, however, if NF-κB undergoes retrograde transport upon activation.

In this study, we examined whether activated NF-κB RelA was transported from distant sites (neurites) to the nucleus in living cells using jellyfish green fluorescent protein (GFP) fusion technology to attach a fluorescent tag to the RelA subunit of NF-κB. To analyze the transport of NF-κB, a fusion protein was constructed that consisted of RelA and a GFP mutant optimized for maximal light emission (EGFP). This new technique offers the opportunity to image both the distribution and interactions of the protein in living cells. In contrast to intracellular antibody staining, using this technique, living cells can be observed over time, and small structures can be labeled, e.g. the label in dendritic spines can be greatly enhanced following overexpression of the GFP protein (31).

EXPERIMENTAL PROCEDURES

Cell Culture—Hippocampal neurons were cultured from embryonic day 17 or 18 rats as described by Banker and Cowan (32) and detailed by de Hoop et al. (33). One day prior to preparation of the hippocampal neurons, neurobasal medium (Life Technologies, Inc.) supplemented with B27 (1:50; Life Technologies, Inc.) and 0.5 mm l-glutamine was conditioned by astrocyte co-culture. Dissected hippocampi were treated
with trypsin (2 mg/ml; Sigma) in Hanks' balanced saline solution without calcium or magnesium and then with soybean trypsin inhibitor (1 mg/ml). Neurons were dissociated by strokes with a fire-polished Pasteur pipette and were suspended in minimal essential medium with Earle's salts containing 1 mM pyruvate, 26 mM NaHCO3, 2 mM L-glutamate, 20 mM KCl, and 10% heat-inactivated horse serum (2 × 106 cells/cm²) were plated on glass coverslips coated with polylysin (1:1000 in borate buffer, pH 7.2; Sigma). After 3 h, the cells adhered and were moved to astrocyte co-cultures with conditioned neurobasal medium. Biostatic experiments were performed after neurons had been maintained in culture for at least 7 days. At this age, the cultures contained mainly N′-methyl-n-aspartic acid type synapses clustered in varicosities (34).

HEK 293 cells (American Type Culture Collection) were seeded on glass coverslips (106 cells/cm²) in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 unit/ml penicillin, and 0.1 mg/ml streptomycin. The following day, calcium phosphate transfection was performed as described previously (26); and 24 h later, cells were fixed with 4% paraformaldehyde. Luciferase assays were performed as described previously (14), and standardization was performed using Renilla reniformis luciferase together with Photoris pyralis luciferase (Dual-Luciferase, Promega, Mannheim, Germany) as suggested by the manufacturer.

Immunostaining—On day 8 in culture, hippocampal neurons were treated with a 1% Triton-X buffer (200 or 500 μg/ml) for 5 min or 100 μg/ml 1.61 glutaraldehyde-conditioned medium for 5 min. After two washing steps with neurobasal medium, cells were incubated at 37°C for 1, 1.5, or 2 h. The cells were then fixed for 2 min in ethanol and for 5 min in 3.7% formaldehyde. After washing with phosphate-buffered saline, the cells were incubated in 5% goat serum for 30 min at room temperature, followed by two 5-min washes with phosphate-buffered saline. Cells were then incubated with anti-RelA monoclonal antibody (1:50 dilution; Roche Molecular Biochemicals, Mannheim). Anti-RelA antibody was detected by Cy3-conjugated anti-mouse IgG (1:400 dilution; Dianova, Hamburg, Germany); staining was performed subsequently; and detection was performed when incubation with the next primary antibody was started. Cells incubated with secondary antibodies, but without primary antibodies, were used as a control. The state of RelA activation in these cultures was tested using the anti-RelA monoclonal antibody because this antibody is commercially available and is specific for the activated form of the RelA subunit (26). Immunoreactivity with this antibody is detectable only after the activation of NF-κB (16, 18, 35, 36).

Plasmids—A pcDNA3 expression vector (Invitrogen, Leek, Netherlands), a pEGFP-1 vector (CLONTECH, Palo Alto, CA), a cytomegalovirus promoter-Renal expression vector (37), and a cytomegavirustransformed human IκB vector (38) were used. A pcDNA3-EGFP expression plasmid was generated using BamHI/NorI restriction of pEGFP-1 and sticky-end ligation of the purified fragment into pcDNA3. The pcDNA3-pEGFP-RelA expression plasmid was constructed by recombinant polymerase chain reaction using PfuTaq DNA polymerases (Expand High Fidelity PCR system, Roche Molecular Biochemicals). pEGFP-1 was used as a template for PCR and the first 30 base pairs from 5′ to 3′ of the EGFP fragment, with the pCMV-RelA fragment carried a stop codon and a NLS of the EGFP fragment, with the pCMV-RelA fragment. This strategy was chosen to overlap with the 3′ end of the RelA fragment, but without TA1 and TA2 (37), was amplified using a 29-base pair primer 3′-end of the RelA fragment and a pCMV-RelA fragment (587 bp). This amplified EGFP fragment (679 base pairs) was cloned into the promoter a GFP fusion containing RelA with its nuclear localization.

Western Blotting—Plasmids—A pcDNA3 expression vector (Invitrogen, Leek, Netherlands), a pEGFP-1 vector (CLONTECH, Palo Alto, CA), a cytomegalovirus promoter-Renal expression vector (37), and a cytomegavirus-transformed human IκB vector (38) were used. A pcDNA3-EGFP expression plasmid was generated using BamHI/NorI restriction of pEGFP-1 and sticky-end ligation of the purified fragment into pcDNA3. The pcDNA3-pEGFP-RelA expression plasmid was constructed by recombinant polymerase chain reaction using PfuTaq DNA polymerases (Expand High Fidelity PCR system, Roche Molecular Biochemicals). pEGFP-1 was used as a template for PCR and the first 30 base pairs from 5′ to 3′ of the EGFP fragment, with the pCMV-RelA fragment. This strategy was chosen to overlap with the 3′ end of the RelA fragment, but without TA1 and TA2 (37), was amplified using a 29-base pair primer 3′-end of the RelA fragment and a pCMV-RelA fragment (587 bp). This amplified EGFP fragment (679 base pairs) was cloned into the promoter a GFP fusion containing RelA with its nuclear localization.

Fluorescent Imaging of Live Cells—Hippocampal neurons were imaged 24 h after biostatic transfection at 30°C in custom-built observation chambers (courtesy of Prof. Dr. Rainer Greger) using an Axiovert (Zeiss, Jena, Germany), high numerical aperture, oil immersion lens, and a fluorescein isothiocyanate filter set (Carl Zeiss). Images were captured using a 2× exposure every 20 min on an Eastman Kodak 3200 ASA color slide films using light from a 50-watt mercury lamp (HB050, Carl Zeiss). Care was taken to minimize exposure to the light. Cellular photodamage was prevented by perfusion via gravity feed with a-tocopherol (1 μM)-supplemented glia-conditioned medium each after exposure (31). Glutamate (100 or 500 μM) or kainate (50 or 100 μM) was added to the glia-conditioned medium for 5 min. Cells were washed twice, and movements of EGFP-RelA and EGFP-(NLSmut)-RelA were observed for 2 h. Quantification of fluorescence intensities was performed as described previously (40), but without background subtraction, using IP-Lab-Spectrum software (Scanalytics, Fairfax, VA). In brief, GFP fluorescence over distance was measured for untreated conditions (U) and after treatment (I) as values obtained after integrating the area under the plotted fluorescence intensities. For statistical evaluation, relative values (I/Un) were obtained from different neurites and experiments were pooled and analyzed as the experimental group containing EGFP-RelA versus the control group containing EGFP-RelA with a mutated NLS, using the Wilcoxon rank sum test. Pseudo-color images were created using NIH Image Version 1.61.

RESULTS

Subcellular Distribution and Biochemical Analysis of NF-κB RelA tagged with EGFP—To analyze the distribution of NF-κB, a GFP fusion protein containing RelA with its nuclear localization.
tion signal was constructed (Fig. 1A). This construct contains only one weak transactivation domain, TA3 (37), to avoid potential toxicity via induction of NF-kB target genes or via squelching (41) of other signal transduction pathways. The feasibility of using this GFP-RelA fusion protein to analyze nuclear transport was tested in HEK 293 cells (Fig. 1B).

The GFP-RelA fusion protein was found exclusively in the nucleus (Fig. 1B), whereas unfused GFP was randomly distributed across all cellular compartments, including the nucleus and cytoplasm (Fig. 1B). Overexpression of IxB-α resulted in the exclusion of GFP-RelA from the nucleus (Fig. 1B), consistent with the idea that IxB-α regulates the location of GFP-RelA in the cell. In contrast, distribution of unfused GFP was not influenced by the coexpression of IxB-α (Fig. 1B).

To biochemically characterize the fusion proteins, transfected HEK 293 cell extracts were separated on SDS gels and analyzed using Western blotting (Fig. 2A). A band corresponding to the predicted protein molecular mass was detected with antibodies directed against either the RelA portion (Fig. 2A, lane 1) or the GFP portion (lane 1) of the fusion protein. A second, faster migrating band was present that might be a degradation product. The specificity of the immunolabeling was tested via the expression of GFP (Fig. 2A, lane 3), which was recognized only by the antibody to GFP and not by the antibody to RelA (lane 7). Similarly, overexpressed RelA was not detected by the antibody to GFP (Fig. 2A, lane 2), but was detected by the antibody to RelA (lane 6). Mock-transfected cells did not react specifically with either antisera (Fig. 2A, lanes 4 and 8).

The DNA-binding characteristics of the EGFP-RelA fusion protein were analyzed by electrophoretic mobility shift assay (Fig. 2B). Using a κ enhancer probe, the binding of EGFP-RelA was examined. The probe bound strongly to EGFP-RelA (Fig. 2B, lane 1). Supershifting with the antibody to RelA verified the identity of the EGFP-RelA complexes (Fig. 2B, lane 2).

Antibody binding to EGFP-RelA was specifically competitively inhibited by unlabeled NF-κB oligonucleotide (Fig. 2B, lane 4), but not by the nonspecific Sp1 oligonucleotide (lane 3).

The transcriptional capability of the EGFP-RelA fusion protein was analyzed using an NF-κB-dependent luciferase vector (Fig. 3, A and B). The fusion protein activated NF-κB-dependent transcription, whereas the control reporter, containing mutated NF-κB sites, was not activated (Fig. 3A). Transcription mediated by EGFP-RelA was inhibited when IxB-α was coexpressed. In general, however, EGFP-RelA exhibited a lower transactivation capability compared with that of RelA (Fig. 3B) due to the fact that this fusion protein contains only one weak transactivation domain (TA3) (37). Deletion of strong transactivation domains was done to avoid potential toxicity via the induction of NF-κB target genes (42) or via squelching (41) of other signal transduction pathways. Taken together, the biochemical and cell biological data indicate that the behavior of the fusion protein was sufficiently similar to that of endogenous RelA to warrant using this EGFP-RelA construct to examine transport in living neurons.
Characterization of Mechanisms of Transport into the Nucleus—We constructed an EGFP-RelA fusion protein with a mutated NLS containing three point mutations (38) (Fig. 4A).

To study the mechanisms of transport into the nucleus of hippocampal neurons (Fig. 4B, left panels) or HEK 293 cells (right panels), cells were transfected as indicated. EGFP-RelA was localized primarily in the nucleus (Fig. 4B), whereas EGFP-RelA with a mutated NLS was localized primarily in the cytoplasm, suggesting that an intact NLS is essential for a nuclear distribution of EGFP-RelA. Low levels of EGFP-RelA with a mutated NLS were detected in the nucleus, which might be due to additional auxiliary nuclear localization sequences (28). This effect was observed in both hippocampal neurons and HEK 293 cells, suggesting that the distribution of NF-κB may be regulated via this mechanism in both cell types.

Coexpression of IκB-α Regulates the Distribution of EGFP-RelA—To activate the reconstituted EGFP-RelA complex via endogenous signal transduction pathways, it was necessary to avoid overloading the system with an overexpression of IκB-α. Therefore, it was essential to determine the least amount of IκB-α necessary to keep the EGFP-RelA fusion protein in the cytoplasm (Fig. 5). The amount of the EGFP-RelA expression plasmid was kept constant, and different amounts of IκB-α expression plasmid were added. The ratios were calculated based on the molecular masses of the expression plasmids. For both hippocampal neurons (Fig. 5, a, c, and e) and HEK 293 cells (b, d, and f), increasing the concentration of IκB-α resulted in changes in the distribution of EGFP-RelA, from primarily nuclear (a and b), to nuclear or cytoplasmic for each specific cell (c and d), and finally to primarily cytoplasmic (e and f). In the cytoplasm, EGFP-RelA was observed within puncta. Based on these data, a ratio of 1:0.2 (EGFP-RelA/IκB-α) was used in the time course experiments described below.

Activation of Endogenous RelA via Glutamate and Kainate—We determined whether endogenous NF-κB was activated by glutamate in a hippocampal neuron/glia co-culture (32, 33). Without treatment, cells exhibited no nuclear staining, whereas following treatment with glutamate, nuclear staining was visible (data not shown). To examine whether treatment with the glutamate agonist kainate activated RelA in these cultured cells, as previously observed in cerebellar granule cells (16), we also examined the effects of kainate. A 5-min treatment with 100 μM kainate resulted in a robust activation of RelA after a delay of 90 min (Fig. 6). The use of a monoclonal

**Fig. 3.** EGFP-RelA activates NF-κB-dependent transcription. HEK 293 cells were transfected with an NF-κB-dependent luciferase indicator vector or an indicator vector without NF-κB sites (tk-luc) together with a Renilla luciferase internal standard vector and additional expression vectors as indicated. Total plasmid amounts were kept constant. Bars represent means ± S.D. of relative light units from three independent determinations. A, experiments characterizing EGFP-RelA; B, control experiments with unfused RelA. CMV, cytomegalovirus.
antibody specific for the activated form of NF-κB allowed us to detect RelA in the neuronal nuclei induced by a brief 5-min kainate treatment. In addition, residual amounts of activated NF-κB could still be detected in neurites 90 min after a kainate pulse (Fig. 6, right panels), similar to the in vivo situation of hippocampal granule cells (10). In processes of hippocampal granule cells, activated NF-κB is present, most likely due to permanent neuronal activity in these cells in vivo. Control cultures incubated with secondary (but not primary) antibody did not exhibit detectable staining (data not shown). Thus, the hippocampal cultures used here are very well suited to study transport of RelA.

Redistribution of EGFP-RelA after Depolarization—To investigate the stimulus-dependent transport of EGFP-RelA from neurites to the nucleus, cultures were treated with 100 mM KCl for 5 min (Fig. 7). Note that under control conditions, EGFP-RelA was localized in long neurites and in puncta resembling varicosities (Fig. 7, upper panel). Ninety min after treatment with KCl, EGFP-RelA was redistributed from neurites and the soma to the nucleus. Thus, we wished to test other stimuli for the capability to induce NF-κB redistribution from neurites to the nucleus. Therefore, we used time-lapse microscopy.

Time-dependent Redistribution of EGFP-RelA—To follow the transport of EGFP-RelA from neurites to the nucleus over time, cultures were treated with 500 μM glutamate after an adaptation time of several minutes following transfer to the imaging chamber (Fig. 8A). After treatment, a gradual transport of EGFP-RelA from neuronal processes to the nucleus was observed (Fig. 8A, panels a–f). By 80 min post-treatment (Fig. 8A, panel e), the EGFP-RelA fusion protein filled the nucleus, consistent with fast retrograde transport. A stimulus-dependent change in the intensity of the EGFP-RelA fluorescence along a thin line through the center of a neuron (Fig. 8A, panel a) is shown Fig. 8B. It is evident that a fraction of EGFP-RelA was sharply redistributed from neurites to the nucleus, suggesting retrograde transport.

Similar transport of EGFP-RelA was observed in cultures stimulated with 100 μM kainate (see below). In contrast to the EGFP-RelA results, the EGFP-RelA fusion protein with mutated NLS was not transported from neuronal processes to the nucleus after either glutamate (Fig. 8C) or kainate (see below) stimulation. Similar results were obtained from five cells recorded on different days.
For better visualization and comparison with controls, pseudo-color images were created from the digitally captured images in Fig. 8 (A, panels a and f; and C, panels a and c) (Fig. 9).

Interestingly, EGFP-RelA was present in puncta resembling varicosities under unstimulated conditions (Fig. 9a), but it was not present in puncta after stimulation with glutamate (Fig. 9b), when also the neurites were free of activated NF-κB (RelA). A strong difference was seen in the NLS controls (Fig. 9, c and d). Here, stimulation with glutamate (Fig. 9d) resulted in the same image as under unstimulated conditions (Fig. 9c); obviously, the blockade of nuclear transport (NLS mutation, see Fig. 4A) interfered also with the retrograde transport from neurites. Only a very weak photobleaching is observed when the images in Fig. 9 (c and d) are compared. To address the significance of the transport described here, we performed a quantitative analysis (Fig. 10).

For this purpose, we randomly selected five neurons from EGFP-RelA experiments or from independent EGFP-(NLSmut)RelA experiments. From these neurons, the GFP fluorescence was measured in several neurites before (I₀) and after treatment (I) with glutamate or kainate along a thin line (as described for Fig. 8B). The \( \frac{I}{I₀} \times 100 \) ratio was depicted as a measure of GFP transport. The strong decrease in fluorescence intensity in neurites from EGFP-RelA-transfected neurons demonstrated the retrograde transport of activated NF-κB from synaptic sites to the nucleus (Fig. 10A and B, left bars). In contrast, this decrease was not observed in neurons transfected with EGFP-(NLSmut)RelA (right columns) since no retrograde transport and no relevant photobleaching occurred. The statistical significance of transported EGFP-RelA in comparison with EGFP-(NLSmut)RelA was analyzed using the Wilcoxon rank sum test. A highly significant difference between the experimental group (EGFP-RelA) and the control group (EGFP-(NLSmut)RelA) was evident with both stimuli glutamate (\( p < 0.0068 \)) (Fig. 10A) and kainate (\( p < 0.00027 \)) (Fig. 10B).
To enable us to analyze the translocation of GFP-tagged RelA in living hippocampal neurons, we fused a GFP tag to the RelA subunit of NF-κB and confirmed that this fusion protein (EGFP-RelA) retained its functionality as a transcription factor. EGFP-RelA was present in the nuclei of neurons; but after overexpression together with the cognate inhibitor of NF-κB, IκB-α, the distribution of the protein changed from nuclear to neuritic (in dendrites and axons). In glutamate-stimulated hippocampal neurons, a return of EGFP-RelA from a neuritic to a nuclear distribution was observed. Interestingly, the redistribution of EGFP-RelA was dependent on a functional NLS. We conclude that NF-κB is capable of being a signal transducer, transmitting information from, for example, active synapses to the nucleus, in addition to its well known role of a transcription factor. Specifically, in mammalian neurons, NF-κB may function as a retrograde messenger, transmitting glutamatergic signals from distant sites to the nucleus.

We found, in untreated hippocampal neurons cultured as astrocyte co-cultures, endogenous RelA immunoreactivity in neuronal processes, the soma, and varicosities, whereas no immunoreactivity was found in the nucleus. No constitutive NF-κB activity was observed in this neuron/astrocyte co-culture paradigm, in contrast to that previously observed in serum-free cultures (14). Previously, we found that the constitutive NF-κB activity was repressed in the presence of glia in the cultures used here (43). Overexpression of EGFP-RelA alone or together with its cognate inhibitor protein IκB was chosen to follow the distribution of both activated (without IκB) and inhibited (with IκB) NF-κB. From a neuritic localization, which was induced by the presence of IκB, the fusion protein migrated after stimulation to the nucleus. In this line, activated endogenous NF-κB was detected in neurites and the nucleus only after glutamatergic stimulation (see Fig. 6). Supporting this result of glutamatergic stimulation, we recently described activated NF-κB present in vivo in the dendrites and nuclei of hippocampal granule cells (10).

The results of this study indicate that NF-κB was activated in hippocampal neurons by glutamate, consistent with its functioning as a signal transducer. This is in accordance with previous reports of the activation of NF-κB in cerebellar granule cells via ionotropic glutamate receptors (7, 16). This observation could be extended to hippocampal neurons (this study).

Also, the stimulation of non-N-methyl-D-aspartic acid receptors via kainate resulted in strong activation of NF-κB in cultivated hippocampal neurons. Activation of NF-κB via non-N-methyl-D-aspartic acid receptors using kainate resulted in strong activation of NF-κB in cultured hippocampal neurons. Activation of NF-κB has been observed using in vivo experiments (7, 16). Initially, it was reported that inhibition of neuronal activity with tetrodotoxin results in an up-regulation of major histocompatibility complex class I expression (16). Initially, it was reported that inhibition of neuronal activity with tetrodotoxin results in an up-regulation of major histocompatibility complex class I expression after intraocular tetrodotoxin injection was observed in vivo (46). Although these data may appear contradictory, the results were obtained using two different experimental paradigms and thus may reflect the complexity of the system. It is tempting to speculate that activation of NF-κB via glutamatergic stimulation may in turn regulate major histocompatibility complex class I expression, which may function as synaptic glue (46). For example, in invertebrates, the inhibition of NF-κB may function as an injury signal, whereas in mammals, injury may activate NF-κB. In this line, Aplysia NF-κB is inhibited after nerve crush, whereas traumatic spinal cord injury activates NF-κB in rats (36). Future experiments may elucidate this issue.

The capability of NF-κB to transmit information from, for example, active synapses to the nucleus is supported by many studies demonstrating the presence of NF-κB in synaptic regions. Synaptosomes can contain presynaptic proteins that are
sealed and stabilized by the postsynaptic density, and latent forms of NF-κB have been found in synaptosomal preparations (6). Low salt extracts prepared from synaptosomes contain NF-κB proteins such as p50 and RelA together with IκB-α. Synaptophysin cofractionates with NF-κB proteins during purification. Colocalization of synaptophysin and NF-κB proteins has also been detected in rat cerebral cortex (6). NF-κB can be activated with the detergent deoxycholate, resulting in two specific DNA-binding complexes with different sensitivities for deoxycholate. Supershifting and inhibition with recombinant IκB-α show a bona fide DNA-binding complex that includes the RelA and p50 subunit. These data have been confirmed using hippocampal synaptosomal preparations (8). In addition, a robust increase in RelA mRNA after long-term potentiation has been reported in vivo (8). It is possible that this is part of a feed-forward mechanism leading to increased DNA binding to NF-κB elements during long-term potentiation. Purkinje cell synapses also were analyzed using light microscopy, and en passant synapses were found to contain NF-κB (7). In addition, an electron microscopy study found NF-κB- and IκB-α-like immunoreactivities within dendrites (10), including dendritic spines and postsynaptic densities, of neurons in the hippocampus and cerebral cortex (9). In Drosophila melanogaster, the NF-κB homolog Dorsal colocalizes with the IκB homolog Cactus within the nervous system. Both proteins are detected at high levels in postsynaptic sites of glutamatergic neuromuscular junctions (47). Therefore, NF-κB and IκB-α and their homologs in other species appear to be present in pre- and postsynaptic regions. To investigate mechanisms involved in the retrograde transport of proteins in neurons, Ambron and coworkers (48, 49) analyzed protein transport from the soma to the axon and vice versa in Aplysia californica. They found that both the retrograde transport of proteins from synapses to the nucleus and the transport of proteins into the nucleus are dependent on an NLS derived sequence that functions in Aplysia. Homologous NLS motifs are located within the basic region of the DNA-binding domain of the activating transcription factor family of transcription factors. Recently, immunostaining and Western blot analysis revealed retrograde transport of the activating transcription factor in nociceptive neurons (52). Here, we found that in mammalian neurons, the retrograde transport of EGF-RelA was dependent on a functional NLS, supporting the idea of an evolutionarily conserved transporting machinery that might be used by different transcription factors. After IκB-α is degraded, the NF-κB subunits p50 and RelA are transported into the nucleus due to the presence of an NLS (38). The molecular basis for this masking of the NLS by IκB-α is now evident from studies of their crystal structure (28) showing that IκB-α covers an α-helix segment of RelA containing the NLS. Using the Aplysia system, Ambron and co-workers (53) have shown that axons contain NF-κB in both its active DNA-binding and inactive forms, possibly complexed with IκB-α. The inactive form may be activated in vitro via treatment with deoxycholate, as described for synaptosomal NF-κB (6). Transport of transcription factors from synapses to the nucleus, e.g., in response to specific stimuli, might be a regulating mechanism of gene expression. In this study, the EGF-Tagged RelA subunit of NF-κB was translocated after glutamate, kainate, or KCl stimulation in hippocampal neurons from neurites to the nucleus. In this line, unidirectional movement of the transcription factor CREB was observed after injection of CREB protein labeled with fluorescence dye into dendrites of hippocampal neurons (54). In accordance with biochemical data (38) and crystal structure studies (28), the results of this study indicate that nuclear translocation of NF-κB was regulated in living hippocampal neurons and HEK 293 cells via interactions with IκB-α. Moreover, we also found that the localization of EGF-RelA to neurites and varicosities was regulated by IκB-α in these living cells, consistent with its being a remote signal transducer. In conclusion, the results of this study support the hypothesis that NF-κB is involved in translating short-term signals from distant sites in neurites into long-term changes in gene expression, which may have a key role in plasticity, development, and survival.

Acknowledgments—We thank Prof. Dr. M. Frotscher for continuous support and helpful suggestions, Dr. Harald Neumann for helpful discussions, Prof. Dr. Carlos Dotti for providing essential protocols, Prof. Dr. Rainer Greger for providing chambers for the time-lapse experiments, and Dr. H. Stockinger for providing the anti-RelA monoclonal antibody. We also thank Titus Sparna for help with quantification and Elmar Bhöm for superb technical assistance.

Note Added in Proof—Recently, Freudenthal and Romano (2000) Brain Res. 855, 274–287 have shown that training activated synaptosomal NF-κB, thus providing a further hint for synapse to nucleus signaling.

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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Methods 9255–9264

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