Regulation of Neuronal Cell Adhesion Molecule Expression by NF-κB

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The neuronal cell adhesion molecule (NCAM) is a key mediator of structural plasticity in the central nervous system, but the mechanisms that control its expression are unknown. Equally, although the transcription factor NF-κB is present in the brain, few NF-κB-regulated genes relevant for central nervous system function have been identified. We have previously demonstrated that NF-κB is activated in neuronal cultures treated with kainic acid or nitric oxide. We show here that kainic acid or nitric oxide also increase the levels of NCAM mRNA and protein in neurons and that this induction of NCAM expression is sensitive to dexamethasone and to antisense, but not missense, oligonucleotides designed to suppress NF-κB synthesis. Nitric oxide also stimulates protein binding to an NF-κB site in the promoter of the NCAM gene. This indicates that NF-κB, which has recently been implicated in synaptic plasticity and also in the etiology of neurodegenerative disease, plays a crucial role in the activity-dependent regulation of NCAM gene expression. In addition, since both NCAM and NF-κB are present in the post-synaptic density, this represents a route allowing direct communication between the synapse and the nucleus.

In all models of long-lasting synaptic plasticity, the later phases are dependent on the synthesis of new proteins. This may at least partially reflect the requirement for durable structural rearrangement of the synapses. Sprouting of new synaptic contacts is associated with long term facilitation in Aplysia (1), whereas an increase in dendritic spine density (2, 3) or alterations in synaptic clustering (4) or spine architecture (5, 6) are observed in the mammalian hippocampus during long term potentiation (LTP)1 or memory formation. The effects on the dendritic spine reflect actions either on the spine itself or on the post-synaptic density (psd), a specialized structure within the dendritic spine that receives and transduces the neurotransmitter signals from the presynaptic terminal. Although it is likely that these structural changes are necessary to sustain long term functional plasticity, there are few indications as to the cellular and molecular mechanisms involved. One possibility is that nitric oxide (NO) plays a central role. In many situations, NO release appears to be necessary for the generation of LTP (7–10), whereas recent evidence has suggested that NO can specifically modulate cytoarchitecture (11, 12).

The psd is composed of a functionally connected network of receptors, channels, enzymes, and scaffolding proteins (13–15). One of the major psd constituents, Ca2+/calmodulin-dependent protein kinase II (CamKII) (16), is known to play a major role in the induction and maintenance of synaptic plasticity (17–22). The level of expression of the α subunit of CamKII is increased a few hours after the induction of hippocampal LTP (23, 24), presumably to maintain the heightened sensitivity. We have reported that exposure to NO increases dendritic CamKIIα mRNA levels (25), an effect likely to be mediated by post-transcriptional mechanisms acting on CamKII mRNA present in the neuronal dendrites (26, 27). Thus glutamate receptors and NO can enhance the synthesis of psd CamKII by acting locally in the dendrite.

The neuronal cell adhesion molecule NCAM is also present in the psd (28, 29, 30). Recent evidence suggests that NCAM also plays an important role in plasticity (31, 32). The maintenance of LTP (33, 34) and learning behavior in the rat (35) and chicken (36) are reduced by antibodies to NCAM. Similarly, inactivation of the NCAM gene in mice results in deficits in spatial learning (37) and in attenuation of hippocampal LTP (38). It has recently been discovered that, in an analogous manner to CamKII, the levels of NCAM in dendritic spines and psds are increased after LTP induction (29), suggesting that NCAM may also be important for the sustained functional change. This hypothesis is supported, in other models by evidence that NCAM levels are increased after learning behaviors (39) by genetic evidence that the Drosophila homologue of NCAM is involved in the regulation of synaptic structure and function at the neuromuscular junction (40) and by the modulation of the Aplysia homologue of NCAM during synaptic plasticity (41).

The mechanisms involved in elevating NCAM expression are likely to be different from the post-transcriptional regulation of CamKII expression, since NCAM mRNA is not found in neuronal dendrites.2 Increased transcription of the NCAM gene is probably involved, since enhanced activation of AMPA/kainate receptors, which facilitate the induction of LTP and enhance learning, elevates the activity of the NCAM promoter (42).

The molecular link between AMPA/kainate receptors and increased NCAM gene transcription therefore becomes of major interest in understanding how the properties of synapses and of the psd, in particular, can be modulated by patterns of afferent activity. In the immune and vascular systems, two nonneuronal homologues of NCAM, the intracellular adhesion molecule (ICAM-1) and the vascular cell adhesion molecule (VCAM-1), show increased expression when cells are exposed to cytokines or lipopolysaccharide (LPS). In both the ICAM-1 gene

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1 The abbreviations used are: LTP, long term potentiation; psd, post-synaptic density; CamKII, Ca2+/calmodulin-dependent protein kinase II; NCAM, neuronal cell adhesion molecule; LPS, lipopolysaccharide; ANOVA, analysis of variance; SNAP. S-nitroso-N-acetylpenicillamine; -ir, immunoreactive; NF-H, high molecular weight neurofilament; iNOS, inducible form of nitric oxide synthase.

2 B. J. Morris, unpublished information.
and the VCAM-1 gene, the promoter region contains an NF-κB site upstream of the transcription start site, and NF-κB activation appears to be required for gene induction (43–47). Inspection of the NCAM promoter reveals a potential NF-κB site in a similar position (48). There is evidence linking activation of NF-κB to neuronal plasticity (49), and interestingly, NF-κB has been detected in the PSD in cortical and hippocampal neurons (50), where the inactive form may be tethered to the PDZ domains of the PSD structural network (51). This location in the PSD would place NF-κB in a unique position to carry transcriptional signals from the synapses to the cell nucleus.

We have recently shown that kainic acid and also NO, which is released in striatal cultures in response to AMPA/kainate receptor stimulation, activate NF-κB in striatal neurons (52). We therefore tested the hypothesis that neuronal NF-κB might be involved in NCAM induction following kainate receptor stimulation or release of NO. If correct, this would identify a novel pathway that links the PSD to the nucleus and acts to couple synaptic stimulation to morphological plasticity.

**EXPERIMENTAL PROCEDURES**

**Primary Neuronal Culture and Drug Treatment**—Neuronal cultures were prepared as described (53) and maintained in supplemented serum-free medium. On day 12 drugs were added, and after 24 h the cells were fixed in 4% paraformaldehyde solution for 10 min. Dexamethasone was added 3 h before drug treatment. When wells were treated with antisense oligonucleotide or control (missense) oligonucleotide, these agents were added 2 days before drug treatments. Cells were plated at equal densities, and samples were treated identically in every experiment. In addition, different treatments were always performed in parallel so that relative staining intensities could be monitored both between groups and also relative to vehicle-treated wells.

**Antisense Treatment**—Antisense oligonucleotides and controls directed to NF-κB p50 have been designed and manufactured by Biognostik, Germany. The sequences were: antisense, atc ctg aaa ccc cac; randomized mismatch control with same AT/GC ratio (missense), gtc cct ata cga cag. Oligonucleotides were introduced into neurons essentially according to published procedures (54).

**Immunocytochemistry**—Immunocytochemistry was performed as described previously (27) with antibodies against p50 and p65 (1:2000, Santa Cruz). Staining was visualized using an ABC peroxidase system (Vector Laboratories).

To check for linearity of staining intensity relative to protein concentration, varying concentrations of NCAM antigen peptide (Santa Cruz) were immobilized on polyvinylidene difluoride membranes and detected as above. The staining intensity was found to be proportional to the amount of peptide present over a range of 2 orders of magnitude, spanning the staining intensities observed in cultured neurons in these experiments (not shown).

**In Situ Hybridization**—In situ hybridization was performed at high stringency as described (27, 53) using an 35S-labeled oligonucleotide probe complementary to the region of NCAM mRNA encoding amino acids 334–347.

**Data Analysis**—Computerized image analysis was used either to measure the intensity of immunocytochemical staining over individual neurons (mean number of pixels/unit area) or the intensity of autoradiographic signal following in situ hybridization (pixel area occupied by silver grains/neuron) using Image NIH 1.52 software (W. Rasband, National Institutes of Health). For each drug treatment, five measurements were taken from different fields of view, and no less than four animals were used per treatment group. Each field of view represented between 50 and 100 neurons, but this small variation in number did not affect the measurements, which are assessed over individual neurons. Pooled data were analyzed for significance using ANOVA with post hoc Fisher’s test for multiple pairwise comparisons (>2 groups) or Wilcoxon (Mann-Whitney U) test (2 groups).

**Electrophoretic Mobility Shift Analysis**—Male Harlan Sprague-Dawley rats (200–250 g) received intraperitoneal injections of 2 mg/kg NOR3 (Calbiochem) or 1 ml/kg vehicle (saline). After 30 min, the rats were killed by anesthetic overdose, and the striatum was dissected out and homogenized. Similarly, cultured neurons were scraped from the culture wells and homogenized. Nuclear extracts were prepared according to standard procedures and incubated for 30 min with radiolabeled NF-κB binding site oligonucleotide (Promega) or an oligonucleotide corresponding to bases 905–926 of the NCAM promoter region. DNA-protein complexes were then resolved on 6% polyacrylamide gels. To control for specificity of shifted bands, a 20-fold excess of NF-κB competitor oligonucleotide (Life Technologies, Inc.) was added to the incubation in some cases.

**Western Blotting**—Cultured neurons were scraped from the culture wells, homogenized in radioimmune precipitation buffer (Roche Molecular Biochemicals), and centrifuged. After a second centrifugation step, proteins in the supernatant were denatured and run on 3–8% polyacrylamide-Tris acetate gels with size markers (Santa Cruz). After blotting onto polyvinylidene difluoride membranes, the proteins under investigation were visualized using primary antibodies as above and alkaline phosphatase-conjugated 2nd antibodies, according to standard procedures.

**RESULTS**

Exposure of cultures of rat striatal neurons to the NO-releasing agent S-nitroso-N-acetylpenicillamine (SNAP) increased the levels of NCAM mRNA (Fig. 1, a and b). The NO scavenger hemoglobin prevented SNAP from increasing NCAM mRNA levels (Fig. 1, a and b). Similarly, increases in immunoreactive NCAM (NCAM-ir) were detected after treatment with SNAP.
Regulation of NCAM Expression by NF-κB

To provide a direct test of the hypothesis that NO enhances NCAM expression via NF-κB, we utilized an antisense oligonucleotide designed to suppress the synthesis of the p50 subunit of rat NF-κB. The utility of antisense oligonucleotides in primary neuronal cultures is usually limited by problems with neurotoxicity due to the oligonucleotides. However, we employed a polyethyleneimine carrier (54) to aid cell penetration and reduce the concentrations required for translational inhibition. Although, in our hands higher concentrations of oligonucleotide (>500 nM) showed neurotoxic effects (not shown), no toxic effects were observed at concentrations of 300 nM or below.

The uptake of oligonucleotide was assessed using a fluorescein-labeled NF-κB p50 oligonucleotide. A very high proportion of cultured striatal neurons showed fluorescent labeling under the conditions used (Fig. 5a). Pretreatment of cultures for 2 days with an antisense oligonucleotide directed against the mRNA encoding NF-κB p50 resulted in a decrease in p50-ir of more than 60% (Fig. 3, b–d). A missense oligonucleotide with the same base composition had no significant effect on p50-ir (Fig. 3, b–d). A smaller decrease in immunoreactivity for the p65 subunit of NF-κB (p65-ir) was detected using the p50 antisense oligonucleotide, although the missense oligonucleotide had no effect (Fig. 3c).

The gene encoding high molecular weight neurofilament (NF-H) is apparently not regulated in association with synaptic plasticity, and the promoter region of the NF-H gene does not contain any potential NF-κB sites (48). Therefore, to ensure that there were no nonspecific effects on protein synthesis occurring, we studied the effect of antisense or missense oligonucleotide treatment on the levels of NF-H-ir. No significant changes in NF-H-ir could be detected with either the antisense or missense oligonucleotides or with any of the drugs used (Fig. 4b). We also monitored the expression of the cell adhesion molecule integrin αv and the transcription factor Elk1 to provide additional information of any nonspecific effects of antisense oligonucleotide treatment. No changes in the levels of integrin αv-ir or Elk1-ir were observed after antisense oligonucleotide treatment (Fig. 4c).

To determine whether the degree of suppression of NF-κB activity following antisense oligonucleotide treatment is sufficient for a functional blockade of NF-κB activity, we studied the induction of the inducible form of nitric oxide synthase (iNOS). Induction of the iNOS gene by cytokine or LPS treatment occurs via activation of NF-κB binding to the promoter region (55, 56). When iNOS protein levels were measured using a specific iNOS antiserum, both kainate and LPS treatment increased iNOS-ir (Fig. 4a). The increased iNOS-ir protein levels were prevented by pretreatment with the NF-κB p50 antisense oligonucleotide but not by pretreatment with the missense oligonucleotide (Fig. 4a). Similar effects were observed when NOS activity in the striatal cultures was monitored by NADPH-diaphorase histochemistry (not shown).

The induction of NCAM-ir by SNAP, LPS, and kainate was completely blocked by pretreatment with the NF-κB p50 antisense oligonucleotide (Fig. 5, a and b) but not affected by the missense oligonucleotide under identical conditions.

**DISCUSSION**

We demonstrate here that kainate, SNAP, and LPS are all able to increase NCAM expression in striatal neurons. In each case the doses of agents used were substantially below the doses that result in neurotoxicity, suggesting that the effect on the NCAM gene is part of the normal repertoire of plasticity-related responses. The lack of toxicity is confirmed by the
absence of any effect of SNAP, kainate, or LPS on NF-H expression. In addition, this illustrates the specificity of the actions of these agents for the NCAM gene, emphasizing that there is not a generalized elevation in the expression of all cellular genes.

The level of immunocytochemical signal is unlikely to be directly proportional to the amount of antigen in the tissue, considering the various amplification steps involved in the detection procedure, and so it is not possible to relate the magnitude of the change in NCAM-ir detected to the magnitude of the increase in NCAM protein. Nevertheless, it is worth noting that even a small increase in NCAM expression is thought to produce a dramatic functional change in cell adhesion properties (57).

Dexamethasone attenuated the increased NCAM expression due to kainate and SNAP treatment. Among other actions, dexamethasone acts to suppress the transactivating potential of NF-κB (55, 58, 59). This inhibition of NCAM induction by dexamethasone is therefore consistent with the hypothesis that NF-κB is involved in the regulation of neuronal NCAM expression. This also provides a likely explanation for the observation that hippocampal NCAM levels and dendritic arborization are enhanced by removal of endogenous glucocorticoids (60), where their absence would remove any inhibition of NCAM expression mediated by this mechanism in vivo. Furthermore, in view of the importance of NCAM expression for learning and memory processes, our results provide a framework to explain the well known suppressive effects of glucocorticoids on acquisition and retrieval of memories (61, 62).

The antisense oligonucleotide directed against NF-κB p50 decreased immunostaining for p50 by more than 50%, whereas no significant suppression of staining was observed with an equivalent missense oligonucleotide. These results suggest that a sequence-specific suppression of neuronal NF-κB p50 levels has been achieved via this approach. The lack of any effect of the oligonucleotide treatments on NF-H immunostaining provides evidence that there is no toxic effect of the treatment and, equally, that there is no generalized, nonselective suppression of protein synthesis within the neurons. Furthermore, the lack of any effect of antisense treatment on the levels of integrin αV-ir suggests that there are no generalized nonspecific effects on cell adhesion molecule synthesis, whereas the lack of any effect of antisense treatment on the levels of Elk1-ir confirms that there is no generalized suppression of transcription factor synthesis and, hence, that the treatment is likely to be selectively inhibiting translation of NF-κB p50.

Interestingly the levels of NF-κB p65-ir were decreased by the p50 antisense treatment but not by the missense treatment. This is probably a reflection of the fact that NF-κB p50 homodimers or p50/p65 heterodimers participate in maintaining the basal levels of neuronal expression of the p65 gene, since the p65 promoter contains a NF-κB site, and dexamethasone shows a greater inhibition of p65 activity relative to p50 activity (52). In consequence, it can be concluded that the antisense oligonucleotide produces a suppression of NF-κB activity by inhibiting the synthesis of both p50 and p65 subunits of NF-κB.

The major influence on expression of the iNOS gene in a variety of cell types is known to be NF-κB (55, 56, 63). We observe that in striatal neurons, kainate and LPS both produced a clear induction of iNOS, providing further evidence that these agents activate NF-κB. This induction of iNOS was not observed after antisense oligonucleotide treatment, con-
firming that the antisense pretreatment produces a functional blockade of NF-κB activity.

The induction of NCAM-ir by SNAP, LPS, and kainate was completely blocked by pretreatment with the NF-κB p50 antisense oligonucleotide (Fig. 4b) but not affected by the missense oligonucleotide under identical conditions. This confirms that the induction of NCAM expression is critically dependent on the activation of NF-κB.

In occasional experiments the basal levels of NCAM-ir were reduced by antisense treatment (Fig. 5b), perhaps reflecting a higher level of endogenous synaptic activity in these cultures. Overall there was no significant effect of antisense treatment on the basal levels of NCAM-ir (Fig. 5a), implying that basal levels are not maintained by the constitutive NF-κB activity in neurons (64) but rather that NF-κB participates in activity-dependent regulation of NCAM expression. Basal levels of NCAM expression are likely to be mainly sustained by other transcription factors. For example, the homeobox-binding proteins have been shown to be important for the developmental regulation of NCAM expression (65).

Activation and nuclear translocation of NF-κB has been detected following the induction of LTP in the hippocampus (49) during neuronal development (66) and as part of the response to neuronal injury (67). Whereas the activation of NF-κB during injury is relatively easy to reconcile with known NF-κB target genes such as cyclooxygenase 2, until now it has not been clear what role NF-κB might play in physiological as opposed to pathological plasticity. Our results suggest that one of the consequences of NF-κB activation will be increased expression of NCAM, which in turn is expected to lead to plasticity in synaptic architecture and, particularly, to sprouting of nerve terminals or dendritic processes (31, 32). The presence of NCAM in the PSD suggests that activity-associated rearrangement of the PSD (5) may involve this pathway. Interestingly, recent evidence suggests that extracellular binding of NCAM will itself activate NF-κB (69). Since NF-κB can be present presynaptically as well as postsynaptically, this provides an elegant mechanism whereby activity-stimulated NCAM induction (via NF-κB) in the PSD could then activate presynaptic NF-κB and, hence, cause a complementary increase in presynaptic NCAM expression. Over a few hours, this would then produce reciprocal stabilization of the new synaptic structure.

It is worth noting that activated neuronal NF-κB has been detected in the immediate vicinity of amyloid plaques in Alzheimer’s disease tissue (70), where there is also extensive aberrant axon terminal sprouting. Furthermore, the cholinergic neurons of the basal forebrain, which show particularly pronounced terminal sprouting during the course of Alzheimer’s disease (71, 72), are also prominent in terms of their high levels of activated NF-κB (73). In the light of a recent report showing...
increased NCAM expression in the regions of abberant sprouting (68), our results suggest that the novel NF-κB-NCAM link reported here contributes to the inappropriate terminal hypertrophy that is a feature of the neuropathology of Alzheimer’s disease.

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