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Basic FGF Regulates the Expression of a Functional 71 kDa NMDA Receptor Protein That Mediates Calcium Influx and Neurotoxicity in Hippocampal Neurons

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Basic fibroblast growth factor (bFGF) was recently found to modulate the outgrowth-regulating effects of glutamate, and protected neurons from several brain regions against excitotoxic/ischemic damage. We provide evidence that the excitoprotective mechanism of bFGF involves suppression of the expression of a 71 kDa NMDA receptor protein (NMDARP-71). NMDARP-71 protein and mRNA levels were reduced in neurons in bFGF-treated hippocampal cell cultures. The levels of the NMDARP-71 were not reduced by NGF or epidermal growth factor, and bFGF did not reduce the level of mRNA for the GluR1 kainate/AMPA receptor, demonstrating the specificity of the effect of bFGF on the NMDARP-71. The reduction in NMDARP-71 expression in bFGF-treated neurons was correlated with reduced vulnerability to NMDA neurotoxicity. A major role for NMDARP-71 in calcium responses to NMDA and excitotoxicity was demonstrated using antisense oligonucleotides directed against NMDARP-71. Northern and Western blot analysis and immunocytochemistry showed that NMDARP-71 antisense oligonucleotides caused a selective suppression of NMDARP-71 mRNA and protein levels during 12–44 hr exposure periods. Elevations in intracellular calcium levels normally caused by glutamate and NMDA were attenuated in neurons exposed to NMDARP-71 antisense oligonucleotide; calcium responses to kainate were relatively unaffected. NMDARP-71 antisense oligonucleotides protected the neurons against excitotoxicity. Thus, NMDARP-71 is a necessary component of an NMDA receptor mediating calcium responses and neurotoxicity in hippocampal neurons. Taken together, these data identify a mechanism whereby bFGF can modify neuronal responses to glutamate, and suggest that regulating the expression of excitatory amino acid receptors may provide a means for growth factors to influence the plasticity and degeneration of neural circuits.

[Key words: antisense oligonucleotide, calcium, excitotoxicity, fura-2, growth factors, mRNA, neuronal death, NMDA]

Glutamate is the major excitatory neurotransmitter in the mammalian brain (Fonnum, 1984), and receptors for glutamate mediate most higher brain functions including learning and memory (Collingridge and Bliss, 1987; Cotman and Iversen, 1987). Glutamate receptors are broadly classified into two categories, namely, NMDA receptors and non-NMDA receptors. Non-NMDA receptors (those responsive to the agonists AMPA, kainate, quisqualate) mediate routine fast synaptic transmission in various cortical and subcortical regions. NMDA receptors, on the other hand, may have more specialized roles in neural plasticity. Thus, in the developing nervous system NMDA receptors are involved in the "fine tuning" of synaptic connections (Mattson, 1988; Mattson et al., 1988a,b; Cline and Constantine-Paton, 1990; McDonald and Johnston, 1990) and at mature synapses NMDA receptors are critical to the process of long-term potentiation (LTP), a cellular correlate of learning and memory. NMDA receptors have the unique property of conducting calcium ions at a high rate (Mayer and Miller, 1990). Calcium influx through the NMDA receptor is essential for LTP (Malenka et al., 1988). Ironically, the calcium-conducting property of NMDA receptors can prove detrimental to neurons, as overactivation of NMDA receptors can result in neuritic degeneration and cell death (Rothman and Olney, 1987; Choi, 1988; Mattson, 1992). Ample evidence supports the involvement of NMDA receptors in the neuronal damage that occurs in stroke, epilepsy, and Alzheimer's disease (for reviews, see Siesjo et al., 1989; Greenamyre and Young, 1989; Mattson, 1992).

Within the last three years, several glutamate receptor proteins have been identified and characterized at the molecular level. A family of AMPA/kainate receptor proteins that has been cloned (Hollmann et al., 1989; Boulter et al., 1990) apparently combines in various permutations to form multisubunit receptors. Recently, a putative NMDA receptor protein complex was isolated from rat brain synaptic membranes (Ikin et al., 1990; Kumar et al., 1991; Ly and Michaelis, 1991) and the cDNA for a 71 kDa subunit of this complex (NMDARP-71) was cloned (Kumar et al., 1991). Protein reconstitution and pharmacological studies suggest that NMDARP-71 is the glutamate-binding subunit of a complex of four proteins that comprise a functional NMDA receptor–ion channel (Kumar et al., 1991; Ly and Michaelis, 1991; Minami et al., 1991). The latter conclusion is consistent with molecular characterizations of the NMDA receptor through the use of conotoxins, as well as electrophysiological characterization of the NMDA receptor (Ascher and Nowak, 1987; Clements and Westbrook, 1991). NMDARP-71 was shown to be localized to postsynaptic sites in hippocampus *in*

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vivo (Eaton et al., 1990) and to dendrosomatic compartments in hippocampal pyramidal-like neurons in cell culture (Mattson et al., 1991). A strong correlation between the presence of NMDARP-71 immunoreactivity and vulnerability to glutamate neurotoxicity was established (Mattson et al., 1991).

In addition to NMDARP-71, the cDNA for a single protein of approximately 100 kDa that possesses electrophysiological properties of an NMDA receptor when expressed in frog oocytes was also cloned (Moriyoshi et al., 1991; Yamazaki et al., 1992). The latter protein, referred to as NMDAR1, has not been purified, but its structure is inferred from the cloned cDNA and exhibits considerable homology to the kainate/AMPA receptor family (Hollman et al., 1989; Boulter et al., 1990; Keinanen et al., 1990). Several homologs of NMDAR1 were recently cloned also and shown to form functional receptor complexes with NMDAR1 (Monyer et al., 1992). The NMDARP-71 subunit does not have such homology to the 100 kDa glutamate receptor proteins (Kumar et al., 1991). The role of the NMDAR1 or the homologs NMDAR2A–C in neurons has not been defined. It is now essential to establish which NMDA receptor proteins are normally functional and mediate the calcium influx that underlies roles of NMDA receptors in neural plasticity and degeneration. In the present study, antisense oligonucleotides directed against the NMDARP-71 were used to establish roles for this protein in NMDA-induced calcium influx and neurotoxicity in hippocampal neurons.

An increasing number of growth factors are being identified that influence neuronal survival and plasticity. Among neuronal growth factors, NGF and basic fibroblast growth factor (bFGF) have been the most intensively studied. Both NGF and bFGF have been shown to influence the outgrowth and survival of developing neurons from specific regions of the CNS; bFGF may have a much broader array of neural targets than does NGF (see Hefti et al., 1989, for review). In addition to influencing the development and plasticity of neural circuits, growth factors may play roles in protecting neurons in a variety of neurodegenerative conditions. For example, bFGF and NGF can protect neurons in cell culture (Cheng and Mattson, 1991) and animal models of ischemia (Berlove et al., 1991; Shigeno et al., 1991). These growth factors may also play a neuroprotective role in more chronic neurodegenerative disorders such as Alzheimer's disease (Hefti et al., 1989; Cheng and Mattson, 1992a). The mechanisms of action of growth factors in protecting neurons against environmental insults are not clear.

In earlier studies, we found that bFGF could protect cultured rat hippocampal neurons against glutamate neurotoxicity (Mattson et al., 1989) and hypoglycemic damage (Cheng and Mattson, 1991). In the present study we explored the possibility that the mechanism of the excitoprotective action of bFGF may be through regulation of gene expression of NMDARP-71. Because of the availability of highly specific monoclonal antibodies against the NMDARP-71, the regulation of expression of this protein in neurons in response to bFGF could be directly examined. Exposure of neurons to bFGF reduced the levels of NMDARP-71 mRNA and proteins, and reduced neuronal vulnerability to excitotoxicity.

Materials and Methods

Cell culture. Procedures for dissociated cell culture of embryonic day 18 rat hippocampus are detailed in our previous reports (Mattson et al., 1988a, 1991). Cultures were plated at a low cell density (70–120 cells/mm^2 of culture surface), and were maintained in a medium consisting of Eagle's minimum essential medium (MEM) supplemented 10% with fetal bovine serum and containing 1 mM pyruvate and 20 mM KCl (0.8 ml/35 mm culture dish). For antisense oligonucleotide experiments cultures were maintained in a defined medium consisting of Eagle's MEM supplemented with 5 μg/ml bovine insulin, 100 μg/ml human transferrin, 100 μg/ml BSA (fraction V), 60 ng/ml progesterone, 16 μg/ml putrescine, 40 ng/ml sodium selenite, 42 ng/ml thyroxine, 33 ng/ml tri-iodo-L-thyronine, 1 mM pyruvate, and 20 mM KCl (all from Sigma). In some experiments cultures were maintained in defined medium from culture day 1 onward. Glial proliferation was greatly reduced in the cultures maintained in defined medium as compared to serum-containing medium. At the time of experimentation (culture days 6–14), non-neuronal cells constituted 10–30% and less than 2% of the total number of cells in the cultures maintained in serum-supplemented and defined medium, respectively. Cultures were maintained in an atmosphere containing 6% CO_2, 94% room air (37°C).

Oligonucleotides. Oligonucleotides were synthesized by the University of Kentucky molecular biology facility using an Applied Biosystems 380B synthesizer. Oligonucleotides were purified using reverse-phase C18 Sep-Pak cartridges (Waters). Oligonucleotide stocks were prepared in sterile water at concentrations of 1–2 mM. The oligonucleotides used in the present study included NMDARP-71 (Kumar et al., 1991) antisense 1 (AS1), 5′-GAGGGCTGGAAAGCGGCCTGT-3′; NMDARP-71 antisense 2 (AS2), 5′-GAAACTCTTTTCATGGTACA-3′; NMDARP-71 sense, 5′-ACAGGCCGCTTTCCAGCCCTC-3′. All experiments involving oligonucleotides were carried out in cultures containing defined medium (composition is given above).

Experimental treatment of cultures and analysis of neuronal survival. L-Glutamate, NMDA, kainate, quisqualate, and aminophosphonovaleric acid (APV; Sigma) were prepared as 100–500× stocks in culture medium (pH 7.2). Basic FGF (bovine recombinant), NGF (2.5S from mouse submaxillary glands), and epidermal growth factor (EGF; from mouse submaxillary glands) were purchased from Boehringer Mannheim and were prepared as 500× stocks in cultured medium. Unless otherwise stated, growth factors were added on culture day 3 and oligonucleotides were added on culture day 6. Basic FGF (10 ng/ml) was added to cultures 24 hr prior to the addition of excitatory amino acids (EAAs). Neuronal survival was assessed by phase-contrast light microscopy as described previously (Mattson et al., 1988a, 1991). Briefly, neurites that normally are uniform in diameter and smooth in appearance become fragmented and "beaded" during the degenerative process. In addition, the soma, which is normally smooth and round to oval in shape, becomes rough, vacuolated, and irregular in shape in degenerating neurons. Viable neurons in premarked microscope fields (each field was approximately 1 mm^2) were counted prior to and 8 hr following exposure to EAAs.

Immunocytochemistry. These methods were similar to those previously reported (Mattson et al., 1991). Cells were fixed for 20–30 min in cold fixative that consisted of a solution of 2% paraformaldehyde, 100 mM D,L-lysine, 10 mM sodium *m*-periodate, and 0.5% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS). Cell membranes were permeabilized by exposure to a 0.2% solution of Triton X-100 in PBS for 5 min. Cells were then incubated for 30 min in the presence of blocking serum (nonimmune horse serum, 1:200), followed by a 3 hr incubation in PBS containing the primary antibody. Two monoclonal antibodies against the NMDARP-71 isolated from rat or bovine brain (clones $27G_8E_{11}$, IgM, and $17G_{11}C_2$, IgG, respectively) were used at a dilution of 1:10. Following incubation for 3 hr in the presence of the primary antibody, the cultures were processed using a Vector Labs ABC kit with goat anti-mouse biotinylated secondary antibody, avidin–peroxidase complex, and diaminobenzidine tetrahydrochloride. Possible nonspecific staining was evaluated by eliminating the primary antibody from the staining procedure, or preabsorbing the primary antibody with a threefold excess of purified antigen (NMDARP-71). The NMDARP-71 was purified from rat brain synaptic membranes according to the procedure described in Wang et al. (1992). Western blots performed with this purified protein and monoclonal antibodies that were preadsorbed with a threefold excess of NMDARP-71 produced no detectable labeling of this protein by the antibodies. The same exposure conditions were used to photograph all cells, and photographic prints were exposed for the same time periods. Comparisons of neuronal immunoreactivity were made by scoring the staining intensity of individual neurons on a scale from 0 to 3 (0, no staining; 1, light; 2, moderate; 3, heavy). Cultures were scored without knowledge of their treatment history. A total of 400 neurons were scored in four separate cultures (100 neurons/culture).

Western blot analysis. These methods are detailed elsewhere (Mattson et al., 1991). Proteins from untreated control cultures, and cultures treated with oligonucleotides or growth factors were separated on 10% SDS-PAGE and electrotransferred to nitrocellulose filters. The nitrocellulose sheets were reacted with NMDARP-71 antibody $27G_8E_{11}$ (IgM) (hybridoma culture supernatant). This was followed by incubation with alkaline phosphatase–conjugated goat anti-mouse IgM antibodies in order to reveal the immunolabeled proteins. The relative amount of immune reaction products formed on Western blots was measured by image analysis procedures using a Universal Imaging System (Balazs et al., 1992). The protein concentration of cell homogenate samples was estimated by the BCA method (Pierce Chemical).

Northern blot analysis. Hippocampal neurons were cultured as described above, including exposure to oligonucleotides and growth factors. Each population of RNA was prepared by the guanidine isothiocyanate–cesium chloride purification method. Total RNA was size-fractionated on a 1% formaldehyde-agarose gel and transferred to Nytran (MSI) membranes. A 550 base pair (bp) Pst 1 restriction fragment of pGBA-2 (NMDARP-71 cDNA), a 570 bp Pst 1 fragment of the cDNA clone p59/2 for GluR1, and the full-length cDNA for NMDAR1 were purified by extracting from low-melting-temperature agarose gels and labeled with ^{32}P-dATP by the random primer labeling method (total activity of 5.2×10^8 dpm in 15 ml hybridization buffer). These probes were hybridized to the RNA blot. RNA concentrations were estimated by measuring absorbance ratios at 260:280 nm. The specific amounts of RNA introduced into each lane are indicated in the figure captions. Agarose gels stained with ethidium bromide were also examined to determine equal loading with RNA. Prehybridization was carried out at 42°C for 3 hr in 5× SSPE (1× = 0.18 M NaCl, 10 mM sodium phosphate, 1 mM EDTA), 5× Denhardt's solution (1× = 0.02% ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 0.1% SDS, and 50 μg/ml of denatured salmon sperm DNA. Hybridization was done at 42°C for 16 hr in the same buffer that included 50% formamide. Filters were washed with 1× SSPE, 0.5% SDS at 37°C followed by 0.1× SSPE, 0.1% SDS at 65°C. The filters were exposed to Kodak X-Omat film with an intensifying screen for 48 hr. The relative amount of RNA labeled by the various probes was quantified by image analysis procedures similar to those used for Western blot analyses. The filters were then stripped and probed with a 27-mer rat β-actin oligonucleotide (Clontech), which was used as a control probe, and the autoradiograms obtained were subjected to image analysis.

Measurements of intracellular free calcium levels. Fluorescence ratio imaging of the calcium indicator dye fura-2 was used to quantify intracellular free calcium levels in the cultured hippocampal neurons. The methods used were identical to those used in our past studies (Mattson et al., 1989; Cheng and Mattson, 1991). Briefly, cells were loaded for 30 min with a 2–4 μM concentration of the acetoxymethylester form of fura-2. The cells were then washed and incubated an additional 1 hr. Prior to imaging the culture maintenance medium was replaced with a medium consisting of Hanks' balanced salt solution (HBSS) containing 10 mM HEPES, 20 mM glucose, and 2 mM $CaCl_2$. Cells were imaged with epifluorescent illumination (xenon UV source) with an inverted Nikon Diaphot microscope (fluoro 40×, 1.3 NA fluorescence objective) coupled to an intensified CCD camera (Quantex), and a Quantex imaging system equipped with QFM software. Intracellular Ca^{2+} levels were determined from the ratio of the fluorescence emission using two different excitation wavelengths (350 nm and 380 nm). Background fluorescence at each wavelength (background images were taken from regions of the culture dish not containing cells) was subtracted from the cell image at that wavelength. The system was calibrated according to the procedures of Grynkiewicz et al. (1985) using the formula $[Ca]_i = K_d[(R - R_{min})/(R_{max} - R)](F_0/F_s)$. For our system, $R_{min} = 0.98$, $R_{max} = 10.40$, $F_0/F_s = 13.99$, and $K_d = 224.0$. Values represent the average free calcium level in the neuronal cell body. Statistical comparisons were made using Student's *t* tests.

Results

bFGF reduces NMDARP-71 protein levels in cultured hippocampal neurons

Levels of NMDARP-71 immunoreactivity were markedly reduced in cultures maintained for 0.5–4 d in the presence of 10 ng/ml of bFGF (Fig. 1). Staining was reduced progressively over a period of 12–48 hr of exposure to bFGF, and remained low through at least 4 d of exposure. After 2 d of exposure, average neuronal staining intensities were 1.98 ± 0.06 in control cultures and 0.56 ± 0.05 in bFGF-treated cultures ($n = 400$ neurons in four separate cultures; $p < 0.001$; see Materials and Methods). Neuronal NMDARP-71 immunoreactivity was not changed in cultures maintained for 2 d in the presence of 10 ng/ml of either NGF or EGF ($n = 4$ separate cultures). The reduction in NMDARP-71 immunoreactivity caused by bFGF apparently resulted from a direct action of bFGF on neurons since bFGF also reduced NMDARP immunoreactivity in cultures maintained in a completely defined medium in which less than 2% of the cells in the cultures were non-neuronal (see Materials and Methods). Under the latter conditions bFGF did not significantly affect astrocyte numbers during a 2 d exposure period (astrocyte density was 1.2 ± 0.21/mm² of culture surface in control cultures and 1.5 ± 2.3/mm² in bFGF-treated cultures; $n = 5$ separate cultures). The bFGF-induced reduction in NMDARP-71 levels was confirmed by Western blot analyses of cultured cell homogenates (Fig. 2). Gels were loaded with equivalent amounts of protein from cell cultures exposed for 4 d to 10 ng/ml of bFGF, NGF, EGF, or no growth factor. Our previous dose–response studies showed that bFGF and NGF at 10 ng/ml were maximally effective in protecting cultured hippocampal neurons against hypoglycemic damage, while EGF was ineffective at this concentration (Cheng and Mattson, 1991). Immunoblots with a monoclonal NMDARP-71 antibody demonstrated a clear decrease in the expression of the 71 kDa NMDARP-71 in bFGF-treated cultures compared with untreated control cultures (Fig. 2). Other bands in Coomassie blue–stained gels were not changed by bFGF (data not shown), suggesting that the actions of bFGF were specific. In lanes that were loaded with 60 μg of solubilized cell proteins, there is the appearance of a second band of protein (60–63 kDa) labeled by the antibodies. This band of protein is probably the result of protein breakdown as was discussed in a previous publication (Eaton et al., 1990). NGF and EGF did not influence the levels of immunolabeled NMDARP-71 on Western blots (Fig. 2). In addition, levels of NMDARP-71 immunoreactivity were reduced by bFGF in neuron-enriched cultures in which glial proliferation was blocked by incubation in a defined medium lacking serum. Average neuronal staining intensities were, for untreated control culture, 2.10; culture treated for 2 d with 10 ng/ml bFGF, 1.27; culture treated for 2 d with 10 ng/ml NGF, 1.96; and culture treated for 2 d with 10 ng/ml EGF, 1.87 ($n =$ 100 neurons scored for each treatment condition). No immunolabeling of cells or isolated proteins was seen in immunocytochemistry or Western blots when nonimmune mouse immunoglobulins were used as primary antibodies (data not shown). In addition, preabsorption of the primary antibody with excess purified NMDARP-71 abolished neuronal staining (see Fig. 7).

bFGF reduces levels of mRNA encoding NMDARP-71

The decrease in levels of NMDARP-71 in neurons treated with bFGF may have resulted from an effect of this growth factor on protein synthesis or degradation. In order to determine whether bFGF affected the expression of mRNA for the NMDARP-71, we analyzed the levels of NMDARP-71 mRNA in control and bFGF-treated hippocampal cultures. The Northern blots shown in Figure 3 demonstrate that the effect of a 4 d exposure of cultured hippocampal neurons to bFGF is a decrease in the level of mRNA that hybridizes with the NMDARP-71 probe. The 1.8 kilobase (kb) RNA species labeled in cultured hippocampal

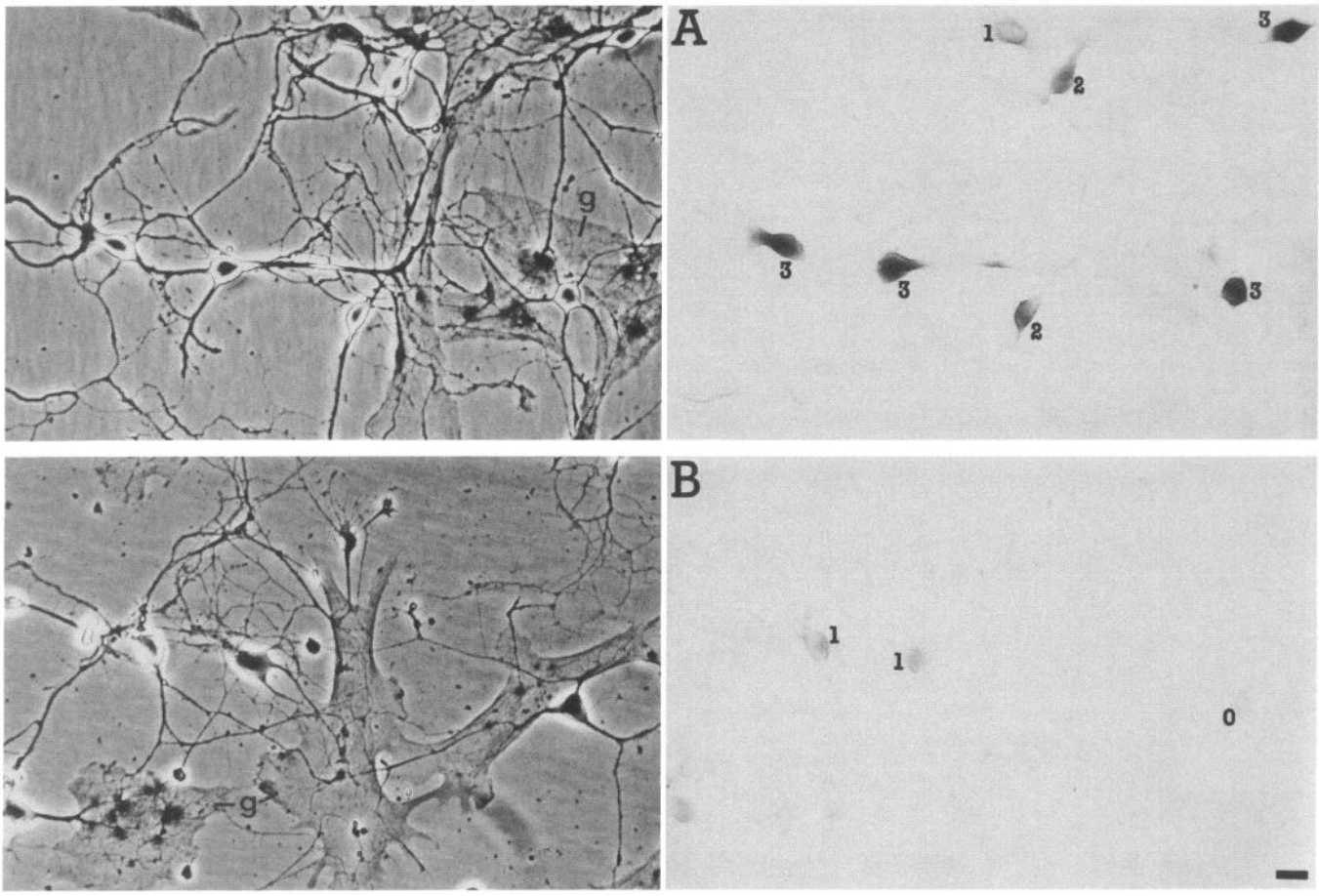

Figure 1. bFGF reduces the level of NMDARP-71 immunoreactivity in hippocampal cell cultures. Neuronal NMDARP-71 immunoreactivity in an untreated 6-d-old culture is intense (*A*). Neuronal NMDARP-71 immunoreactivity in a 6-d-old culture to which bFGF (10 ng/ml) was added on culture days 4 and 5 is markedly reduced (*B*). *Numbers* adjacent to neurons indicate staining intensity on a relative scale: *0*, no staining; *1*, light; *2*, moderate; *3*, intense. *g*, glial cell. These results are representative of those obtained in eight separate control and bFGF-treated cultures. Scale bar, 10 μm.

neurons appears to be identical to that labeled in RNA extracted from whole rat brain (Fig. 3) or from various brain regions (Kumar et al., 1991). The effect of bFGF on the RNA for the NMDARP-71 appears to be very selective, as RNA from the same cells labeled with a cDNA probe for GluR1, the clone for the 100 kDa kainate/AMPA receptor (Hollman et al., 1989; Keinanen et al., 1990), was not decreased in bFGF-treated neurons (Fig. 3). Densitometric analysis of Northern blots demonstrated a time-dependent reduction in NMDARP-71 mRNA levels in bFGF-treated cultures (Fig. 4). NMDARP-71 mRNA levels were reduced to 91% and 73% of control levels within 24 and 48 hr of exposure to bFGF, respectively, and continued to decrease to 21% of control levels after 4 d of exposure. The data shown in Figures 3 and 4 were derived from three separate experiments, each Northern blot analysis performed at least twice.

bFGF protects neurons against excitotoxicity

We previously reported that bFGF protected cultured hippocampal neurons against glutamate neurotoxicity (Mattson et al., 1989), but we did not establish whether the protection was specific for activation of a particular class of EAA receptor. In the present study we examined whether the protective effect of bFGF was specific for NMDA-induced excitotoxicity. Neuronal survival in cultures exposed to NMDA was significantly enhanced in cultures maintained in the presence of bFGF (Fig. 5). In addition, calcium responses to NMDA (measured using fluorescence ratio imaging of the calcium indicator dye fura-2) were reduced in bFGF-treated neurons (data not shown; cf. Mattson et al., 1989). Thus, the reduced expression of NMDARP-71 was reflected in reduced neuronal vulnerability to NMDA neurotoxicity. Neuronal survival in cultures exposed to kainate or quisqualate was also significantly enhanced by bFGF, suggesting that bFGF might also influence the expression or function of non-NMDA receptors. On the other hand, the Northern blot analysis above indicated that kainate/AMPA GluR1 receptor levels were not reduced by bFGF. In order to resolve this problem, we tested the hypothesis that the reduced vulnerability to kainate and quisqualate in bFGF-treated cultures resulted from reduced expression of NMDA receptors. This possibility was suggested by the results of recent studies demonstrating that activation of non-NMDA receptors, which results in membrane depolarization, can induce glutamate release from the cells, which then activates NMDA receptors (Sucher et al., 1991). Hippocampal cultures incubated in the presence of the NMDA receptor antagonist APV were exposed to kainate or quisqualate and neuronal survival in these cultures was compared to that in cultures exposed to the agonists in the absence of APV. APV significantly reduced neuronal death in cultures exposed to kainate or quisqualate (Fig. 5), indicating that the neurotoxicities

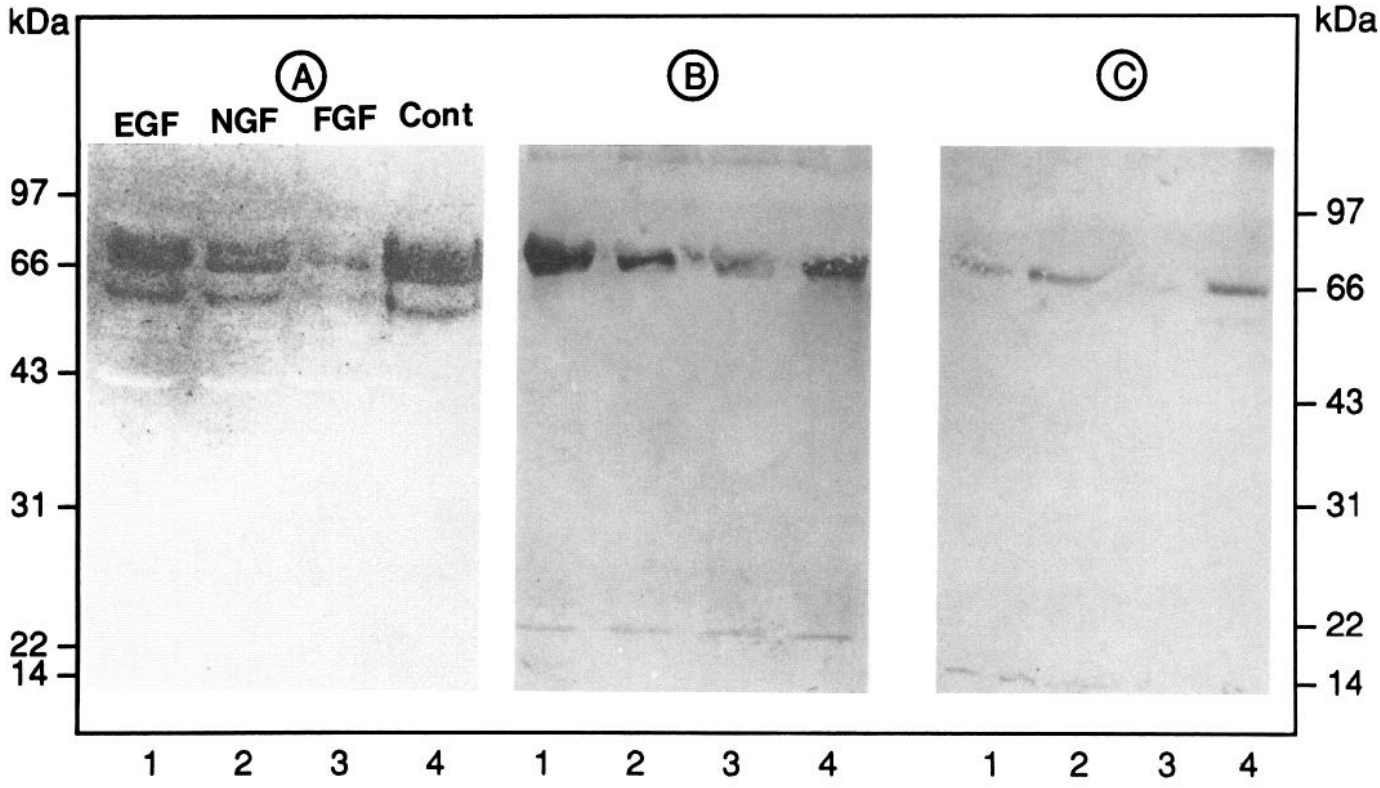

Figure 2. Western blot analysis of NMDARP-71 in growth factor-treated cultures. *Lanes 1–4* in *A–C* represent proteins from cultures treated for 4 d with EGF, NGF, bFGF, and an untreated culture, respectively. Solubilized proteins (60, 40, and 20 μg) were loaded into each lane in A–C, respectively. Note that bFGF reduced the level of NMDARP-71–immunoreactive band in all three cases. These results are representative of those obtained in three separate experiments.

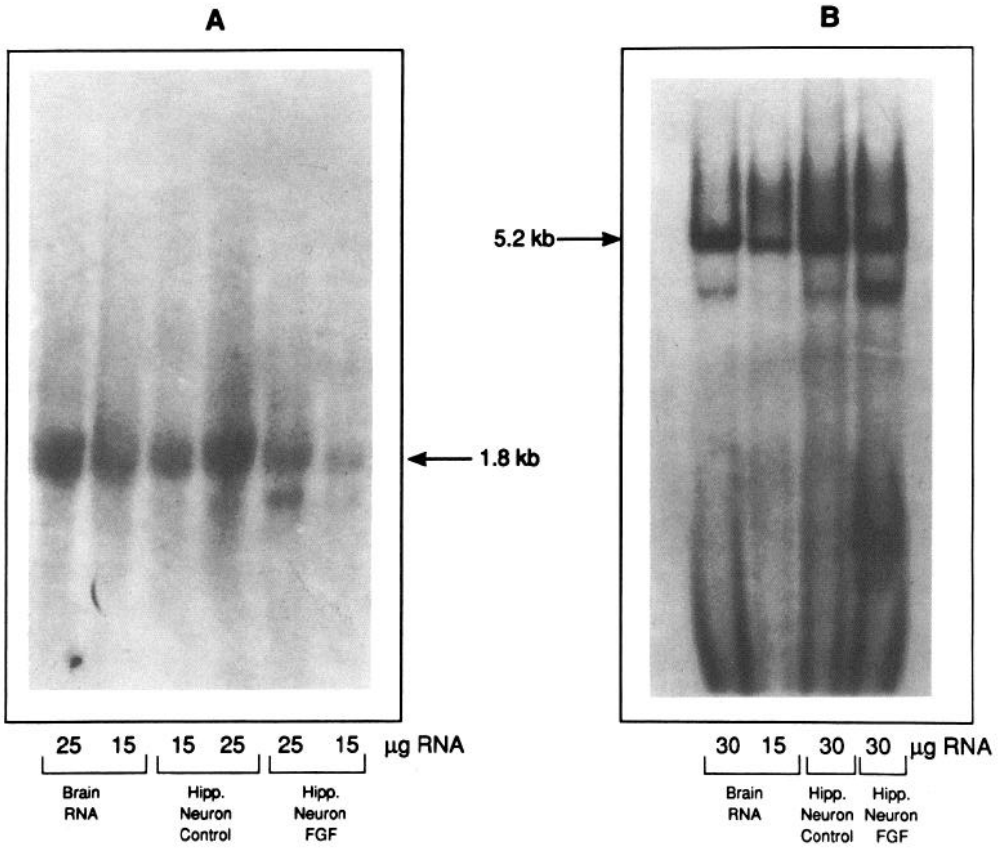

Figure 3. bFGF causes a selective decrease in levels of mRNA for the NMDARP-71. Shown are Northern blots using restriction fragments of the cDNAs for the NMDARP-71 (pGBA-2; Kumar et al., 1991) and the kainate/AMPA receptor (GluR1; Hollman et al., 1989; Keinanen et al., 1990). RNA was prepared from either whole rat brain or rat hippocampal cell cultures that either were not treated (control) or were treated with 10 ng/ml bFGF for 4 d. The amounts of RNA loaded into each lane are indicated at the *bottom*. The results of Northern hybridization with pGBA-2 are shown in *A*; those with GluR1 are in *B*. Note that bFGF caused a reduction in the level of mRNA for NMDARP-71 but did not decrease the level of GluR1 mRNA.

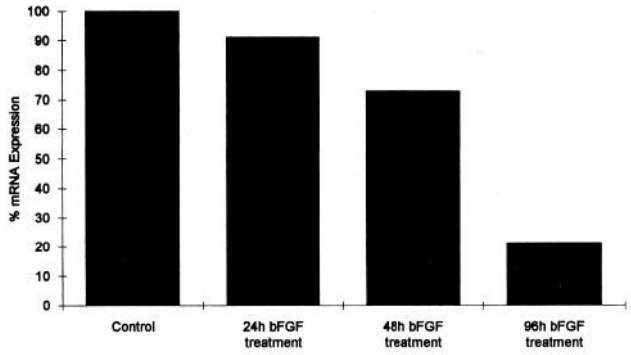

Figure 4. Time course of reduction in NMDARP-71 mRNA levels in cultures exposed to bFGF. Parallel hippocampal cell cultures were untreated (control) or were exposed to 10 ng/ml bFGF for the indicated number of days. NMDARP-71 mRNA levels were quantified by densitometric analysis of the 1.8 kb RNA species on Northern blots probed with the PstI fragment of pGBA-2. The levels for the treated cultures are expressed as percentage of the respective control culture. In each case, 15 μg of RNA was loaded onto each lane.

of these agonists were mediated, at least in part, via NMDA receptors.

We previously demonstrated that neurons contacting glia in hippocampal cultures were protected against glutamate neurotoxicity and that astrocyte-derived bFGF played a role in this excitoprotective action (Mattson and Rychlik, 1990). Taken together with the data above, the latter observation suggested that neurons contacting glia should exhibit reduced levels of NMDARP-71. This possibility was tested by comparing levels of NMDARP-71 immunoreactivity in neurons contacting astrocytes with those not contacting astrocytes (essentially all of the non-neuronal cells in these cultures were immunoreactive toward glial fibrillary acidic protein antibodies and were therefore presumed to be type I astrocytes; cf. Mattson et al., 1988a; Mattson and Rychlik, 1990). NMDARP-71 immunoreactivity was significantly less in neurons contacting astrocytes as compared to neurons not contacting astrocytes (average staining intensity of 0.98 ± 0.32 in neurons contacting an astrocyte and 2.28 ± 0.09 in neurons not contacting an astrocyte; mean ± SEM of determinations made in four separate cultures with 100 neurons scored/culture; $t = 12.9$, $p < 0.001$, Student's t test). An example of reduced NMDARP-71 immunoreactivity in neurons contacting astrocytes is shown in Figure 6. In some experiments cultures were exposed to glutamate for 8 hr and then immunostained with NMDARP-71 antibodies. In such cultures, neurons contacting astrocytes were protected against glutamate toxicity and generally showed low levels of NMDARP-71 immunoreactivity. As previously described (Mattson and Rychlik, 1990), neurons not contacting astrocytes were more vulnerable to excitotoxicity, and stained more intensely with NMDARP-71 antibodies (Fig. 6). These data are consistent with our previous work in which a correlation between expression of NMDARP-71 and vulnerability to excitotoxicity was established (Mattson et al., 1991). In order to probe more directly the relationship between NMDARP-71 and the vulnerability of hippocampal neurons to excitotoxicity elicited by NMDA, we utilized the approach of pretreating neurons with antisense oligonucleotides to the NMDARP-71 to block selectively the expression of this protein.

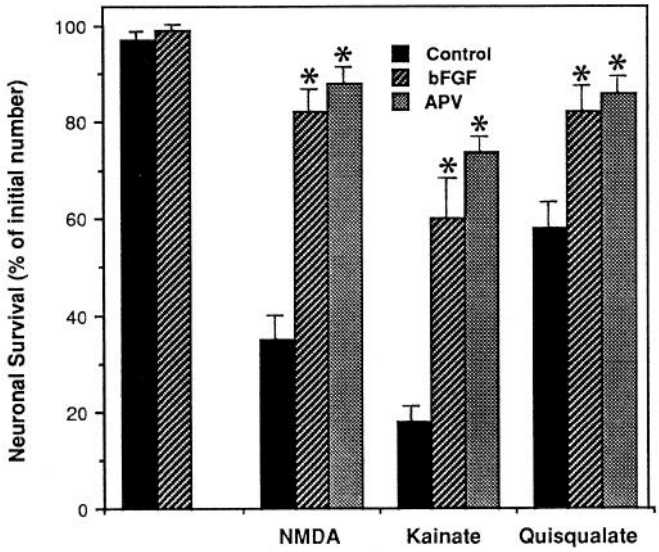

Figure 5. bFGF protects against NMDA receptor–mediated neurotoxicity in hippocampal cell cultures. Hippocampal cells (6 d in culture) were exposed to the indicated EAA agonists and/or antagonists for 8 hr and the percentage of neurons surviving the exposure period was determined. Values represent the mean and SEM of determinations made in four separate cultures. *, significant difference from control value ($p < 0.01$ for NMDA and kainate; $p < 0.05$ for quisqualate; paired Student's t test).

NMDARP-71 receptor protein and mRNA levels are reduced in neurons exposed to antisense oligonucleotides

Hippocampal cell cultures either were left untreated or were exposed to a 10–50 μM concentration of NMDARP-71 sense, antisense 1 (AS1), or antisense 2 (AS2) oligonucleotide. Cultures were fixed at 12, 20, and 44 hr following treatment, and were immunostained using monoclonal antibodies to NMDARP-71 (Mattson et al., 1991). The neuronal immunoreactivity was eliminated by absorption of the primary antibody with excess NMDARP-71 protein (Fig. 7). NMDARP-71 immunoreactivity was greatly reduced in cultures exposed to AS1 or AS2 for 12–20 hr relative to untreated cultures or cultures exposed to NMDARP-71 sense DNA (Figs. 7, 8). The greatest reduction in NMDARP-71 immunoreactivity was observed in cultures exposed for 20 hr to AS1 and AS2 (50 μM), and levels of immunoreactivity subsequently increased between 20 and 44 hr of exposure. The effects of AS1 and AS2 were concentration dependent, as a greater reduction in immunoreactivity was observed following a 20 hr exposure to 50 μM NMDARP-71 AS1 or AS2 than in parallel cultures exposed to 10 μM NMDARP-71 AS1 or AS2 (Fig. 8).

Western blot analyses of proteins extracted from cultures exposed to sense or antisense oligonucleotides for 22 hr confirmed that a reduction in levels of NMDARP-71 did, in fact, occur in cultures exposed to NMDARP-71 antisense oligonucleotide (Fig. 9). The levels of NMDARP-71 in untreated cultures were nearly identical to those in cultures exposed to sense NMDARP-71 oligonucleotide. Treatment of these primary neuronal cultures with NMDARP-71 antisense oligonucleotide brought about a 48% decrease in the levels of NMDARP-71 detected on Western blots by image analysis. Northern blot analysis revealed that treatment of the cultures with NMDARP-71 antisense oligonucleotide also reduced levels of mRNA for NMDARP-71. NMDARP-71 mRNA levels were reduced to 80% of control within 12 hr of exposure to NMDARP-71 AS1 and continued to decline to 62% of control after 20 hr of exposure (Fig. 10).

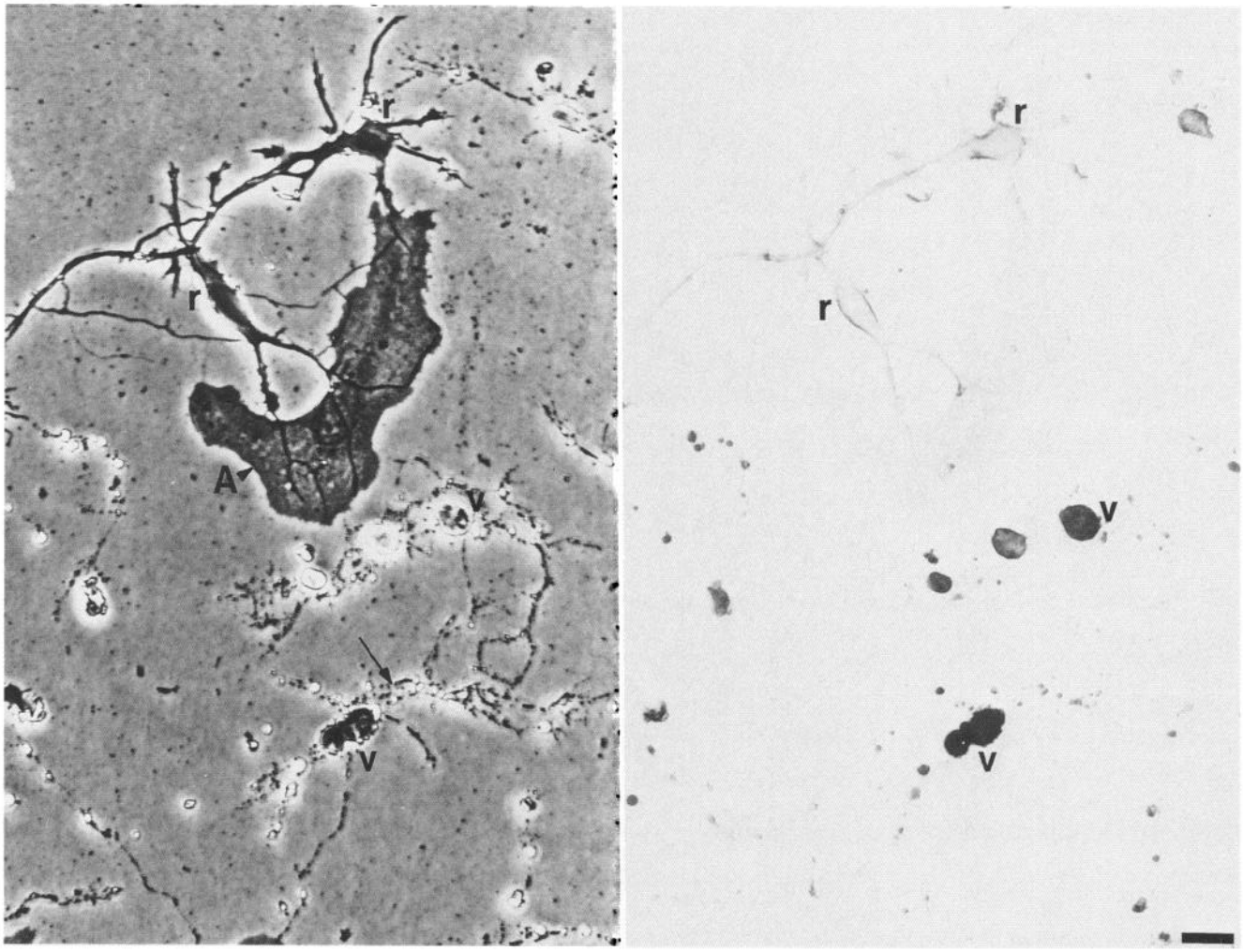

Figure 6. Neurons contacting astrocytes exhibit reduced NMDARP-71 immunoreactivity and reduced vulnerability to glutamate neurotoxicity: phase-contrast (*left*) and bright field (*right*) micrographs of a field of cultured hippocampal cells exposed to 500 μM glutamate for 8 hr and then immunostained with NMDARP-71 antibody. The two neurons contacting the astrocyte (*A*; *top*) were resistant (*r*) to glutamate neurotoxicity and exhibited a very low level of NMDARP-71 immunoreactivity. In contrast, the neurons not contacting astrocytes (*bottom*) were vulnerable (*v*) to glutamate toxicity (note fragmented neurites and irregular somata) and exhibited considerable NMDARP-71 immunoreactivity. Scale bar, 10 μm.

The effects of the NMDARP-71 antisense oligonucleotides were specific in that levels of mRNA for both the NMDAR1 and GluR1 were unaffected by NMDARP-71 antisense oligonucleotides (levels for these two mRNA species remained at 98–104% of control following 12 to 20 hr exposure to AS1). Furthermore, mRNA levels for β-actin remained at 100% of control following similar treatment of these cultures, an indication of equal loading of RNA across different lanes.

Calcium responses to NMDA are reduced in neurons exposed to NMDARP-71 antisense oligonucleotide

Fluorescence ratio imaging of the calcium indicator dye fura-2 was used to measure intracellular free calcium levels in hippocampal neurons. Changes in intracellular calcium levels following exposure to NMDA were examined in neurons that had been exposed to NMDARP-71 antisense or sense oligonucleotides for 20 hr (Figs. 11, 12). In neurons pretreated with 50 μM NMDARP-71 sense oligonucleotide, NMDA caused a progressive rise in intracellular calcium levels during a 10 min exposure period; levels were elevated two- to fivefold above pretreatment levels after 10 min of exposure (Fig. 11). In contrast, calcium responses were markedly attenuated in cultures pretreated with a 50 μM concentration of either of two antisense oligonucleotides (Fig. 11). Examples of calcium images in neurons prior to and following sequential exposures to NMDA and kainate are shown in Figure 12. Whereas neurons treated with NMDARP-71 antisense showed only very small calcium responses to NMDA, responses to kainate were much larger, indicating that the effect of the oligonucleotides was specific for NMDA responses.

NMDARP-71 antisense oligonucleotides protect neurons against excitotoxicity

Cultures that had been pretreated with NMDARP-71 sense or antisense oligonucleotides (50 μM) for 20 hr were exposed to 200 μM NMDA or kainate and neuronal damage was assessed 6 and 24 hr later. In cultures exposed to sense oligonucleotide, NMDA caused the degeneration of approximately 65% of the neurons during a 24 hr exposure period (Figs. 13, 14). Excitotoxic damage was characterized by neurite fragmentation and vacuolation of the cell body. Neurons in cultures pretreated with NMDARP-71 antisense oligonucleotides were relatively resistant to NMDA neurotoxicity.

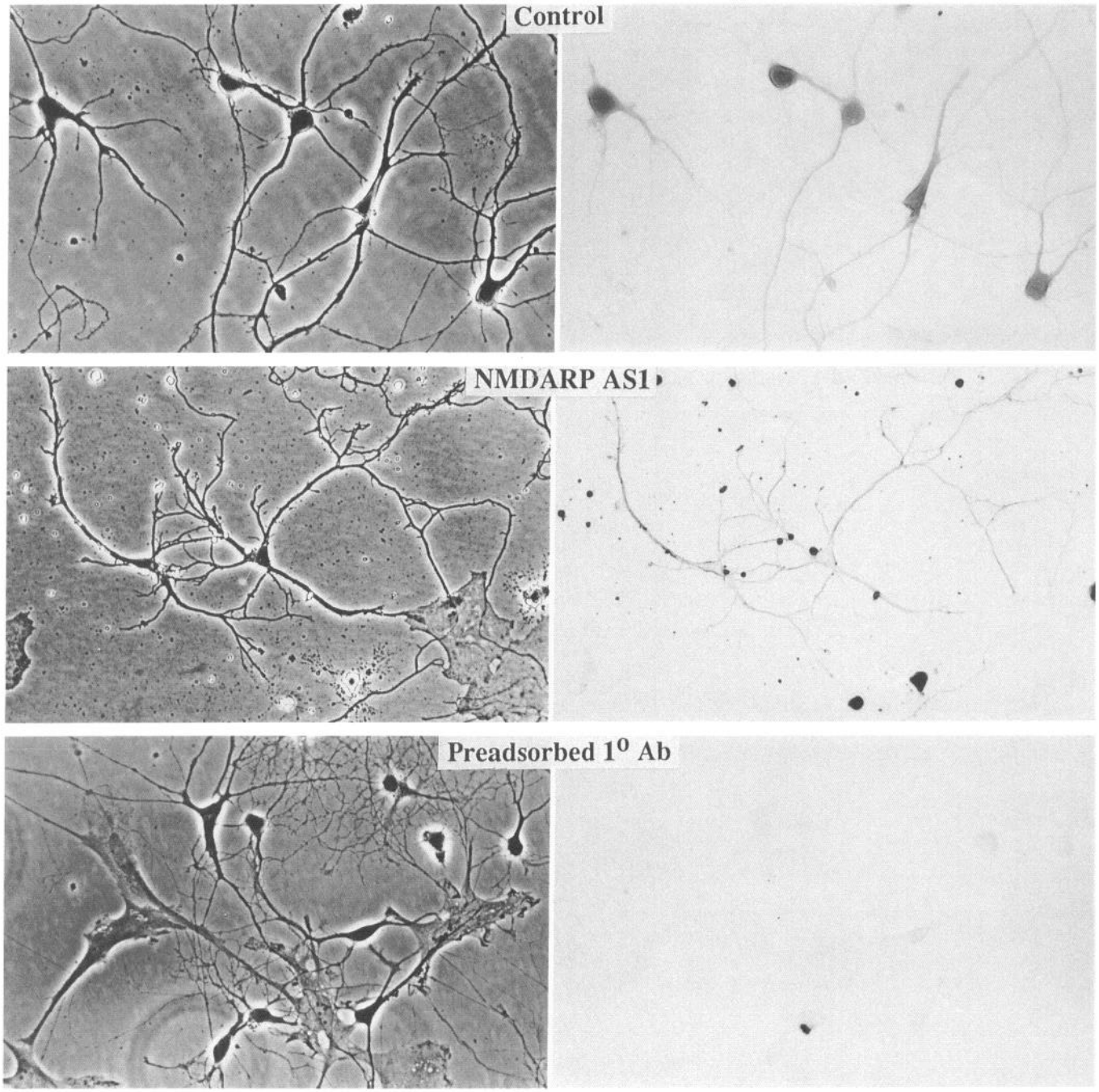

Figure 7. NMDARP-71 antisense oligonucleotides reduce NMDARP-71 immunoreactivity in hippocampal neurons: phase-contrast (*left*) and bright-field (*right*) micrographs of cultures immunostained with an antibody (clone $17G_{11}C_2$; see Mattson et al., 1991) to NMDARP-71. *Top,* Culture that had been exposed to NMDARP-71 sense DNA (50 μM) for 20 hr. *Middle,* Culture that had been exposed to NMDARP-71 antisense (AS1) DNA (50 μM) for 20 hr. *Bottom,* Culture immunostained with primary antibody that had been preabsorbed with excess NMDARP-71 protein (see Materials and Methods).

Interestingly, NMDARP-71 antisense oligonucleotide also partially protected hippocampal neurons against kainate neurotoxicity (Fig. 14). Whereas more than 80% of the neurons were vulnerable to 50 μM kainate in control cultures, only 60% were vulnerable in cultures pretreated with 50 μM NMDARP-71 AS1. These data suggested that the NMDA receptor may participate in the neurotoxicity caused by kainate as has been previously suggested (cf. Sucher et al., 1991). Consistent with this possibility, we found that the NMDA receptor antagonist APV greatly reduced kainate neurotoxicity (Fig. 5).

Discussion

Our data indicate that NMDARP-71 is present in hippocampal neurons, where it plays a role in regulating calcium influx through an NMDA-stimulated channel. Selective suppression of the levels of NMDARP-71 using either antisense oligonucleotides or bFGF resulted in a marked reduction in the calcium-elevating and neurotoxic actions of NMDA. We had previously shown that NMDARP-71 immunoreactivity localizes to the dendrites and cell bodies of cultured hippocampal neurons (Mattson et

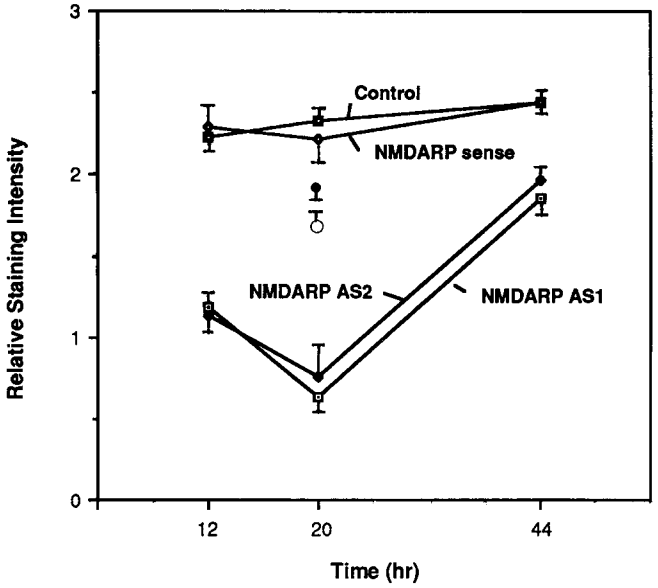

Figure 8. Time course of reduction in NMDARP-71 immunoreactivity in hippocampal neurons. Parallel cultures were exposed to no DNA (control), NMDARP-71 sense (50 μM), NMDARP-71 antisense 1 (AS1, 50 μM), or NMDARP-71 antisense 2 (AS2, 50 μM). Cultures were fixed at 12, 20, and 44 hr following treatment. Additional cultures were exposed for 20 hr to 10 μM NMDARP-71 AS1 (*solid circle*) or AS2 (*open circle*). Fixed cells were immunostained in parallel using a monoclonal antibody to NMDARP-71. Neuronal staining intensities were scored on a relative scale (*0*, no staining; *1*, light; *2*, moderate; *3*, heavy). Values represent the mean and SEM of determinations made in four separate cultures (100 neurons scored/culture). The overall reduction in NMDARP-71 immunoreactivity in antisense-treated cultures was highly significant (control vs AS1, $t = 7.1$, $p < 0.0001$; control vs AS2, $t = 6.5$, $p < 0.0001$; sense vs AS1, $t = 8.4$, $p < 0.0001$; sense vs AS2, $t = 6.7$, $p < 0.0001$).

al., 1991) and hippocampal neurons *in vivo* (Eaton et al., 1990). Neurons that expressed high levels of NMDARP-71 were particularly vulnerable to glutamate neurotoxicity, and a polyclonal antibody to NMDARP-71 prevented NMDA neurotoxicity (Mattson et al., 1991). In addition, data obtained in cerebellar granule cell cultures indicate that the expression of NMDARP-71 is well correlated with the development of enhanced granule cell sensitivity to NMDA and with depolarization-induced neu-

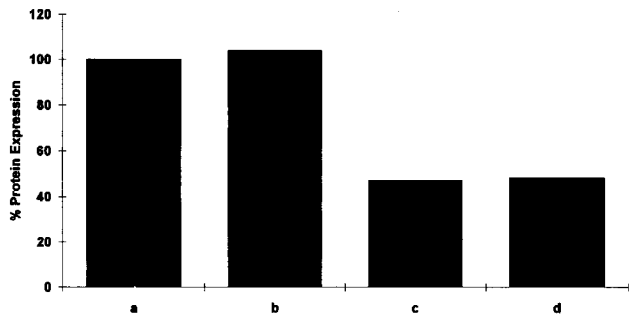

Figure 9. Levels of NMDARP-71 detected by Western blot analysis are reduced in hippocampal cultures exposed to antisense oligonucleotides. Parallel cultures were left untreated (*a*) or were exposed to 50 μM NMDARP-71 sense (*b*), NMDARP-71 AS1 (*c*), or NMDARP-71 AS2 (*d*) for 22 hr. Protein was separated by polyacrylamide gel electrophoresis (50 μg of solubilized protein/lane), transferred to nitrocellulose, and immunoblotted using a monoclonal antibody to NMDARP-71. The blots were subsequently analyzed by image analysis and densitometry. The densitometric values are expressed as percentage of the control.

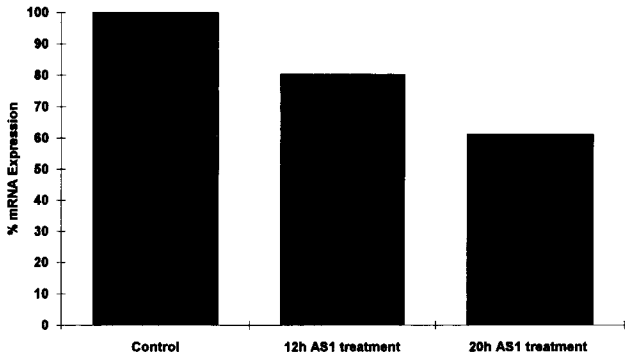

Figure 10. Time course of reduction in NMDARP-71 mRNA levels in cultures exposed to NMDARP-71 antisense oligonucleotide. Parallel hippocampal cell cultures were treated for 20 hr with NMDARP-71 sense oligonucleotide (control, 50 μM) or with 50 μM NMDARP-71 AS1 for 12 or 20 hr, and mRNA levels were quantified by densitometric analysis of the 1.8 kb RNA species on Northern blots probed with the ^{32}P-labeled PstI fragment of pGBA-2. Densitometric values are expressed as percentage of control (i.e., the sense oligonucleotide–treated culture).

ronal survival (Van der Valk et al., 1991; Balazs et al., 1992). Protein reconstitution studies demonstrated that while NMDARP-71 alone is not sufficient to form a functional NMDA receptor, it can associate with three additional proteins to form functional NMDA-activated ion channels and that the activation of this channel by glutamate or NMDA is blocked by pretreatment of the proteins with polyclonal antibodies to NMDARP-71 (Kumar et al., 1991; Ly and Michaelis, 1991; Minami et al., 1991). We have not tested preparations of the hetero-oligomeric complex of NMDA receptor proteins for ion

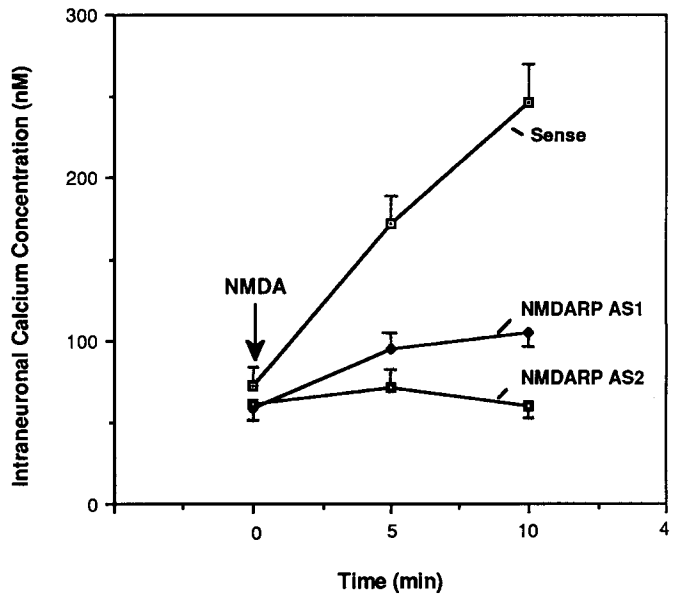

Figure 11. NMDA-induced elevations in intraneuronal free calcium levels are reduced in neurons pretreated with NMDARP-71 antisense oligonucleotides. Parallel cultures were exposed to 50 μM NMDARP-71 sense, AS1 or AS2 for 20 hr. Calcium measurements were made in the same neurons ($n = 14$–23) prior to and 5 and 10 min following exposure to 200 μM NMDA. Values represent the mean and SEM. The effect of AS1 and AS2 on the NMDA response (compared to sense-treated neurons) was highly significant at both the 5 min (sense vs AS1, $t = 7.8$, $p < 0.0001$; sense vs AS2, $t = 7.5$, $p < 0.0001$) and 10 min (sense vs AS1, $t = 9.3$, $p < 0.0001$; sense vs AS2, $t = 8.5$, $p < 0.0001$) time points (paired Student's t test).

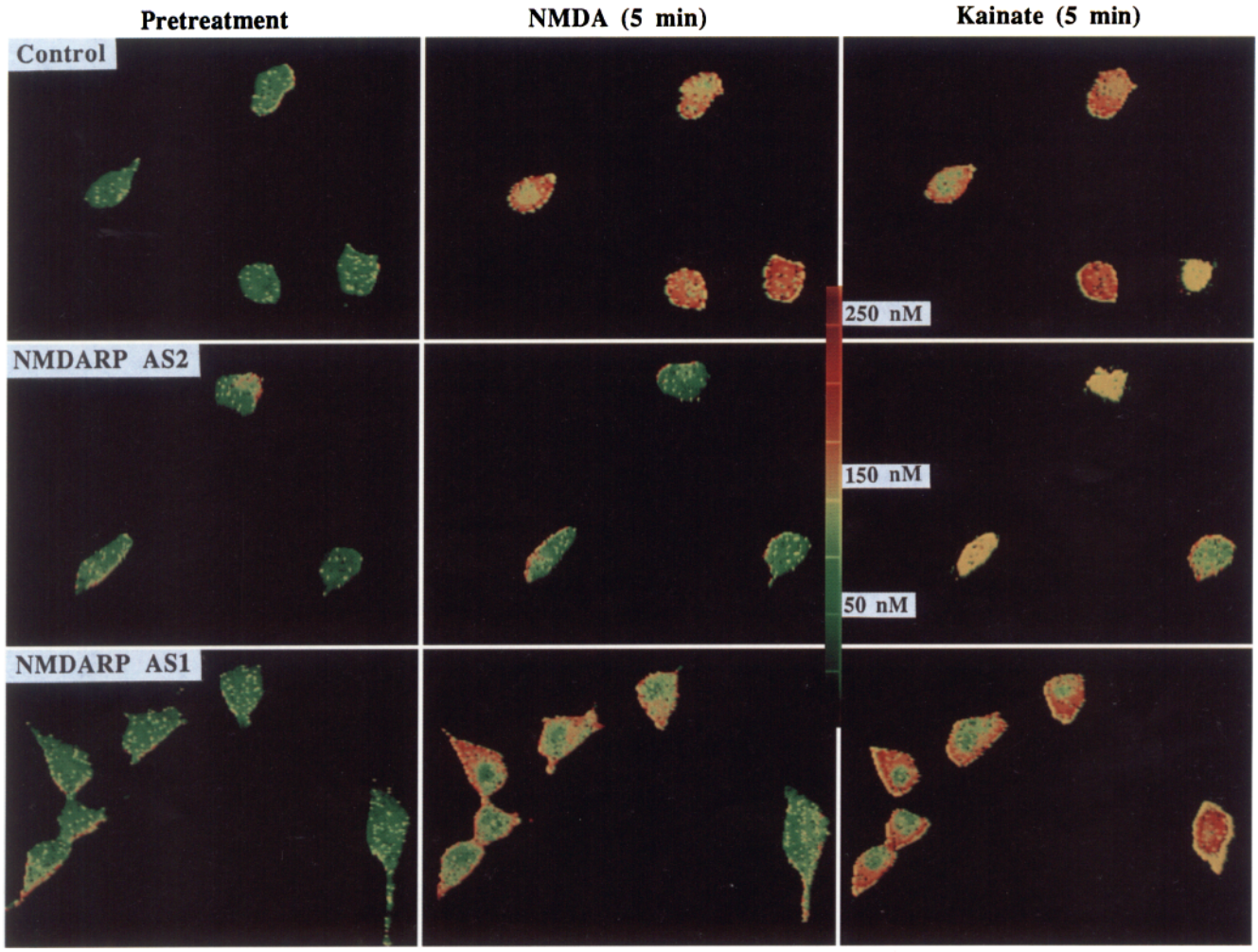

Figure 12. Calcium responses to NMDA are selectively reduced in hippocampal neurons exposed to NMDARP-71 antisense oligonucleotides. Fluorescence ratio images of the calcium indicator dye fura-2 in cultured hippocampal neurons are depicted in pseudocolor according the calcium concentration scale shown. *Top panels,* A field of neurons in culture that had been exposed for 20 hr to 50 μM NMDARP-71 sense oligonucleotide is shown prior to treatment (*left*), 5 min following exposure to 200 μM NMDA (*middle*), and 5 min following a subsequent exposure to 200 μM kainate (*right*). *Middle panels,* Neurons that had been exposed for 20 hr to 50 μM NMDARP-71 antisense oligonucleotide (AS2) are shown prior to treatment (*left*), 5 min following exposure to 200 μM NMDA (*middle*), and 5 min following exposure to 200 μM kainate (*right*). *Bottom panels,* Neurons that had been exposed for 20 hr to 50 μM NMDARP-71 antisense oligonucleotide (AS1) are shown prior to treatment (*left*), 5 min following exposure to 200 μM NMDA (*middle*), and 5 min following exposure to 200 μM kainate (*right*). Note that calcium responses to NMDA are greatly reduced in the cultures pretreated with NMDARP-71 antisense oligonucleotides relative to the culture pretreated with sense oligonucleotide.

channel activity when only three of the four proteins are reconstituted, that is, when the 71 kDa NMDARP is absent. By extension of our observations of inhibition of the function of this complex produced by antibodies that are specifically reacting with the 71 kDa protein, we assume that the absence of this protein will produce a nonfunctional receptor–ion channel complex.

The time course of suppression of levels of NMDARP-71 by antisense oligonucleotides and bFGF suggests that NMDARP-71 has a half-life of approximately 12–24 hr in cultured hippocampal neurons. This estimation is based upon the observation that a single exposure of cultures to NMDARP-71 antisense resulted in a transient reduction in NMDARP-71 levels such that levels were markedly reduced after 12–24 hr of exposure and then recovered by 48 hr of exposure. In addition, levels of NMDARP-71 immunoreactivity were markedly reduced after 12–24 hr of exposure to bFGF. These data are consistent with our previous study in which we showed that the neuroprotective effect of bFGF against glutamate neurotoxicity required pretreatment with the growth factor for 12–24 hr (Mattson et al., 1989). It is of interest that the excitoprotective effect of bFGF may require mRNA and protein synthesis since the protective effect is abolished when neurons are incubated in the presence of either actinomycin D or cycloheximide (Mattson et al., 1989). Taken together with the finding that bFGF suppresses the expression of NMDARP-71, the data are consistent with a scenario in which activation of bFGF receptors leads to production of proteins involved in the regulation of the gene encoding NMDARP-71. It would be of interest to determine whether protein synthesis is required for the suppressive effect of bFGF on NMDARP-71 levels.

Hippocampal neurons exposed to antisense oligonucleotides to NMDARP-71 were protected against NMDA neurotoxicity. These results demonstrate a role for this NMDARP-71 in excitotoxicity. Our data also showed that NMDARP-71 mediates calcium influx caused by glutamate and NMDA. It is possible that the effects of the antisense oligonucleotides were produced through suppression of the expression of NMDARP-71 and a

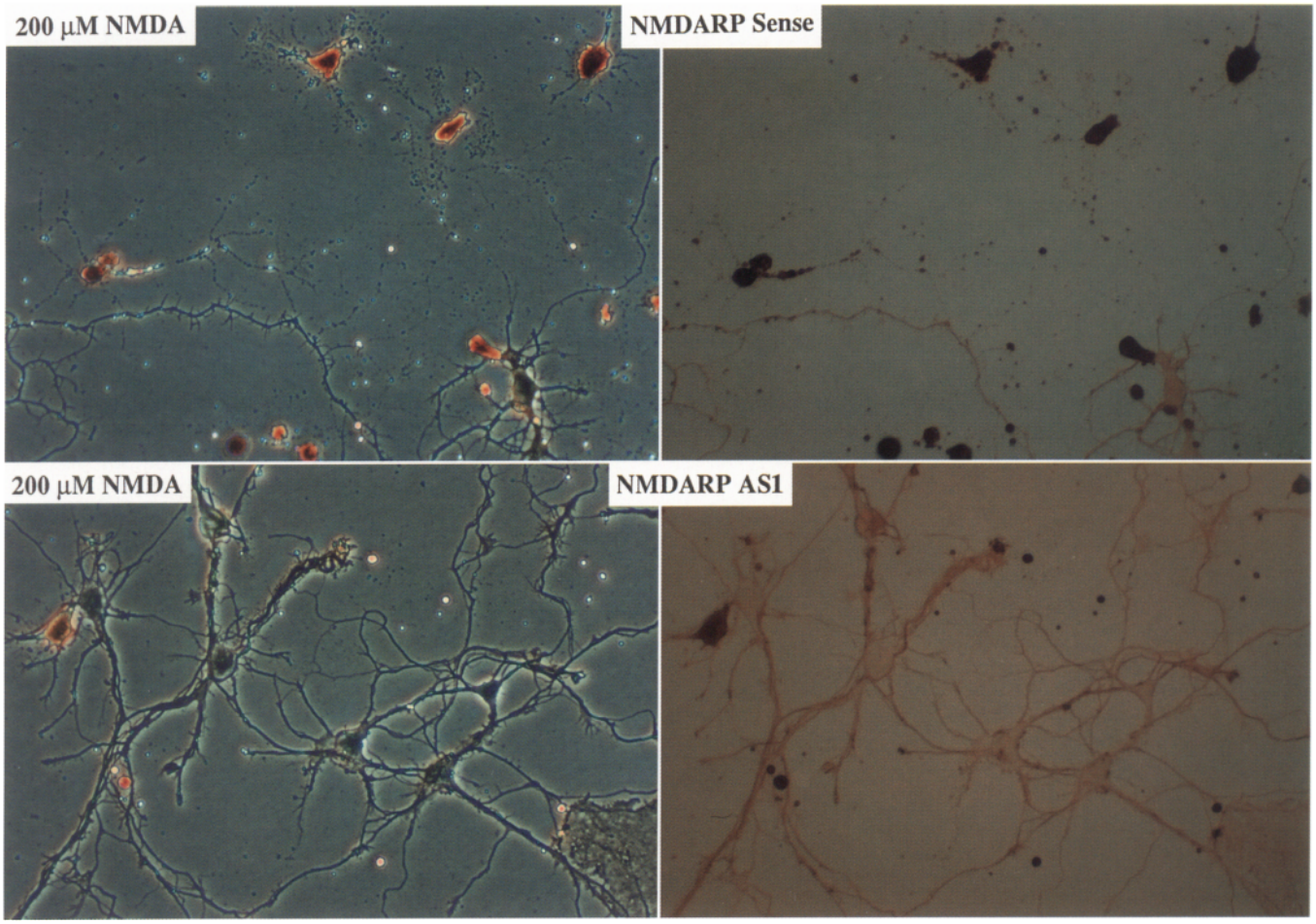

Figure 13. Effects of NMDARP antisense oligonucleotides on NMDARP-71 immunoreactivity and vulnerability to NMDA neurotoxicity. Cultures were exposed to 50 μM NMDARP-71 sense or antisense oligonucleotides for 20 hr. The cells were then exposed to 200 μM NMDA for 6 hr, at which time they were fixed and immunostained with NMDARP-71 antibody. Phase-contrast (*left*) and corresponding bright-field (*right*) micrographs are shown. Note that NMDA caused degeneration of the majority of neurons pretreated with sense oligonucleotide (*upper left*); these neurons stained intensely with the NMDARP-71 antibody (*upper right*). In contrast, the majority of neurons in cultures pretreated with NMDARP-71 antisense were resistant to NMDA toxicity (*lower left*) and stained lightly with the NMDARP-71 antibody (*lower right*).

subsequent diminution of the expression of other glutamate receptor proteins. However, NMDARP-71 antisense oligonucleotides did not reduce levels of GluR1 or of NMDAR1 mRNA. In additional studies, we have found that a GluR1 antisense oligonucleotide significantly reduced neuronal damage caused by kainate, but did not protect neurons against NMDA neurotoxicity (M. P. Mattson, unpublished observations). Thus, antisense technology may prove valuable in defining roles for different glutamate receptor proteins in physiological and pathophysiological processes.

Whereas NMDARP-71 antisense oligonucleotides caused a marked reduction in levels of the 71 kDa protein, they caused a more moderate reduction in levels of NMDARP-71 mRNA. The suppression of NMDARP-71 mRNA was specific for the antisense oligonucleotides since sense oligonucleotide did not reduce mRNA levels. Although the mechanisms whereby antisense oligonucleotides reduce protein levels are not completely understood, data suggest that the oligonucleotides can enhance degradation of mRNA and/or block translation without enhancing mRNA degradation (Colman, 1990). Our data are consistent with effects of NMDARP-71 antisense oligonucleotides on both the level of NMDARP-71 mRNA and blockade of translation from extant NMDARP-71 mRNA. The data indicate that the suppression of NMDARP-71 mRNA and protein by bFGF was apparently due to a selective effect on this protein in neurons. Thus, NMDARP-71 mRNA levels were reduced within 24 hr of exposure to bFGF and continued to decline through 96 hr of exposure. In contrast, levels of GluR1 mRNA were not reduced by bFGF. Western blot analysis showed a clear reduction in NMDARP-71 levels in bFGF-treated cultures, whereas EGF and NGF did not reduce NMDARP-71 levels. Immunocytochemical analysis confirmed the Western blot results.

Previous studies indicated that neuroprotective action of bFGF in hippocampal cell cultures is due to a direct action on neurons (Mattson and Rychlik, 1990; Cheng and Mattson, 1991). However, since bFGF is a mitogen for astrocytes, it was possible that an increase in astrocyte numbers might have contributed to the apparent reduction in NMDARP-71 levels in bFGF-treated cultures. However, several findings argue against the latter possibility, including the following: levels of NMDARP-71 were clearly reduced in neurons as shown by immunocytochemical analysis; levels of NMDARP-71 protein were not reduced in EGF-treated cultures despite the fact that EGF is a potent mitogen for astrocytes (Han et al., 1992); astrocytes represented only approximately 20% of the cells in cultures incu-

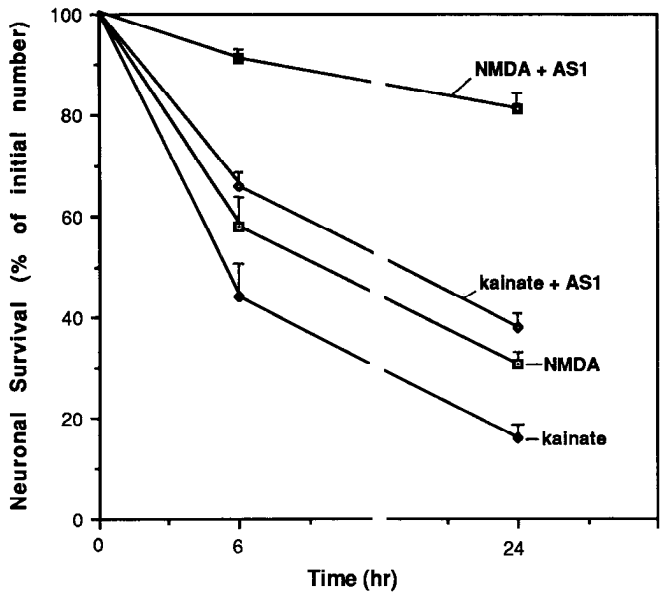

Figure 14. Vulnerability to excitotoxicity is reduced in hippocampal cultures pretreated with antisense oligonucleotides to the NMDARP-71. Parallel cultures were pretreated for 20 hr with 50 μM NMDARP-71 AS1 (*NMDA+AS1, kainate+AS1*) or 50 μM NMDARP-71 sense oligonucleotide (*NMDA, kainate*) and were then exposed to 200 μM NMDA or 200 μM kainate. Neuronal survival was determined at 6 and 24 hr following exposure to the EAAs. Values represent the mean and SEM of determinations made in four separate cultures. Values for cultures pretreated with NMDARP AS1 or NMDARP AS2 were significantly greater than values for control cultures or cultures exposed to NMDARP sense at both the 6 hr (NMDA vs NMDA+AS1, $t = 5.8$, $p < 0.01$; kainate vs kainate+AS1, $t = 5.3$, $p < 0.02$) and 24 hr (NMDA vs NMDA+AS1, $t = 10.8$, $p < 0.002$; kainate vs kainate+AS1, $t = 5.5$, $p < 0.02$) time points (paired Student's t tests).

bated in serum-containing cultures and less than 2% of cells in cultures maintained in serum-free medium; the reduction in NMDARP-71 mRNA and protein levels occurred within 24 hr of exposure to bFGF, a time period too short to allow significant astrocyte proliferation. In addition, bFGF did not affect astrocyte numbers significantly during a 48 hr exposure period in culture medium lacking serum, but nevertheless reduced NMDARP-71 levels. Finally, since GluR1 levels were not altered following treatment with bFGF, it is unlikely that substantial proliferation of hippocampal astrocytes had occurred.

Several observations suggest that the suppressive action of bFGF on the NMDARP-71 mRNA and protein levels demonstrated in the present study may have relevance to the intact developing and adult brain. First, bFGF has been shown to promote the survival and outgrowth of neurons from many brain regions including the hippocampus (Morrison et al., 1986; Walicke et al., 1986; Mattson et al., 1989). Second, glutamate can inhibit dendritic growth cones and cause dendritic regression in hippocampal neurons, and these effects of glutamate can be prevented by bFGF (Mattson et al., 1989). Third, bFGF can protect central neurons against EAA neurotoxicity (Mattson et al., 1989; present results), and hypoglycemic damage that involves NMDA receptor activation (Cheng and Mattson, 1991). Fourth, bFGF can provide trophic support to central neurons in the adult brain *in vivo* (Anderson et al., 1988). Fifth, bFGF can protect hippocampal neurons against ischemic damage *in vivo* (Berlove et al., 1991). Furthermore, FGF receptors have been localized to a large number of neuronal populations in the brain including hippocampal neurons (Wanaka et al., 1990; Cheng and Mattson, 1991). Our data indicate that one mechanism whereby bFGF might influence neuronal plasticity and survival is by suppressing the expression of an NMDA receptor. We recently reported that bFGF prevents the loss of calcium homeostasis that normally mediates hypoglycemic excitotoxic damage (Cheng and Mattson, 1991). The present data provide an explanation for this calcium-stabilizing action of bFGF. That is, by reducing the expression of an NMDA receptor, bFGF presumably reduces calcium influx and therefore prevents the sustained elevations in intracellular calcium that normally mediate excitotoxicity.

The suppressive effect of bFGF on the expression of NMDARP-71 was specific for this growth factor. NGF and EGF did not reduce NMDARP-71 levels. These data are consistent with our previous findings that NGF did not protect hippocampal neurons against glutamate neurotoxicity (Mattson et al., 1989), and that EGF did not protect rat hippocampal or human cortical neurons against hypoglycemic damage that is NMDA receptor mediated (Cheng and Mattson, 1991). On the other hand, NGF was reported to protect hippocampal neurons against ischemic damage *in vivo* (Shigeno et al., 1991) and against hypoglycemic damage *in vitro* (Cheng and Mattson, 1991). It therefore appears to be the case that NGF and bFGF differ in their mechanisms of action. The suppressive effect of bFGF on NMDARP-71 was specific for this type of EAA receptor since GluR1 mRNA levels (i.e., the mRNA for the kainate/AMPA receptors) were not decreased by bFGF. Since reduction of expression of NMDARP-71 protected neurons against NMDA-induced cell damage, we conclude that the neuroprotective effect of bFGF was due in part to suppression of expression of NMDARP-71. Whether bFGF and other neuroprotective growth factors including NGF (Cheng and Mattson, 1991) or the insulin-like growth factors (Cheng and Mattson, 1992b) affect the expression of other EAA receptors remains to be established.

The NMDA receptor probably plays an important role in a variety of neurodegenerative conditions including stroke, epilepsy, Alzheimer's disease, Huntington's disease, and Parkinson's disease (Sloviter, 1987; Choi, 1988; Greenamyre and Young, 1989; Graham et al., 1990; Otto and Unsicker, 1990; Mattson, 1992). Growth factors have also been proposed to play roles in each of these disorders (Appel, 1986; Hefti et al., 1989). The increasing number of reports of neuron-damaging effects of NMDA receptor activation, and neuroprotective effects of growth factors (NGF and bFGF in particular), in animal and cell culture models of neurodegenerative disorders (see introductory remarks) strongly suggests that the mechanism of neuroprotective action of bFGF documented in the present study may have relevance to these diseases. Work is now in progress to determine whether bFGF influences the expression of NMDARP-71 *in vivo*. If a reduction in expression of NMDARP-71 and resultant protection against excitotoxicity by bFGF also occur in humans, then this growth factor may be potentially useful in preventing the neuronal damage in the myriad of disorders that involve NMDA receptors. Clearly, a better understanding of the neuroprotective mechanism of action of bFGF may provide information valuable in developing preventative and therapeutic strategies for these diseases.

Although the present study did not directly examine the effects of bFGF on processes of neural plasticity such as neurite outgrowth, synaptogenesis, and LTP, it is certainly worth considering the implications of the present findings for these NMDA

receptor–mediated processes. Data from an increasing number of laboratories have demonstrated roles for EAA receptors in general, and the NMDA receptor in particular, in the development of neural circuits. For example, EAA receptors regulate neurite outgrowth in hippocampal neurons (Mattson et al., 1988a) and promote survival of cerebellar neurons (Balazs et al., 1988). The EAA receptors apparently play a major role in the process of synapse formation in many brain regions including the visual system (Kleinschmidt et al., 1987; Cline et al., 1990) and hippocampus (Mattson et al., 1988b). In the hippocampus, glutamate released from axons may activate EAA receptors on dendritic targets, stabilize their outgrowth, and thereby promote synaptogenesis (Mattson et al., 1988b; Mattson and Hauser, 1991). Since bFGF suppressed the expression of the NMDA receptor in the embryonic hippocampal neurons in the present study, it is reasonable to consider that bFGF might play an important role in regulating synaptogenesis in the hippocampus. At the mature synapse, NMDA receptors are clearly involved in LTP (Collingridge and Bliss, 1987). Previous data suggested that growth factors may play roles in processes such as learning and memory. For example, Sastry and coworkers have shown that NGF and other unidentified proteins can modify LTP in the hippocampus (Sastry et al., 1988; Xie et al., 1991). The present data provide evidence that bFGF can influence a transmitter receptor believed to play a central role in the postsynaptic calcium influx involved in LTP (Malenka et al., 1988). The precise consequences of such an action of bFGF in the context of circuits involving many neurons are not clear, and may be quite complex.

Receptors for the EAAs may also be involved in the process of natural cell death during development (Mattson and Hauser, 1991; Oppenheim, 1991). It has been recognized for some time that growth factors play a role in regulating both cell numbers and synaptic connections in developing neural circuits (Purves, 1986). Neural activity also plays a prominent role in shaping neural circuitry (Frank, 1987). Interactions between activity-regulating neurotransmitter systems and growth factor systems have been demonstrated (Landis, 1990). For example, seizure activity increases the expression of NGF in hippocampal neurons (Gall and Isackson, 1989). The present data provide a direct demonstration of regulation of the expression of a neurotransmitter receptor by a growth factor. We propose that the regulation of neurotransmitter receptors by target-derived growth factors may provide a feedback mechanism to "adjust" activity in the involved neural circuits and modify connections adaptively. In the case of the NMDA receptor, a reduction in its level by bFGF may be at least part of the mechanism for determining which neurons live during development. The latter possibility is consistent with the "trophic theory" of neural connections (Purves, 1986) in that the availability of target-derived trophic factor plays an important role in determining which neurons live and which die. Reciprocal interactions between neurotransmitter and growth factor systems may provide a fundamental mechanism of regulating synaptic connections and neuronal survival.

If bFGF does play a role in regulating NMDA receptor expression *in vivo,* then it is important to identify the cellular sources of bFGF that are important in this process. bFGF is believed to be produced by both neurons and glia *in vivo* (Pettman et al., 1986; Ferrara et al., 1988; Mattson and Rychlik, 1990). We previously found that neurons contacting astrocytes were protected against glutamate neurotoxicity, and that bFGF antibodies abolished the excitoprotective effect of astrocytes (Mattson and Rychlik, 1990). Those data suggested that bFGF associated with astrocytes could reduce neuronal vulnerability to EAAs. In the present study, we found that levels of NMDARP-71 immunoreactivity in neurons contacting astrocytes were lower than in neurons not contacting astrocytes. Furthermore, the reduced levels of NMDARP-71 immunoreactivity correlated with reduced vulnerability to glutamate neurotoxicity. These data demonstrate that some types of glial cells can influence the expression of EAA receptors, and suggest that astrocytes may play important neuromodulatory/neuroprotective roles *in vivo.* The involvement of target-derived and glia-derived growth factors in regulating processes such as synaptogenesis, LTP, and cell death that involve the NMDA receptor clearly merits further study.

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