Nerve Growth Factor Up-regulates the N-Methyl-D-aspartate Receptor Subunit 1 Promoter in PC12 Cells*

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The N-methyl-D-aspartate (NMDA) subtype of glutamate receptor plays important roles in synaptic plasticity, the induction of long term potentiation, and excitotoxicity. Mechanisms governing the regulation of expression of its subunit genes remain largely unknown. The promoter of the essential subunit of the NMDA receptor heteromer, NMDAR1, contains DNA binding elements recognized by the nerve growth factor-inducible/early growth reaction factor (NGFI/Egr) family of transcription factors that are rapidly induced by neurotrophins, such as nerve growth factor (NGF). This study examined the effect of NGF on the activity of the N-methyl-D-aspartate receptor subunit 1 (NMDAR1) promoter/luciferase reporter constructs in PC12 cells, which contain the high affinity TrkA receptor for NGF and the low affinity p75NTR receptor for neurotrophins. NGF up-regulated the activity of the NMDAR1 promoter by 3–4-fold in a time- and dose-dependent manner. 5′ deletional analysis of the promoter indicated that the responsive element(s) resides in the proximal region containing GSG and Sp1 sites. Mutational analysis of these sites revealed that both were important for NGF regulation. Transient expression of Egr-1 increased activity of the wild type promoter but failed to increase activity of a GSG mutant promoter. Other neurotrophins did not activate the promoter, while K-252a inhibited the action of NGF. These results suggest that the NGF effect is mediated by the high affinity NGF receptor, TrkA and that neurotrophin binding to the low affinity neurotrophin receptor, p75NTR, alone does not affect the promoter activity. Our results suggest that NGF is able to up-regulate the activity of the NMDAR1 promoter and may play a role in controlling the expression levels of NMDA receptors.

The NMDA1 subtype of glutamate receptor is a unique ligand-gated calcium channel exhibiting voltage-dependent magnesium block, which suggests important roles for this receptor in developmental neuronal survival, synaptic plasticity, and long term potentiation (1–3). Overactivation of this receptor may be responsible for the glutamate-induced neuronal excitotoxicity in the postischemic brain, trauma-induced brain injury, and possibly chronic neurodegenerative disorders of aging such as Alzheimer’s and Huntington’s diseases (2).

Recent evidence suggests that functional NMDA receptors in neurons are heteromeric molecules assembled from an essential subunit, NMDAR1, and one or more of a second family of subunits termed NMDAR2-A–D (4–6). The NMDAR1 gene transcript undergoes alternative splicing to produce eight variants (7, 8). Different variants show distinct physiological and pharmacologic properties (9). Numerous studies have shown that the level of total NMDAR1 mRNA changes during development and maturation of the central nervous system (10–12), that there are region-specific changes in expression after experimentally induced ischemia (13–15), and that mRNA levels change after prolonged antagonist treatment (16). It also has been reported that message levels change due to 6-hydroxydopamine lesions (17), chronic dopamine antagonist treatment (18), estrogen treatment (19), epileptic seizures (20), and a diurnal rhythm (21). These changes in message levels may potentially influence the amount of the NMDAR1 subunit protein and result in the differential assembly of heteromeric NMDA receptors or a change in the number of functional channels. However, the mechanisms governing these changes in message levels remain largely unknown. Only recently have insights been obtained into the transcriptional regulation of the NMDAR1 gene (22).

Neurotrophins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurophin 3 (NT-3), and neurophin 4/5 (NT-4/5), classically were found to support neuronal survival, growth, and differentiation (23–26). Recent studies revealed that they also are involved in other neuronal activities such as synaptic plasticity (27, 28) and possibly neurotoxicity (29). Interestingly, NMDA receptors also are involved in these processes (2). Activation of the NMDA receptor is able to up-regulate BDNF and NGF levels in hippocampal neurons (30), and most recently BDNF was shown to be required for the formation of LTP in hippocampal brain slices (26) and for the maturation of NMDA receptors in cultured cerebellar granule cells (31). Little evidence is available on the effect of neurotrophins on the expression of NMDA receptor genes.

We have previously isolated and analyzed a 3-kb promoter region of the NMDAR1 gene (22, 32). A DNase I footprinting experiment showed protection of a proximal GC-rich sequence including a consensus GSG motif (GCG-GC) recognized by an immediate early gene family of transcription factors, i.e. nerve growth factor-inducible factors (NGFI)s or early growth reaction (Egr) factors (33–36). Neurotrophins induce a rapid increase in this family of factors both in vitro and in vivo (37). Several reports documented little or no change in NMDAR1
message after ischemic insult, while others showed marked increases (13–15). In this experimental paradigm, elevated levels of neurotrophins, NGFI-A, NMDA receptor activity, and subsequent cell loss also were documented (2, 30, 38–41). These findings prompted us to examine the effects of neurotrophin treatment on activity of the 3-kb promoter region of the NMDAR1 gene in hopes of better understanding the interrelationship between neurotrophic factor activity and NMDA gene regulation.

EXPERIMENTAL PROCEDURES

Plasmid Construction

Site-directed Mutagenesis—A megaprimer PCR method was used to create point mutations of GSG and Sp1 elements in the proximal 356-bp promoter of the NMDAR1 gene (42). A 356-bp fragment of the NMDAR1 gene promoter was retrieved from pG356 (22) by digestion with BsmI and removal of overhang ends and then digestion with SacI. The isolated fragment was subcloned into Hincl/SacI-digested pGEM3Zf(+), to form pG356. A Ndel-linearized pG356 served as a template in all of the following PCR reactions. An upstream primer, GB 463 (5) oligonucleotide TGCCCATCAGGCGCAAGTT (TACAG-3) and a downstream primer, GB 399 (5) GTTCTACCCCTCCCACCGCCCCCGCTTCCTG, were used to generate the megaprimer with the following mutated primers in antisense sequence: GB57 for S1a site, 5′-CTGTGCTACTGA-TGCGACCGCCCCGGTTCCTGC; GB41 for Sp1 site, 5′-CTGTTTACACCCCTCATGACCCTGCTCTGG; GB58 for GSG site, 5′-CTGTTTACACCCCTCATGACCCTGCTCTGG; GB40 (5′-CGTGTACACCCCTCATGACCCTGCTCTGG) for riboprobe 1 (Invitrogen, San Diego, CA). All oligonucleotides were synthesized with an ABI 392 DNA/RNA synthesizer (Foster City, CA) and purified with G5 columns.

Reporter Genes—The mutated 356-bp promoter constructs were excised from pGEM-T vector with HinclI/SacI and ligated into Smal/Not-digested pGL2-Basic, a luciferase reporter gene vector (Promega) to form pNRL356. A luciferase plasmid was included in some reactions to control for transfection efficiency. NMDAR1 promoter/reporter constructs for transient expression were prepared by subcloning the promoter/luciferase gene fragments from the pGEM-Basic constructs into pCDNAI/neo (Invitrogen, San Diego, CA). All mutagenized NMDAR1 promoters were linearized with SacI and ligated into pGEM3Zf(+). In some experiments, pGL2-Basic was linearized with EcoRI and ligated into pCDNAI/neo (Invitrogen, San Diego, CA). To generate plasmids containing luciferase reporter gene constructs, pNRL356 (22) was digested with SacI and Blunted with T4 DNA polymerase. The constructs were further digested with BamHI. The pOV80BamHI fragments containing a SV40 polyadenylation signal/NMDAR1 promoter/luciferase gene cDNA/SV40 small intron/SV40 polyadenylation signal were isolated. These fragments were then cloned into EcoRV/SacI-digested pGEM3Zf(+)-digested pCDNAI/neo vector to yield pNRL356Sp1a/neo, pNRL356Sp1b/neo, pNRL356GSG/Sp1x2/neo, pNRL356GSG/Sp1x2/neo, pNRL356GSG/Sp1x2/neo, and pNRL356GSG/Sp1x2/neo. All DNA plasmids were purified from host Escherichia coli using Qiagen (Chatsworth, CA) maxi-prep kit. Plasmids pCBEGr-1, containing full-length Egr-1 cDNA driven by the CMV promoter, and pCBEGr-13531–374, lacking the DNA binding domain of Egr-1, were kindly supplied by Dr. Vakas P. Sukhatme.

Template Plasmids—To generate riboprobes for NMDAR1 mRNA, plasmid pG37K7S was linearized with PstI for transcribing riboprobe 1 (32). A SacI/BamHI sequence with nucleotides 2328–2660 (complementary to a common region of all NMDAR1 mRNA splicing variants was subcloned from pN60 (43) into pGEM3Zf(+), to form pN356, which was linearized with EcoRI for riboprobe 4. To transcribe an antisense riboprobe of luciferase mRNA, pGEM-luc (Promega) was linearized with EcoRV. For in vitro transcription/translation, Egr-1 or Egr-13531–374 DNA including 5′- and 3′-untranslated regions was retrieved with EcoRI from pCBEgr-1 and subcloned into pGEM3Zf(+), to generate pEgr-1 and pEgr-13531–374 with inserts at 3′ → 5′ orientation in the vector multiple cloning sites. A Stn1 digestion of pEgr-1 and pEgr-13531–374 and religation of vector/cDNA containing fragments removed most of the 5′-untranslated region and an outside ATG and formed pEgr-13531–374-em and pEgr-13531–374-em.

NGF Regulation of the NMDAR1 Promoter in PC12 Cells

Transfections of PC12 Cells and Reporter Gene Assay

PC12 cells were cultured and maintained as described previously (22). PC12 cells were transfected with constructs using Lipofectin (Life Technologies, Inc.). For transient expression, the constructs were cotransfected with pCMVβ (Clontech, Palo Alto, CA). Twenty hours after transfection, NGF (2.5 S; Promega) was added at 100 ng/ml. The cells were harvested at different times after adding NGF, and both luciferase and β-galactosidase activities in the cell lysates were measured (22). In these experiments, the DNA content in the cell lysates was measured with Hoechst Dye 33258 (Polyscience, Warrington, PA) to correlate the relative reporter activity to cell number in individual samples (44). In some experiments, pNRL356 or pNRL556mGSG was co-transfected into PC12 cells with pCBEGr-1 or pCBEGr-1/3313–374 in addition to pCMVβ. Two days after transfection, activities of luciferase and β-galactosidase in cell lysates were measured. To establish stable transfecants, only luciferase-containing constructs were used, and 2 days after transfection the cells were cultured with Geneticin (G-418, Life Technologies) at 300 μg/ml. Each construct was transfected in duplicate transfactions in 100-mm culture dishes, and all transfectedants for the same constructs were pooled together after a 3-week selection. The level of luciferase in the transfectants was measured as described below (22). The total genomic DNA in the lysate was quantitated with Hoechst Dye 33258, and the integrated NMDAR1 promoter/luciferase genes were measured as described previously.7 The stable transfecants were subcultured on 60-mm dishes coated with collagen and then treated with NGF. At various times, cells were collected for a luciferase assay. In some experiments, stable transfecants were pretreated with either actinomycin D at 1 μg/ml or K-252a at 50 nM for 30 min and treated with or without NGF. Some stable transfecants or wild type PC12 cells were cultured and treated with NGF for RNA extraction.

Measurement of mRNA Level

Total RNA in stable transfecants or wild type PC12 cells was extracted with an RNAzol kit (Cinna/Biotecx, Friendswood, TX) and quantitated by measuring the absorbance at 260 nm. A RNase protections assay was used (21). In some experiments, a single-stranded oligonucleotide corresponding to the 3′ untranslated region of endogenous NMDAR1 mRNA and the expressed luciferase mRNA (32). Briefly, two riboprobes for NMDAR1 mRNA were used: riboprobe 1 for the 5′-untranslated region was described before (22). Riboprobe 4 was transcribed from EcoRI-linearized pGMR1–4 by Sp6 RNA polymerase and has a 330-nucleotide sequence complementary to NMDAR1 mRNA. For the luciferase mRNA assay, an antisense probe was synthesized from EcoRI-linearized pGMR1–4 by Sp6 RNA polymerase and was used in a 413-nucleotide probe containing 356 nucleotides complementary to luciferase mRNA. Ten μg of total RNA were hybridized with riboprobes overnight at 42 °C and digested with RNase ONE (Promega) for 30 min for NMDAR1 and 20 min for luciferase mRNA. Protected RNA probes were resolved on 7M urea/polyacrylamide gel and exposed to x-ray film as well as to a PhosphorImager plate. The amount of mRNA was analyzed on a PhosphorImager SI system (Molecular Dynamics, Sunnyvale, CA).

Cell Mobility Shift

Transcription factor Egr-1 or Egr-1/3313–374 was synthesized in vitro from pGEGr-13531–374-em using the TNT Sp6 coupled reticulocyte lysate system (Promega) following the manufacturer’s instructions. A luciferase plasmid was included in some reactions to determine the efficiency and specificity of reporter gene transactivation from templates. The gel mobility shift assay was performed as described previously (22). The probe was used as a double-stranded oligonucleotide GB63/87 encompassing the coding region of the NMDAR1 promoter, and its sequences are as follows: GB63 for sense strand, 5′-CAGGAAGCAGGGGGGCGGAGGGGTAGAACG; GB67 for antisense strand, 5′-GTCACTCCCTCCACCGCCCAGCCTTCTGC; GB27 for sense strand, 5′-TACAGGAAGGGCGGGCGGTTAGAACG; GB28 for sense strand, 5′-TCCCATCGGCCCCCGGTTCTCGA. These probes were labeled at the 5′-end with 32P by T4 polynucleotide kinase. For competition, in addition to nonlabeled GB63/67, oligonucleotide GB27/28 containing a GSG core sequence and GB68/52 carrying a mutated GSG element were synthesized. GB58 is the same as the one used in site-directed mutagenesis, and the others have sequences as follows: GB62 for sense strand, 5′-CAGGAAGCGGGGGGCGGAGGGGTAGAACG; GB67 for antisense strand, 5′-GTCACTCCCTCCACCGCCCAGCCTTCTGC; GB58 for sense strand, 5′-TACAGGAAGGGCGGGCGGTTAGAACG; GB27 for sense strand, 5′-TCCCATCGGCCCCCGGTTCTCGA. In some experiments, a GSG consensus

2 D. Krienc, G. Bai, J. W. Kusiaik, R. Brent, and S. A. Lipton, submitted for publication.
(Santa Cruz Biotechnology, Santa Cruz, CA) was labeled at the 5'-end with $^{32}$P by T<sub>4</sub> DNA polynucleotide kinase and used as a probe to examine the activity of synthesized Egr-1 protein. Polyclonal antibodies against Egr-1 or Sp1 protein (Santa Cruz Biotechnology) were used to confirm the specificity of Egr-1 binding. In a typical reaction, a slowly migrating band was formed after 1 ml of synthesized Egr-1 protein reacted with 25 fmol of labeled GSG consensus. This band was abolished by preincubation with a 20-fold excess of nonlabeled probe but not by an 80-fold excess of Sp1 consensus (Promega). The same band was supershifted only by Egr-1 antibody and not by Sp1 antibody. Synthesized Egr-1<sub>D</sub>331–374 failed to form this band with labeled GSG consensus.

RESULTS

We previously cloned and characterized a 3-kb promoter of the NMDAR1 gene. In luciferase reporter assays, this promoter exhibited high level expression in PC12 cells. This activity resided in a proximal, GC-rich region of the promoter near multiple transcriptional start sites (22). To test whether this promoter was sensitive to neurotrophic factor regulation, we initially transfected the full-length NMDAR1 promoter, fused to a luciferase gene (pNRL3029), into PC12 cells and added NGF to the cells 20 h after transfection. Transfection efficiency was monitored by co-transfecting a lacZ gene driven by a CMV promoter. We also measured the DNA content of each transfected plate of cells to control for differences in proliferation (slowed) and differentiation (enhanced) of the NGF-treated PC12 cells versus untreated cells. NGF treatment increased both the luciferase activity and the β-galactosidase activity of transfected cells. The luciferase activity increased with time and reached a maximum 48 h after the addition of NGF (Fig. 1A), while β-galactosidase activity was elevated at the earliest time point and remained above untreated cells throughout the experiment (Fig. 1B). These results suggested that NGF may up-regulate the NMDAR1 promoter and that the CMV promoter may have NGF-responsive elements. This result precluded the use of this lacZ construct as a control for transfection efficiency. We then tested the effect of NGF on PC12 cell

FIG. 1. Up-regulation of NMDAR1/luciferase reporter by NGF in PC12 cells. A and B, NGF effect on pNRL3029 in transiently transfected PC12 cells. PC12 cells were transfected with pNRL3029 as described under "Experimental Procedures." Twenty hours after transfection, some cells were treated with 100 ng/ml NGF and then collected at the indicated times for luciferase, β-galactosidase, and DNA assays as described under "Experimental Procedures." DNA content was used to correct the luciferase activities (A) and β-galactosidase activities (B). Open squares, nontreated cells; solid circles, NGF-treated cells. C, time course of NGF on NMDAR1 promoter/reporter activity in stably transfected PC12 cells. Constructs, pNRL3029/neo and pNRL356/neo, were separately transfected into PC12 cells, and the stable transfectants were selected with G-418 as described under "Experimental Procedures." Transfectant cells were treated with 100 ng/ml NGF 1 day after plating. Cells were collected, and the luciferase in cell lysates was measured. Open bars, pNRL356 transfectants; solid bars, pNRL3029 transfectants. D, dose effect of NGF on the NMDAR1 promoter in stably transfected PC12 cells. Stable transfectants as described in C were treated with the indicated dose of NGF for 4 h and collected for the luciferase assay. Open bars, pNRL356; solid bars, pNRL3029 transfectants.
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FIG. 2. Effect of NGF on the transcription of the luciferase gene directed by the NMDA promoter. A, luciferase mRNA level in pNRL3029 transfectants after NGF treatment. Stable PC12 transfectants carrying pNRL3029 were treated with 100 ng/ml of NGF for the indicated times. Total cellular RNA was extracted, and the luciferase mRNA was measured with a RPA using a 32P-labeled riboprobe as described under “Experimental Procedures.” The dried gel containing protected bands was exposed to both x-ray film and a PhosphorImager plate for quantitation. B, blockade of NGF effect by actinomycin D. Stable PC12 transfectants carrying pNRL356 and 3029 were pretreated with actinomycin D for 15 min and exposed to 100 ng/ml NGF for an additional 4 h. As controls, transfectant cells were treated with actinomycin D, NGF, or Me2SO vehicle alone. Open bars, pNRL356 transfectants; solid bars, pNRL3029 transfectants.

lines stably transfected with either a 3029- or a 356-bp proximal NMDAR1 promoter fused to a luciferase gene. NGF caused a time- and dose-dependent increase in luciferase activity (Fig. 1, C and D). Maximal activity, ~3.5-fold over untreated cells, was reached in 4 h, and NGF was effective at concentrations of 5 ng/ml with a maximum effect at about 50 ng/ml. This is in the range of its differentiating effect on PC12 cells (45). Similar NGF effects were seen in cell lines expressing either promoter fragment. The decline in luciferase activity after peak activity may be due to exhaustion of NGF from the culture medium. To confirm that the increase was due to transcriptional up-regulation and not translational modification of luciferase reporter, we measured luciferase mRNA levels by an RNase protection assay. Luciferase message levels increased as early as 30 min after NGF treatment and reached a maximal elevation of ~3.2-fold above untreated cells about 4 h after treatment (Fig. 2A). Actinomycin D totally abolished the NGF effect while having no effect on activity in untreated cells (Fig. 2B). These results suggest that the NGF effect was at the NMDAR1 promoter and was due to enhanced transcription of the reporter gene.

Since the 356-bp proximal promoter construct showed an NGF response similar to that of the 3-kb promoter (Fig. 1, C and D), we mutated the GSG and the two tandem Sp1 elements on this shorter fragment to test the importance of these sites in mediating the NGF effect. This fragment has basal activity similar to the 3-kb promoter in unstimulated cells (22). In transient transfections, mutagenesis of any one of the sites reduced basal activity to about 50% that of wild type promoter (Fig. 3A). In PC12 cell lines stably expressing these same mutant constructs, single mutations led to similar reductions in activity (Fig. 3B). However, mutations in both Sp1 sites (one of which overlaps the GSG site) reduced activity to about 10% of wild type in both transfection assays. We then examined the NGF responsiveness of these cell lines stably expressing the various mutations. Mutagenesis at each site alone reduced, but did not abolish, NGF responsiveness (Fig. 3B). However, mutagenesis at both Sp1 sites greatly reduced the NGF responsiveness.

The role of the GSG site in the NGF response was tested by transiently co-transfecting Egr-1/NGFI-A protein with the NMDAR1 promoter/luciferase reporter gene in PC12 cells. In these cells, overexpression of Egr-1/NGFI-A increased basal activity by about 2-fold. Transfection of a deletional mutant of Egr-1/NGFI-A, lacking the DNA binding domain failed to enhance activity. In another experiment, co-transfecting Egr-1/NGFI-A did not increase the activity of the GSG mutant luciferase construct (Fig. 3C). Also, we noticed that increasing the amount of co-transfected Egr-1 construct resulted in a reduced reporter activity. This reduction also was seen with increased deletional mutant of Egr-1/NGFI-A. Therefore, we believe that this reduction was due to a nonspecific inhibition due to excess DNA in the transfection. To correlate the transfection results seen in Fig. 4 with a molecular mechanism, we examined the interaction of synthesized Egr-1 protein with the GSG element in the NR1 promoter. In a gel shift experiment (Fig. 3D), an NR1 promoter oligonucleotide probe containing the GSG element formed a specific band with synthesized Egr-1 protein. The specificity of this band was confirmed with a GSG consensus probe, competitors, and antibody supershift (see “Experimental Procedures”). In Fig. 3D, mutated GSG oligonucleotide failed to compete the binding compared with strong competition by both nonlabeled probe and a short oligonucleotide containing the GSG core sequence. These experiments demonstrate that both the GSG and Sp1 sites play important roles in both basal and NGF-regulated activity of the NMDAR1 promoter.

To examine how NGF mediates its effect, we examined the role of low affinity neurotrophin receptor, p75NTR, and high affinity Trk A receptor. PC12 cells express both of these receptors but lack high affinity Trk B and C receptors for BDNF and NT-3 (46). Treatment of cells with BDNF, NT-3, and NT-4 at concentrations (100 ng/ml) sufficient to activate the p75NTR failed to increase luciferase activity in cell lines stably expressing the 356-bp proximal NMDAR1 promoter construct (Fig. 4A). Furthermore, the NGF effect was completely blocked by preincubation with K-252a (Fig. 4B), a potent inhibitor of Trk receptor kinase activity (47). These results suggest that NGF was having its effect by activation of Trk A receptors and not via the p75NTR low affinity receptors.

PC12 cells endogenously express low levels of NMDAR1 message in one predominant transcript and a very low level of receptor subunit protein (48). We examined whether NGF could regulate the expression of the endogenous gene by RNase protection assays with two probes to the NMDAR1 transcript. Even after 4 days of treatment with NGF, when neurite extension had occurred, PC12 cells did not show any changes in the levels of endogenous NMDAR1 message (Fig. 5). This experiment was repeated on several different PC12 cell lines with similar results. The half-life of the endogenous message was not different in cells treated with and without NGF (data not shown). Also, we did not detect any differences in the sequence
of the PC12 cell NMDAR1 gene promoter relative to the rat sequence that we originally characterized and used in the reporter constructs described here.

**DISCUSSION**

Neurotrophins and glutamate are thought to be involved in neuronal survival, growth, and plasticity (1–3, 23–25, 49–51). We therefore examined the effects of NGF treatment on activity of the previously isolated 3-kb promoter region of the NMDAR1 gene in PC12 cells. In this study, we observed that NGF, the prototype of neurotrophins, up-regulated the activity of NMDAR1 promoter/luciferase reporter constructs in both transient and stable transfection experiments. The dose response for this effect paralleled the neurite outgrowth and differentiation effects of NGF on PC12 cells and, like the continuous requirement for NGF to maintain a viable differentiated state in these cells (52), NMDAR1 promoter activation also required the continual presence of NGF. These characteristics of the NGF effect on the NMDAR1 promoter are in agreement with the effects of neurotrophins in establishing and maintaining the neuronal phenotype (52, 53). The expression of neurotrophins and their receptors is elevated during central nervous system development and declines in adulthood (54). Interestingly, expression of the NMDAR1 gene follows neurotrophin expression and also is elevated during early development compared with adult levels (10, 11). The NMDAR1 gene and several neurotrophin genes have an overlapping regional expression; high levels of both types of genes are found in the hippocampus, striatum, and cortex (4, 5, 23). Therefore, the preexistence of neurotrophins in the developing brain may be, in part, responsible for the high level of NMDAR1 message in the late postnatal period. Our result showing a gradual decline in promoter activity, apparently paralleling a decline in NGF from the culture medium, suggests that the continual presence of NGF is required for stable NMDAR1 message levels. This result also agrees with the continual need for trophic factor to support neuronal growth and maintain a differentiated state in

![Sp1 and GSG sites are important for the basal and NGF activities at the NMDAR1 promoter.](image-url)
vivo and in vitro (24, 52, 53). However, in models of severe ischemia, when neurotrophic factor expression is greatly elevated, NMDAR1 gene expression also can be induced in the hippocampus (see below) (13, 38, 39).

The proximal region (see Fig. 3A) of the NMDAR1 promoter contains a consensus GSG site that overlaps with a consensus Sp1 site, which, in turn, is separated by a single nucleotide from a second Sp1 site (5/6 consensus). Using site-directed mutagenesis experiments, we found that both the GSG-overlapping Sp1 and the downstream Sp1 sites were required for the NGF up-regulation of activity (Fig. 3B). Mutation of each site individually reduced the NGF effect by about 50%, while mutation of both sites almost completely abolished the NGF effect. Our previous results showed that deletion of this region of the promoter abolished basal activity of the promoter (22); the double mutation reported here also dramatically reduced basal activity.

The GSG element is a consensus binding motif for a group of NGF-inducible transcription factors termed NGFI/Egr transcription factors (i.e. NGFI-A/Egr-1, NGFI-C, Egr-2, and Egr-3) that are immediate early genes (33–36). Their expression is rapidly and transiently induced by a number of factors including serum, growth factors, and glutamate (35, 55, 56). They are thought to be the first wave of genes induced after receptor activation (24, 53). In PC12 cells, NGF-induced expression of NGFI-A and -C reaches a peak at 30 min (33, 34). In the present study, NGF up-regulated NMDAR1 promoter activity with a maximum at 4 h, following the NGFI-A response and may represent an example of a second wave of gene expression initiated by NGF. Furthermore, co-transfection of a cDNA for Egr-1 enhanced activity about 2-fold. Also, in gel mobility shift experiments, an oligonucleotide probe containing the intact GSG element of the NMDAR1 promoter was able to form a shifted complex with in vitro synthesized Egr-1. These results suggest that NGF-inducible genes play a role in activation of NMDAR1 gene expression.

The double mutation of the GSG/Sp1 and Sp1 sites abolished the NGF effect, suggesting that in addition to the GSG binding factors, Sp1 protein may be important in the NGF response. Sp1 is a ubiquitous transcription factor that may recruit TATA-box binding proteins or initiation complexes to the initiation site in promoters lacking consensus TATA-boxes (57, 58). The possibility exists that Sp1 proteins are required for NMDAR1 promoter activity and may interact with NGFI proteins to maximally activate NMDAR1 transcription.

PC12 cells express the high affinity NGF receptor Trk A but not Trk B and Trk C (46). Our results suggest that the NGF effect is due to activation of the Trk A receptor rather than the low affinity p75NTR receptor. The NGF effect was blocked by K-252a, an inhibitor of Trk tyrosine kinase activity (47). BDNF, NT-3, and NT-4, all of which interact with the p75NTR (23, 25, 46), did not induce activity of the NMDAR1 promoter. Since the Trk receptors share similar signal transduction pathways (46), the results reported here in PC12 cells may be extrapolated to the central nervous system, where several other neurotrophic factors including BDNF in the cortex may regulate expression of NMDA receptor genes (23). In global ischemia models, BDNF expression is elevated in the penumbral region and is seen in hippocampal neurons (38, 39). Maximal expression of BDNF is seen in severe ischemia, a condition under which NMDAR1 gene expression also is elevated (13, 38, 39). These increases are seen in CA1 pyramidal neurons of the hippocampus, which are highly sensitive to glutamate-induced toxicity (2, 41). These results suggest that high levels of neurotrophins may be required to up-regulate the NMDAR1 gene. Interestingly, an in vitro study of cultured primary cortical neurons that contain Trk B receptors (23) showed that a high dose of BDNF potentiated NMDA-induced necrosis (29).

Neurotrophins are generally thought to protect neurons from various stimuli (23, 46). In fact, toxic activation of NMDA receptors is known to increase expression of NGF, BDNF, TrkB, and TrkC (30). Our results with NGF activation of
NMDAR1 expression suggest a potential for a lethal cycle of trophic factor up-regulation increasing NMDA receptor expression. This overexpression of NMDA receptors may then be responsible for neurotoxicity. However, it is possible that the levels of neurotrophins elicted by various insults may be lower than that required to induce expression of NMDA receptor genes; alternatively, these neurotrophins may induce other gene cascades that are protective of neurons. Interestingly, cerebellar granule neurons require NMDA receptor activity in addition to trophic factor support for survival and differentiation (49–51). Granule cells express Trk B receptors (23). BDNF may accelerate the maturation (expression) of NMDA receptor genes normally seen in embryonic neurons in culture (31). It may be that low levels of a positive feedback loop are necessary for optimum survival of neurons, but a major insult that dramatically increases expression of one of these players may induce an accelerated neurotoxic feedback loop (13, 38, 39).

PC12 cells express one of eight possible isoforms of the NMDAR1 gene and low levels of NMDAR2-A, -C, and -D (48, 59, 60). However, several studies have shown that these cells have very little functional NMDA channel activity. Sucher et al. (48) reported barely detectable NMDAR1 protein levels relative to the level of transcripts. Interestingly, in several studies and in results reported here, NGF failed to increase the levels of endogenous NMDAR1 message (48). This resistance to NGF treatment was not due to a change in mRNA stability or mutations in the endogenous promoter sequence. As we have reported previously, the proximal region of the NMDAR1 promoter is GC-rich and has a high frequency of CpG islands, the endogenous gene promoter is rich in CpG islands yet appears to be GC-poor (63) observed that the NGF Regulation of the NMDAR1 Promoter in PC12 Cells.