Neuronal κB-binding Factors Consist of Sp1-related Proteins

FUNCTIONAL IMPLICATIONS FOR AUTOREGULATION OF N-METHYL-D-ASPARTATE RECEPTOR-1 EXPRESSION*

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Neurons contain a protein factor capable of binding DNA elements normally bound by the transcription factor NF-κB. However, several lines of evidence suggest that this neuronal κB-binding factor (NKBF) is not bona fide NF-κB. We have identified NKBF from cultures of neocortical neurons as a complex containing proteins related to Sp1. This complex was bound by antibodies to Sp1, Sp3, and Sp4 and was competed from binding to an NF-κB element by an oligonucleotide containing an Sp1-binding site. This Sp1 oligonucleotide detected an abundant factor in neuronal nuclei that migrated in electrophoretic mobility shift assays at a position consistent with NKBF. Expression of transfected Sp1 stimulated transcription in a manner dependent upon a κB cis-element. Similar to our previous reports for NKBF (Mao, X., Moerman, A. M., Lucas, M. M., and Barger, S. W. (1999) J. Neurochem. 73, 1851–1858 and Moerman, A. M., Mao, X., Lucas, M. M., and Barger, S. W. (1999) Mol. Brain Res. 67, 303–315), the activity of the Sp1-related factor was reduced by activation of ionotropic glutamate receptors, consistent with proteolytic degradation of all three Sp1-related factors. Expression of the N-methyl-D-aspartate receptor-1 (NR1) subunit of glutamate receptors correlated with the activity of the Sp1-related factor, specifically through an Sp1 element in the NR1 promoter. These data provide the first evidence that Sp1-related family members are responsible for κB-binding activity and are involved in a negative feedback for NR1 in central nervous system neurons.

Agonists often stimulate down-regulation of their receptors as a mechanism providing negative feedback. Glutamate plays a major role in many fundamental aspects of neurotransmission in the central nervous system (1). On the other hand, it is also well known that glutamate contributes to many neuropathological states. Glutamate is able to kill many types of neurons through the phenomenon of excitotoxicity (2). By activating its ionotropic receptors, especially those of the NMDA1 class, glutamate can generate an ion flux and free radical production so robust that cellular homeostatic mechanisms are overwhelmed. In a population of cortical neurons challenged with glutamate, a fraction die by rapid osmotic stress; however, others die 18–24 h later (2). This later cell death bears signs of apoptosis (2, 3) and is dependent on an early commitment phase. The commitment appears to involve calcium influx (4) and a mitochondrial membrane permeability transition (5, 6), but is otherwise incompletely understood.

The delayed aspect of excitotoxicity permits consideration of changes in gene expression. The number of genes altered by glutamate receptor activation is also consistent with transcriptional contributions to the delayed component excitotoxicity. Unfortunately, the influence of abusive glutamate receptor stimulation on transcription is poorly characterized. Some reports have suggested that the transcription factor NF-κB is an important target for glutamate (7–9). However, no defined role of NF-κB in neuronal death has been demonstrated in excitotoxic paradigms, and emerging data suggest that NF-κB might play completely different roles under different neurotoxic conditions (10–13).

Glutamate is able to activate NF-κB in mouse and rat cerebellar cultures maintained in serum-containing medium (7–9). However, by rigorously controlling glial numbers, we found that the prominent κB-binding factor in cultured cortical neurons is composed of proteins distinct from bona fide NF-κB (14, 15). This neuronal κB-binding factor (NKBF) is distinct from NF-κB with regard to immunoreactivity, DNA binding preferences, and electrophoretic mobility under both denaturing and non-denaturing conditions. An additional distinction is that glutamate application diminishes NKBF activity; NF-κB is activated by glutamate only in cultures containing significant numbers of glia (14, 15). NMDA receptors and calcium were involved in the diminution of NKBF by glutamate, and other data provided correlations between NKBF suppression and excitotoxicity (14).

In light of the fact that Sp1 can compete with Rel factors for κB enhancers (16), we explored the contribution of Sp1 family transcription factors to NKBF. Antibody reactivity and probe competition assays indicated that Sp1, Sp3, and Sp4 contribute to NKBF binding activity. Glutamate was able to evoke a substantial reduction in Sp1-related factors in a time- and dose-dependent manner, as detected by DNA binding assays and immunoblot analysis of the Sp1 family polypeptide levels. More importantly, glutamate was capable of influencing expression of NMDA receptor-1 (NR1) expression through the

HIV, human immunodeficiency virus; IL6, interleukin; RBP-Jκ, recombination signal binding protein Jκ; RT, reverse transcription; EMSA, electrophoretic mobility shift assay.

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modulation of Sp1-related factors, consistent with previous demonstrations of a role for Sp1 in NR1 transcriptional regulation (17).

EXPERIMENTAL PROCEDURES

Materials—Ig/HHV-β, Sp1, and AP-1 oligonucleotides used in DNA binding assays were supplied by Promega (Madison, WI). Phosphorothioate-modified oligonucleotides used for decoy experiments were synthesized by Oligos Etc. (Wilsonville, OR) with the following sequences: decoy, 5'-TTGCGGAGGCTCTCCCTACAG-3' (Sp site is underlined); and scrambled control, 5'-GACATGTCGCTCAGTCCG-3'. Invitrogen supplied all other oligonucleotides, including Ig/HHV-β in assays where it was not synthesized internally. The plasmid pNRL239 was generously provided by Dr. G. Bai (17, 21). These reporters are derivatives of plasmids pNRL239, pNRL356, and pNRL356mtSp1 (State University) (19); pPAC-Sp4 was obtained from Dr. G. Suske (University of Hamburg, Germany) (20). Luciferase reporter plasmids pNRL239, pNRL356, and pNRL356mtSp1 were generously provided by Dr. G. Bai (17, 21). These reporters are derivatives of pGL2-Basic (Promega) with insertions from the NR1 gene promoter. The pNRL239 plasmid contains NR1 promoter region −239 to −1 (translation initiation site as +1). The pNRL356 plasmid contains NR1 promoter region −356 to −1. Two Sp1 sites located at −299 to −267 have been specifically mutated in pNRL356mtSp1. Cell Cultures—N9 cells were generously provided by Prof. P. Ricciardi-Castagnoli (22); they were maintained in minimal essential medium with Earle's salts (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). Drosophila melanogaster SL2 cells (obtained from Dr. J. C. Azizkhan, Roswell Cancer Institute, Buffalo, NY) were maintained in minimal essential medium containing 3 μg/ml of Neurobasal/B27 medium per well. The dual-luciferase reporter assay system was used to determine luciferase activities following the manufacturer's instructions in a Turner TD-20e luminometer. Transient Transfections of Primary Neocortical Neurons—SL2 cells were transfected with plasmids prepared from QIAGEN maxiprep kits by calcium phosphate coprecipitation. Cells were plated in 24-well plates at 104/ml with 0.5 ml of medium/well the day before transfection. Each well received 50 μl of a DNA/calcium phosphate suspension that contained 0.2 μg of reporter plasmid (pNRL series or pR1E1-GL3), 40 ng of pPAC-series plasmids, 0.2 μg of pRL-prom, and an appropriate amount of inert carrier DNA to equalize to 2 μg of DNA. After 48 h, cells were harvested. The dual-luciferase reporter assay system was used to determine luciferase activities following the manufacturer's instructions in a Turner TD-20e luminometer. Extensive tests of plasmid/calcium phosphate concentrations and cell density were conducted to further optimize this protocol for our culture conditions. Ultimately, neurons were plated in 24-well plates at 2 × 104/cm2 and treated with cysteine arabinoside in Neurobasal/B27 medium to restrict glial cell numbers. After 8 days in vitro, neurons were transfected with DNA/calcium phosphate suspension (at 40 μg/ml) containing 0.2 μg of pNRL series plasmid, 0.2 μg of pRL-CMV, and 1.6 μg of carrier DNA. After a 3-h transfection, the medium containing DNA/calcium phosphate was removed, and the neurons were washed three times with HEPES-buffered Hanks' balanced salt solution and then maintained in fresh Neurobasal/B27 medium. In control neurons, 10 μM cytosine arabinoside was present during the first 4 days to suppress glial growth. All neuronal cultures were 8–10 days old at the time of treatment.

RESULTS

Sp1 Family Factors Are the Prominent β-binding Factors in Neurons—In our investigations of the influence of glutamate on the activity of neuronal β-binding factors, we found that the prominent factor binding a β sequence in neurons is not composed of bona fide NF-κB proteins (15). This factor, which we termed NKBF, was found to have target sequence specificities that are subtly distinct from those of NF-κB, and its

Nuclear Protein Extraction and Electrophoretic Mobility Shift Assay (EMSA)—The specific protocols have been described previously (15). Antigens and unlabeled oligonucleotides were added to the binding reaction prior to addition of the radiolabeled NF-κB or Sp1 consensus oligonucleotide. For EMSAs including antibodies, the 15-min incubation period was extended to 60 min at 4 °C in the presence of antibody.

Western Blot Analysis of Sp1-related Factors and NR1—For Western blot analysis, highly enriched neocortical neurons were treated with glutamate (50 μM) for the times indicated. After glutamate treatment, cultures were lysed in 80 mM Tris-HCl (pH 6.8) and 2.5% SDS, and total proteins were harvested for Western blot analysis as described previously (15).

Decoy Administration and RNA Harvesting—The decoy protocol has been described previously (23). Briefly, 500 μl of minimal essential medium containing 3 μg decoy or scrambled oligonucleotide was dispensed into 500 μl of minimal essential medium containing 70 μl of LipofectAMINE suspension dropwise with constant mixing. The DNA/LipofectAMINE mixture was mixed vigorously for 15 s and then incubated for 30 min at room temperature before use. Fifty microliters of the DNA/LipofectAMINE mixture was added dropwise to each well of 24-well plates containing 450 μl of Neurobasal/B27 medium per well. The final concentration for decoy and scrambled oligonucleotides was 150 nm. Only marginal toxicity could be detected after long-term (>72 h) decoy administration. After 24 h of decoy treatment, total RNA was harvested by Trireagent according to the manufacturer's protocol. The RNA concentration was quantified by absorbance at 260 nm. RNA was immediately used for RTP-PCR or stored at −80 °C.

RT-PCR—The RT-PCR procedure was performed according to the manufacturer's protocols and has been described previously (24). For NR1, the forward primer was 5'-CCCCCTGACGAGAAGTTCT-3' and the reverse primer was 5'-CCCGTGGAATCTGGGAGTGA-3', creating a 333-bp amplier. For β-actin, the forward primer was 5'-GTCCTCTGCATGTTGTTC-3' and the reverse primer was 5'-GTCGGCCCTCTGTAATTTG-3', creating a 439-bp amplier. The PCR was performed at 94 °C for 30 s, 61 °C for 45 s, and 72 °C for 30 s for denaturation, annealing, and extension, respectively (27 and 24 cycles for NR1 and β-actin, respectively).
DNA-binding activity is diminished by toxic glutamate treatments in primary neuronal cultures (14). Sp1 and related factors can compete with NF-κB for binding to a DNA sequence (16). Combined with the target DNA specificity of NKBF, this finding suggested that NKBF might include Sp1-related proteins. To test this possibility, EMSA analyses were performed utilizing antibodies directed against Sp1-related factors. Four such factors have been cloned: Sp1 and Sp3 are ubiquitously expressed in most cell and tissue types; Sp2 and Sp4 have more restricted expression patterns (26), but high levels of Sp4 expression have been reported in the developing central nervous system (27). Therefore, Sp1, Sp3, and Sp4 were investigated in extracts from primary cultures of neocortical neurons probed with an oligonucleotide probe containing an NF-κB-binding site. Antibodies against Sp1 family factors either supershifted or inactivated NKBF (Fig. 1A), and the combination of multiple

**Fig. 1. Identification of the prominent κB-binding factors in primary neurons.** Nuclear extracts from primary neocortical neurons were prepared, and the DNA-binding activities were analyzed by EMSA. A, antibodies against Sp1 family factors (Sp1, Sp3, and Sp4) or NF-κB subunits (p50 and p65) were included in EMSA reactions using the HIV-IκB probe sequence. The total antibody amount was equalized to 1.8 μg in all conditions, except 0.9 μg of antibody was used in lanes 2, 4, and 6. For the conditions with more than one antibody, the total amount of antibody was consistent across groups. A longer exposure of the autoradiograph is included at the bottom to show the supershifted bands more clearly. B, a complex (arrow) comigrating with NKBF was detected when an Sp1 probe was used. The nature of binding complexes was tested with antibodies against Sp1 family factors (Sp1, Sp3, and Sp4) or NF-κB subunits (p50 and p65). Nuclear proteins were excluded from reactions in lanes 12–14, where probe was incubated with antibodies alone. Again, a longer exposure of the autoradiograph is included at the bottom to show the supershifted bands more clearly. C, different amounts (0.46–7.8 ng) of anti-RBP-Jκ antibody were included in these assays of nuclear proteins extracted from activated N9 cells. The IL6-IκB probe was used to exclude the binding activity from Sp1-related factors, which had a mobility similar to that of the supershifted band. No antibody was added in the 0 lane. Of note, this antibody did not interfere with the interaction between NF-κB (p50/p65) and this probe. S.S., supershifted probe; F.P., free probe.
antibodies was more efficient than an equivalent amount of a single antibody. For example, the combination of 0.9 μg of anti-Sp1 antibody and 0.9 μg of anti-Sp4 antibody (Fig. 1A, lane 9) altered NKBFF more effectively than did 1.8 μg of anti-Sp1 antibody alone (lane 3) or 1.8 μg of anti-Sp4 antibody alone (lane 7). The combination of antibodies against Sp1, Sp3, and Sp4 almost completely abrogated the detection of NKBFF (Fig. 1A, lane 11). These data indicate that NKBFF consists of Sp1 family factors. We previously observed an abundant, fast-migrating xB-binding activity (Complex III) in several cell and tissue populations (15); this complex is insensitive to anti-Sp1 family antibodies. An Sp1-binding activity with the same mobility as NKBFF was observed when an Sp1 consensus probe was used in EMSA (Fig. 1B). With this probe, Complex III was absent, but a separate fast-migrating band appeared (Fig. 1B, arrowhead). Antibodies against Sp1 family factors affected the NKBFF-comigrating complex similarly to their influence on reactions with the NF-xB probe. The fast-migrating band observed with this probe was sensitive to anti-Sp3 antibody. No interactions between antibodies and the probe were observed when nuclear proteins were omitted in the reactions (Fig. 1B, lanes 12-14). An unlabeled, double-stranded oligodeoxynucleotide containing an Sp1-binding site was able to compete NKBFF from a labeled NF-xB probe (data not shown).

On the basis of its target sequence specificity and its mobility, we speculated that Complex III might include RBP-Jx. The core sequence for RBP-Jx binding is 5'-CTCTGGGAAA-3', which matches the sequence of the 3'-end of the Ig/HIV-xB probe, extending beyond the 3'-end of the xB consensus sequence. Furthermore, mutations in this portion of the probe disrupted Complex III binding (data not shown). Initial assays indicated that a monoclonal antibody (K0043) against RBP-Jx altered NF-xB activities in EMSA (Fig. 1B). With this probe, Complex III was absent, but a separate fast-migrating band appeared (Fig. 1B, arrowhead). Antibodies against Sp1 family factors affected the NKBFF-comigrating complex similarly to their influence on reactions with the NF-xB probe. The fast-migrating band observed with this probe was sensitive to anti-Sp3 antibody. No interactions between antibodies and the probe were observed when nuclear proteins were omitted in the reactions (Fig. 1B, lanes 12-14). An unlabeled, double-stranded oligodeoxynucleotide containing an Sp1-binding site was able to compete NKBFF from a labeled NF-xB probe (data not shown).

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To determine whether Sp1 or related factors can act as transactivating factors at xB cis-elements, we cotransfected an Sp1, Sp3, or Sp4 expression vector with a luciferase reporter plasmid containing four copies of a xB element. These experiments were performed in the Drosophila SL2 cell line, which lacks detectable Sp1-related activity. The reporter construct utilized the xB element from the promoter of the human β-amyloid precursor protein, as this sequence lacks the element responsible for RBP-Jx binding (15). Separate cultures were transfected with the above expression vectors alone, and nuclear extracts were prepared to confirm active protein production by EMSA analysis (Fig. 2A). When Sp1 was cotransfected with the xB-dependent reporter plasmid, luciferase activity was elevated over 2-fold (Fig. 2B). Expression of Sp3 alone did not affect transcription from this particular xB element, and Sp3 did not appreciably affect the induction by Sp1.

However, Sp4 expression significantly suppressed basal activity, and the combination of Sp3 and Sp4 significantly suppressed the induction by Sp1.

Specific Inhibition of the Activity of Sp1-related Factors by Toxic Glutamate Treatments—We previously found that glutamate evokes reduction of NKBFF DNA-binding activity in a time- and dose-dependent manner (14). This effect is correlated with glutamate neurotoxicity. To further characterize the relationship of Sp1 to NKBFF, we tested the influence of glutamate on the DNA-binding activity of Sp1 and related factors in extracts from neocortical neurons. Glutamate influenced the gel retardation of an Sp1 consensus target probe in a time- and dose-dependent manner (Fig. 3, A and B), reminiscent of its effects on NKBFF binding to the Ig/HIV-xB probe. Substantial suppression of Sp1-related binding activity was observed only after long-term or high-dose glutamate exposure. This effect appeared to be specific to Sp1-related factors, as glutamate did not have a similar influence on the activities of other transcription factors tested. First, using a similar time course paradigm, we found that the DNA binding of transcription factor AP-1 was inhibited by short-term glutamate exposure and enhanced at longer times of glutamate treatment (Fig. 4A), behavior that was essentially opposite that of the Sp1-related factors. Second, no significant influence (p = 0.9175) of glutamate on RBP-Jx was observed (Fig. 4B). On the other hand, glutamate treatment significantly inhibited the DNA-binding activity of Sp1-related factors (p = 0.0002, glutamate-treated versus untreated). Surprisingly, the activity of Sp factors was fully restored after prolonged glutamate exposure (Fig. 5), whereas
glutamate inhibited the activity of Sp1-related factors for relatively short-term treatments (<18 h).

Interestingly, the diminution of the activity of Sp1-related factors was accompanied by the appearance of a fast-migrating species binding the Sp1 site probe (Fig. 5, lanes 2 and 3, arrowheads). The possibility that this band arose from a fragment of an Sp1-related factor could not be confirmed by super-shift, perhaps due to changes in the antigenic determinant. However, degradation of Sp1 family factors was suggested by Western blot analysis. When proteins from neocortical cultures were analyzed with an antibody against Sp1, a 78-kDa immunoreactive band was detected in neuronal cultures. The levels of this species were reduced by short-term glutamate exposure and recovered after long-term treatments (Fig. 6B). Concomitant with the diminution of the 78-kDa band by glutamate, a smaller species (~68 kDa) was initially enhanced. Its levels were also diminished by 12 h, perhaps due to loss of the 78-kDa precursor. The levels of Sp3 and Sp4 experienced a similar transient decline, although the precise time course was different for these proteins (Fig. 6B). Specifically, the lowest level of Sp3 was at 1 h, whereas for Sp4, it was at 6 h. Parallel cultures were subjected to EMSA (Fig. 6A), confirming a correlation between Sp1-related DNA-binding activity and the described changes in Sp1 polypeptide levels.

**Role for Sp1-related Factors in Regulation of NR1 Expression**—Sp1-binding sites appear to be important for expression of the NR1 glutamate receptor subunit (17). Together with our demonstration of the attenuation by glutamate of Sp1-related DNA-binding activity, this relationship suggested a potential feedback inhibition of NR1 expression. Therefore, we assayed NR1 polypeptide levels by Western blot analysis in the same samples analyzed for Sp1-related factors. Application of glutamate to neocortical cultures resulted in a reduction of detectable NR1 protein, reaching ~50% of its control level after a 3-h treatment (Fig. 6B). Thus, reductions in NR1 expression correlated with the reduction of Sp1-related proteins by glutamate.

As a more direct test of the role of Sp1-related proteins in control of NR1 expression, we sought to inhibit Sp1-related DNA-binding activity by loading the cells with decoy oligonucleotides containing a binding site for Sp1 factors. These oligonucleotides were delivered to primary neocortical neurons.
After 24 h, total RNA was isolated, and RT-PCR was performed to detect NR1 mRNA levels. An expected, a 333-bp band was detected for NR1 amplification, and the levels of NR1 mRNA were significantly diminished by decoy treatments compared with application of a scrambled control oligonucleotide (p < 0.05, decoy versus scrambled oligonucleotide) (Fig. 7, A and B).

Surprisingly, the scrambled oligonucleotide reduced NR1 mRNA levels as well. This may reflect unintended binding of Sp1-related factors due to the inescapable high GC content of the scrambled oligonucleotide, as this control sequence competed with the canonical Sp1-binding sequence in EMSA, albeit with a lower efficiency compared with the decoy oligonucleotide sequence (data not shown). The effect of Sp1 factor decoy treatment on NR1 mRNA was specific; no changes were observed for β-actin mRNA levels after decoy or scrambled oligonucleotide administration. These data indicate that in primary neurons, Sp1-related factors may function as feedback regulators of NR1 expression in response to glutamate.

The NR1 Promoter Is Activated by Sp1 Family Factors in Drosophila SL2 Cells—The results of decoy experiments indicated that Sp1-related factors may be necessary for normal levels of NR1 expression. Two putative Sp1 cis-elements have been identified in the NR1 promoter, and these sites are important for NR1 expression in PC12 cells (17). We investigated the influence of the individual Sp1-related factors on NR1 promoter activity in Drosophila SL2 cells. SL2 cells were transfected with pNRL356, a reporter construct containing the NR1 promoter coupled to a luciferase coding region, or with pNRL239, a reporter construct containing a shorter portion of the upstream regulatory region, lacking the Sp1 sites. Cells were cotransfected with either of these reporters and vectors encoding Sp1 or Sp3 (Fig. 8). Twenty-four hours later, cells were harvested, and the luciferase activities were determined.

No significant activation or inhibition was observed for pNRL239 by Sp factors (Sp1, Sp3, or Sp1/Sp3). By contrast, the activity of pNRL356 was enhanced by Sp1 (p < 0.001, pPAC versus pPAC-Sp1 transfection). Sp3 did not affect the activity of pNRL356, but it repressed the promoter activity induced by Sp1 (p < 0.005, pNRL356/Sp1 versus pNRL356/Sp1/Sp3). It is not clear that this inhibitory function of Sp3 involved interactions with the Sp1 cis-elements, as the activity of Sp1/Sp3 cotransfection was lower than that of Sp1 alone in pNRL239 transfectants as well (p < 0.001, pNRL239/Sp1/Sp3 versus pNRL239/Sp1). Each transfection of Sp1-related factors (Sp1,
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We previously found distinctions between NF-κB and the factor(s) responsible for prominent κB-binding activity in primary neurons, which we termed NKBF (14, 15). We have now characterized NKBF as a complex containing members of the Sp1 family of transcription factors. The proteins binding to a κB enhancer sequence were influenced by antibodies against members of the Sp1 family, and the binding was competed by an oligonucleotide sequence more conventionally used as an Sp1 probe. Furthermore, expression of Sp1 in cells deficient in detectable Sp1 activity stimulated expression from a reporter construct in a manner dependent upon κB enhancers. Although RBP-Jκ was also a major factor interacting with the Ig/HV-κB probe, its binding depended on nucleotides beyond the 3'-end of the core κB consensus bases, and thus it is unlikely to include most other κB elements, as evidenced by its inability to bind the β-amyloid precursor protein κB element (15). Therefore, Sp1-related factors appear to be the only detectable κB-binding factors in primary neurons.

This study has conclusively demonstrated that the prominent κB-binding factor in nearly pure cultures of neocortical neurons is composed of proteins antigenically related to Sp1. However, the proportions of different Sp1-related factors in the active transactivating factor remain difficult to discern. Because of the high levels of endogenous Sp1 family members in cortical neurons, our functional assays concentrated on the SL2 cell line, in which the Sp1-related activity could be more rigorously controlled. And although the data therein confirmed that Sp1 itself could stimulate transcription from a κB enhancer, Sp3 and Sp4 appeared to be either inactive or inhibitory in this system. Sp3 and Sp4 play modulatory roles at typical Sp1-binding sites in the human alcohol dehydrogenase-5 gene promoter, dampening Sp1-evoked transcriptional enhancement in proportion to their DNA-binding activity (28). Inactivity or suppressive actions of Sp3 and Sp4 at typical Sp1 sites are partially dependent upon the DNA sequence of the cis-element (28–30). Therefore, it is not surprising that the interaction of various Sp1 family members with κB elements would show variability. However, it is also possible that the differences between mammalian cortical neurons and the SL2 cell line (including potential differences in components of the RNA polymerase II complex) account for the apparent inactivity of Sp3 and Sp4 that we observed. The final assessment of the roles of Sp3 and Sp4 will await more elaborate experiments involving depletion of endogenous Sp3 and Sp4 in mammalian neurons, as well as a survey of their transactivating functions on cis-elements of varying sequence.

The identification of Sp1 and/or related factors as the prominent κB-binding elements in neurons calls into question the mechanism of action of NF-κB in neurons. Other studies have found an induction of NF-κB by glutamate in primary neuronal cultures (7–9). We have detected the p50 and RelA subunits of NF-κB in our highly enriched neuronal cultures both by Western blot analysis and by immunocytochemistry,2 but we did not detect DNA-binding activity by EMSA (this study and Refs. 14 and 15). Transfection studies have shown an influence of NF-κB in individual neurons (31). One possible resolution of this apparent conflict is suggested by interactions of NF-κB with other nuclear proteins, including glucocorticoid receptors, CAAT/enhancer-binding protein, the progesterone receptor, and p300/cAMP-responsive element-binding protein-binding protein (32–35). Perhaps, glutamate or other stimuli can alter the interaction between other transcription factors and NF-κB, independently of the latter's own DNA binding.

2 X. Mao, A. M. Moerman, and S. W. Barger, unpublished data.

DISCUSSION

![Fig. 9. Role of Sp1 sites in NR1 promoter activity and influence of glutamate.](image)

Sp3, or Sp1/Sp3) elicited significantly higher activity from pNRL356 than from pNRL239 (p < 0.0001, pNRL239/Sp1 versus pNRL356/Sp1, p < 0.01, pNRL239/Sp3 versus pNRL356/Sp3, and p < 0.0001, pNRL239/Sp3 versus pNRL356/Sp1/Sp3). Sp4 did not affect these promoter activities either positively or negatively (data not shown)

NR1 Promoter Activities in Neurons and Influence of Glutamate—Transient transfection of neocortical neurons was employed to explore the activities of NR1 promoters in mammalian primary neurons. Sp1 and related factors are expressed abundantly in primary neocortical neurons, so these cultures were transfected by reporter genes (pNRL239 or pNRL356) alone. After transfection, the neurons were allowed to recover overnight from the calcium phosphate exposure and then treated with glutamate (50 μM, 60 min). Glutamate exposure was followed by a second overnight chase phase; then cells were lysed, and the luciferase activities were determined. In these cultures, the relative activity of pNRL356 was at least 50-fold higher than that of pNRL239 (Fig. 9) suggested, that neurons provide a better cellular environment for NR1 promoter activation than do SL2 cells. The promoter activity of pNRL356 was significantly reduced by 60-min glutamate treatment (50 μM) (p < 0.005, pNRL356 versus pNRL356/glutamate), whereas the same glutamate treatment did not result in a significant activity change for pNRL239 (p = 0.78, pNRL239 versus pNRL239/glutamate). The 117-nucleotide difference between pNRL356 and pNRL239 could potentially include other active cis-elements. To test the role of the Sp1 site more specifically, we used a more refined site-specific mutation (pNRL356mtSp1×2). This inactivation of the Sp1 site lowered expression to a point equivalent to that of pNRL356 after glutamate treatment (p = 0.58, pNRL356/glutamate versus pNRL356mtSp1×2), and glutamate had no effect on the mutant reporter (p > 0.05, pNRL356mtSp1×2 versus pNRL356mtSp1×2/glutamate). These data indicate that toxic glutamate treatments can specifically diminish the activity of the NR1 promoter in primary neurons through Sp1 cis-elements.
The data presented here are consistent with our previous observations that the DNA-binding activity resulting from Sp1-related factors is sensitive to toxic levels of glutamate (14, 15). Furthermore, our findings extend this phenomenon to the possibility that proteolysis of Sp1-related proteins is responsible for the effects of glutamate. This relationship indicates that glutamate could be involved in the negative regulation of one of its receptor subunits that depends on Sp1-related factor(s) for expression. This hypothesis was lent credence by our demonstrations that 1) suppression of NKBF activity with decay oligonucleotides depressed NR1 expression; 2) the activity of the NR1 promoter in SL2 cells was dependent upon cotransfection of Sp1; and 3) Sp1 sites in the NR1 promoter were required for normal expression levels and for depression of NR1 promoter activity by glutamate.

The activity of the NR1 promoter has not been investigated in primary neurons before. Our data indicate that Sp1-related factors are involved in NR1 gene expression in neurons, which is consistent with earlier studies utilizing the PC12 cell line (17). Analysis of both endogenous gene expression and transfected reporter constructs explicates the vital role of Sp1-related factors in NR1 gene expression in primary neurons. It is possible that the suppression by glutamate of a transcription factor required for expression of a critical receptor subunit reflects a programmed feedback inhibition of glutamate bioactivity. Glutamate enhanced the DNA-binding activity of AP-1 within 1 h, consistent with earlier reports (36–38). At that time, Sp1-related activity was suppressed, a specificity suggesting that the influence of glutamate on the Sp1 complex may involve a defined signaling pathway, as indicated previously (14). Consistent with earlier reports (39), no increases in NR1 mRNA levels were detected after prolonged treatments with the NMDA antagonists AP-5, MK-801, and dexamethasone (data not shown). This may indicate that basal glutamatergic neurotransmission does not activate the relevant mechanism to the extent required to exert a tonic influence on Sp1-related activity. Thus, this pathway may exist only as a “safety valve” responding to pathologically high levels of glutamatergic stimulation. It is notable that the activity of the Sp1 complex and the levels of related polypeptides were restored during an overnight glutamate exposure, which implies attenuation either of the cells’ glutamatergic responsiveness or of the specific pathways leading to Sp1 inactivation. Under our culture conditions, 24 h of continuous exposure to 50 μM glutamate kills only ~15% of neurons (14). Apparently, many neurons are able to survive this insult and replenish their Sp1 and related factors. However, it is not clear whether the inactivation of Sp1-related activity is beneficial (through the blunting of NR1 activity) or contributes to cell death through suppression of the vast number of genes dependent upon Sp1 and its family members. In the cells that die, loss of Sp1-related activity might be a major early event in subsequent delayed excitotoxicity. Our previous study showed that the rapid effect of glutamate on the activities of Sp1-related factors is correlated with excitotoxicity manifest 12–24 h later (14). One break in this correlation is the failure of nitric-oxide synthase inhibitors to prevent the rapid loss of Sp1-related activity, whereas such inhibitors are quite effective at blocking glutamate neurotoxicity. This suggests that neurons can tolerate a temporary loss of Sp1 activity. However, the kinetics of Sp1 and related factors in individual cells under these conditions remain unknown.

Identification of mechanisms by which glutamate inhibits Sp1-related factors will be aided by the clues at hand. One consequence of activation of glutamate receptors (of the NMDA class, in particular) is a large calcium influx. Elevated intracellular calcium appears to be essential for the ability of glutamate to diminish the activity of Sp1-related factors (14). Toxic levels of glutamate can elevate the production of reactive oxygen species (1), and Sp1 is known to be sensitive to redox changes (40). For instance, a diminution of Sp1 activity in aged rat tissues can be restored by the reducing agent diithreitol, and Sp1 DNA-binding activity can be inhibited by hydrogen peroxide (41). We found that exposing cells to ferrous sulfate inactivates NKBF (14), which generates hydroxyl radicals via the Fenton reaction.

The reduction of Sp1-related DNA-binding activity by glutamate was accompanied by the appearance of a fast-migrating species in EMSA. It is possible that Sp1-related factors are cleaved by a calcium-activated protease into a fragment that retains specific DNA-binding activity. Unfortunately, none of the anti-Sp1 antibodies we tested could retard these fast-migrating species in EMSA. However, Western blot analysis of glutamate-treated cultures did reveal an Sp1-immunoreactive 68-kDa band that may have been derived from intact Sp1. Presumably, this immunoreactive fragment is distinct from the DNA-binding fragment apparent in EMSA, consistent with the mobility of the latter being greater than that of RBP-Jκ, which has a denatured mobility suggesting ~27 kDa (15). Rickers et al. (42) found that Sp1 could be cleaved by caspases during B-cell apoptosis. The ability of cyclosporin A to block the glutamate-evoked loss of Sp1-related activity (14) suggests that a similar mechanism could be at work here, as cyclosporin A can inhibit the mitochondrial permeability pore transition and thereby block activation of some caspase cascades (43).

Several lines of data indicate that Sp1 is a major regulator for NR1 glutamate receptor expression. By analyzing the NR1 promoter, Bai and Kusiak (17) identified two Sp1-binding sites whose mutation could greatly reduce the promoter activity in PC12 cells. The ubiquitous expression profile of Sp1 and Sp3 and the high expression levels of Sp4 in neurons during early developmental stages might explain why NR1 is expressed broadly in neurons. Interestingly, the Sp1-binding sites in the NR1 promoter might also confer regulation by single-stranded DNA-binding proteins (44). That study found the binding patterns of single-stranded DNA-binding proteins to be different between glia and neurons. These single-stranded DNA-binding proteins may participate in the restriction of high NR1 expression to neurons. Unfortunately, the precise identity of the relevant single-stranded DNA-binding protein(s) remains unknown. Our Drosophila transfection results showed that in SL2 cells, Sp1-related factors could activate the NR1 promoter, but could not confer the same level of activity seen in neurons. This indicates that Sp1-related factors alone may be sufficient to generate only low activity from the NR1 promoter, as seen in non-neuronal cells (17). It is unknown whether SL2 cells contain single-stranded DNA-binding factors similar to those present in mammalian non-neuronal cells.

REFERENCES
