Journal of Neurochemistry
Lippincott–Raven Publishers, Philadelphia

Glia Modulate NMDA-Mediated Signaling in Primary Cultures of Cerebellar Granule Cells

Carol M. Beaman-Hall, J. Clancy Leahy, Saloua Benmansour, and Mary Lou Vallano

Department of Pharmacology, SUNY/Health Science Center, Syracuse, New York, U.S.A.

Abstract: Excessive activation of *N*-methyl-D-aspartate (NMDA) receptor channels (NRs) is a major cause of neuronal death associated with stroke and ischemia. Cerebellar granule neurons in vivo, but not in culture, are relatively resistant to toxicity, possibly owing to protective effects of glia. To evaluate whether NR-mediated signaling is modulated when developing neurons are cocultured with glia, the neurotoxic responses of rat cerebellar granule cells to applied NMDA or glutamate were compared in astrocyte-rich and astrocyte-poor cultures. In astrocyte-poor cultures, significant neurotoxicity was observed in response to NMDA or glutamate and was inhibited by an NR antagonist. Astrocyte-rich neuronal cultures demonstrated three significant differences, compared with astrocyte-poor cultures: (a) Neuronal viability was increased; (b) glutamate-mediated neurotoxicity was decreased, consistent with the presence of a sodium-coupled glutamate transport system in astrocytes; and (c) NMDA- but not kainate-mediated neurotoxicity was decreased, in a manner that depended on the relative abundance of glia in the culture. Because glia do not express NRs or an NMDA transport system, the mechanism of protection is distinct from that observed in response to glutamate. No differences in NR subunit composition (evaluated using RT-PCR assays for NR1 and NR2 subunit mRNAs), NR sensitivity (evaluated by measuring NR-mediated changes in intracellular Ca^{2+} levels), or glycine availability as a coagonist (evaluated in the presence and absence of exogenous glycine) were observed between astrocyte-rich and astrocyte-poor cultures, suggesting that glia do not directly modulate NR composition or function. Nordihydroguaiaretic acid, a lipoxygenase inhibitor, blocked NMDA-mediated toxicity in astrocyte-poor cultures, raising the possibility that glia effectively reduce the accumulation of highly diffusible and toxic arachidonic acid metabolites in neurons. Alternatively, glia may alter neuronal development/phenotype in a manner that selectively reduces susceptibility to NR-mediated toxicity. **Key Words:** Calcium—Excitotoxicity—Glutamate—NMDA—RT-PCR—Neurons—Astrocytes.
J. Neurochem. **71,** 1993–2005 (1998).

The *N*-methyl-D-aspartate (NMDA) type of glutamate receptor channel (NR) has an essential role in neuronal development (for review, see Scheetz and Constantine-Paton, 1994), plasticity (for review, see Collingridge and Bliss, 1995), and pathology (for reviews, see Teichberg, 1992; Choi, 1994). Brief exposure of cultured neurons to high concentrations of glutamate results in substantial neuronal death within ~24 h, which is mediated by activation of and subsequent influx of Ca^{2+} through NRs. Accordingly, use of NR antagonists or Ca^{2+}-free media during glutamate (or NMDA) exposure is neuroprotective (Teichberg, 1992; Choi, 1994). This delayed form of excitotoxicity has generated intense interest among investigators because it represents an in vitro model for the neuronal loss associated with acute trauma and focal ischemia, epilepsies, and some neurodegenerative diseases (for reviews, see Choi and Rothman, 1990; Meldrum and Garthwaite, 1990; Beal, 1994; Schousboe and Frandsen, 1995).

In vivo, certain brain regions and neuronal types exhibit a selective vulnerability to NR-mediated neurotoxic damage. Hippocampal pyramidal neurons, for example, are exquisitely sensitive to ischemic insult, whereas cerebellar granule cells are much less sensitive (Brierly and Graham, 1984; Pulsinelli, 1985). Selective vulnerability to excitotoxicity is likely due to several factors, including regional differences in the density or composition of neuronal NRs, that would influ-

Received February 9, 1998; revised manuscript received May 20, 1998; accepted May 27, 1998.

Address correspondence and reprint requests to Dr. M. L. Vallano at Department of Pharmacology, SUNY/Health Science Center, Syracuse, NY 13210, U.S.A.

The present address of Dr. J. C. Leahy is Department of Biology, Lynchburg College, Lynchburg, VA, U.S.A.

The present address of Dr. S. Benmansour is Department of Pharmacology, University of Texas Health Science Center, San Antonio, TX, U.S.A.

Abbreviations used: AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate; AP-5, DL-2-amino-5-phosphonovaleric acid; AraC, cytosine arabinoside; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; DIV, days in vitro; FDA, fluorescein diacetate; GFAP, glial fibrillary acidic protein; KA, kainate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NDGA, nordihydroguaiaretic acid; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; NOS, nitric oxide synthase; NR, *N*-methyl-D-aspartate receptor channel; PI, propidium iodide; *Taq* polymerase, *Thermus aquaticus* polymerase; TTX, tetrodotoxin; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

ence the initial phase of neurotoxicity by altering the amount of glutamate-mediated Ca^{2+} influx. Experimental evidence indicates that most NRs are oligomers composed of two distinct subunits: an obligatory NR1 subunit and at least one type of NR2 subunit (for reviews, see Hollmann and Heinemann, 1994; McBain and Mayer, 1994). Up to eight forms of NR1 have been identified, resulting from alternative splicing of three exons on a single gene (for review, see Zukin and Bennett, 1995). Four NR2 subunits exist (in the rat, designated as NR2A–D), each the product of a separate gene. Mapping studies indicate that developmental and regional variability exists for the multiple forms of NR1 and NR2 subunits throughout the brain (Monyer et al., 1992, 1994; Watanabe et al., 1992; Laurie et al., 1995). Differences in subunit composition result in functionally distinct NR responses (Durand et al., 1992; Traynelis et al., 1995; Sucher et al., 1996).

The neuronal environment also influences susceptibility to excitatory amino acids. In particular, glia modulate neuronal responsiveness at all stages of development (for reviews, see Barres, 1991; Vernadakis, 1996) in a region-specific manner (Westergaard et al., 1991). In cerebral cortical neurons, the degree of glutamate- but not NMDA-mediated toxicity is substantially reduced in astrocyte-rich cultures relative to astrocyte-poor cultures, owing to glial-mediated glutamate uptake (Rosenberg and Aizenman, 1989; Rosenberg et al., 1992). Similar effects are observed in hippocampal cultures (Sugiyama et al., 1989). As a consequence of their catalase activity, astrocytes protect against H_2O_2-mediated toxicity in cultured striatal neurons if at least one astrocyte per 20 neurons is present (Desagher et al., 1996). Conversely, neurotoxic responses of cerebral cortical neurons to α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) are exacerbated in astrocyte-rich versus astrocyte-poor cultures, possibly owing to AMPA receptor-mediated glial glutamate release (Dugan et al., 1995). Although microglia can also enhance cytotoxicity in vitro, their primary role in vivo may be to protect the integrity of the CNS (for review, see Banati and Graeber, 1994). Collectively, these studies indicate that multiple mechanisms contribute to the ability of glia to protect against or, in some cases, exacerbate neurotoxicity in forebrain neurons. In cerebellar cortex, introduction of a transgene that conditionally ablates cerebellar astrocytes in postnatal mice results in marked granule cell death in a manner that can be reversed by treatment with an NR antagonist (Delaney et al., 1996). This in vivo study suggests that NR-mediated excitotoxicity is a significant factor contributing to granule cell loss during cerebellar neurogenesis and that astrocytes are protective. In dissociated culture, but not in vivo, cerebellar granule neurons are highly sensitive to excitatory amino acid-mediated toxicity (Lysko et al., 1989; Cox et al., 1990; Schramm et al., 1990), but information is lacking on effects of glia. In the present report, the influence of cerebellar glia on NR-mediated signaling and neurotoxicity in astrocyte-rich versus astrocyte-poor cultures of granule neurons was evaluated.

MATERIALS AND METHODS

Materials

Thermus aquaticus polymerase (*Taq* polymerase) was purchased from Perkin-Elmer. DNA standards (123-bp ladder), Moloney murine leukemia virus (MMLV) reverse transcriptase, basal Eagle's medium with Earle's salts, and L-glutamine were purchased from GibcoBRL. Ribonuclease inhibitor and random primers were obtained from Promega. [α-^{32}P]dCTP (~3,000 Ci/mmol) was purchased from NEN. Glial fibrillary acidic protein (GFAP) antibodies for immunocytochemistry (mouse anti-GFAP), NMDA, L-glutamic acid, indomethacin, and nordihydroguaiaretic acid (NDGA) were purchased from Sigma. *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) and cyclosporin A were purchased from Calbiochem. FK506 was a generous gift from Dr. I. Bekersky (Fujisawa, Inc.). Other reagents for RT-PCR and tissue culture were molecular biology grade and tissue culture grade, respectively, and were obtained from commercial sources.

Tissue culture

Granule cell-enriched cultures were obtained essentially as described by Levi et al. (1989) and previously reported by our laboratory (Leahy et al., 1994). In brief, cerebella from 7–9-day-old Sprague–Dawley rats were minced, trypsinized, and triturated to dissociate cells. The cells were plated at a density of $2.0–2.5 \times 10^6$ cells/2 ml of medium onto six-well or 35-mm-diameter Corning dishes (for neurotoxicity or RNA extraction) or $6–6.3 \times 10^5$ cells/0.5 ml of medium in 24-well Corning dishes containing glass coverslips (for fura-2/Ca^{2+} measurements) precoated with poly-L-lysine and incubated at 37°C in a humidified atmosphere containing either 5% CO_2/95% air (pH 7.4) or, in some cases, 10% CO_2/90% air (pH 7.1) (see Leahy et al., 1994). Astrocyte-rich cultures were grown in basal Eagle's medium with Earle's salts, supplemented with (final concentrations) heat-inactivated fetal calf serum (10%), gentamicin sulfate (100 μg/ml), and L-glutamine (2 m*M*). Because cerebellar granule cell differentiation and survival in culture are activity-dependent, the growth medium was also supplemented with KCl (25 m*M*). In some cases, serum-free defined medium was used (Leahy et al., 1994). Astrocyte-poor cultures were obtained by adding 10 μ*M* cytosine arabinoside (AraC) to the culture medium 24 h after plating. Production of astrocyte-rich cultures grown in the absence of AraC was confirmed by daily observation using phase-contrast microscopy and immunocytochemical staining of astrocytes using antibodies against GFAP. Cells were used for experiments at 7–10 days in vitro (DIV). Medium was not exchanged during this time, but 5.5 m*M* glucose was typically added at 6 DIV to enhance long-term survival (Schramm et al., 1990). Cell viability was assessed routinely throughout the culture period, and long-term survival was quantified by determining the number of days in culture to reach <10% viability, using phase-contrast microscopy. Typically, large-scale cell death occurred over a 24-h period once cell viability reached 50–60% (assuming 100% viability at 1 DIV). Survival of granule cells cocultured with glia was improved compared with survival in glial-poor cultures (data not shown).

Toxicity

Neurotoxicity was routinely measured by differential fluorescent staining (Jones and Senft, 1985). The growth medium was collected, and cells were washed twice with standard Locke's solution containing 154 m*M* NaCl, 5 m*M* KCl, 2.3 m*M* $CaCl_2$, 1.0 m*M* $MgCl_2$, and 8.6 m*M* HEPES, pH 7.4. Two milliliters of a nominally Mg^{2+}-free Locke's solution containing L-glutamate (100 μM) or NMDA (50–500 μM), in the presence or absence of 10 μM glycine, was added, and dishes were left in room air (22–23°C) for 15 (glutamate) or 30 min (NMDA). In kainate (KA) studies, cells were incubated for 30 min in standard Locke's solution. After incubation with drug, cells were washed twice with standard Locke's solution, the original (preconditioned) growth medium was replaced, and cells were returned to the incubator. After 24 h, cells were washed with standard Locke's solution before and after incubation with fluorescein diacetate (FDA; 10 μg/ml) and propidium iodide (PI; 4.6 μg/ml) for 10 min and then examined using fluorescent microscopy, as reported previously (Leahy et al., 1994). In one series of experiments as indicated, the total number of living cells was compared using FDA staining only, as glial-mediated phagocytosis eliminated dead cells (authors' unpublished data).

To examine the pathways involved in NMDA-mediated neurotoxicity in astrocyte-poor cultures, the effects of the following pharmacological agents were compared: indomethacin (10 μM) to inhibit the cyclooxygenase pathway of arachidonate metabolism; NDGA (10 μM) to inhibit the lipoxygenase pathway of arachidonate metabolism; cyclosporin A (100 n*M*) or FK506 (100 n*M*) to inhibit the Ca^{2+}- and calmodulin-dependent phosphatase calcineurin; and W-7 (20 μM) to inhibit calmodulin. In these toxicity assays, cells were either coincubated with drug and 100 μM NMDA/10 μM glycine for 30 min (NDGA, indomethacin, or FK506) or preincubated with drug for 15 min in Locke's solution before addition of NMDA/glycine for 30 min and then postincubated with drug for 60 min in Locke's solution after NMDA removal (W-7 and cyclosporin A). Concentrations and conditions of incubation were chosen to be maximally effective based on previously published information (Rothman et al., 1993; Okuda et al., 1994; Marcaida et al., 1995; Ankarcrona et al., 1996). The culture medium was replaced, and the cells were returned to the incubator for 24 h and then assayed for toxicity using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). MTT is selectively converted by viable cells to a blue formazan product that is then quantified spectrophotometrically (Carmichael et al., 1987). Results obtained using this assay and FDA/PI are comparable (Ankarcrona et al., 1995; authors' unpublished data).

RNA isolation and RT

Whole-cell RNA was isolated from cerebellar granule cells in culture (8–10 DIV) by the guanidinium isothiocyanate method described by Chomczynski and Sacchi (1987). After the final ethanol precipitation, the RNA pellet was resuspended in sterile water. Concentration and purity of RNA were assessed with a spectrophotometer using nucleotide absorption at 260 nm and ratio at 260/280 nm (nucleotide/protein), respectively. RNA was used in a RT reaction to produce cDNA for use as template in PCR as previously described (Vallano et al., 1996). In some cases, the RT reaction was performed in the absence of either RT enzyme or RNA as controls.

PCR

PCR of NR1 subunit mRNAs was performed essentially as previously described (Vallano et al., 1996) in a final volume of 100 μl containing 1–5 ng of input RNA (following RT) and the following components (final concentrations): *Taq* buffer [10 m*M* Tris-HCl (pH 8.8), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, and 0.001% gelatin]; *Taq* polymerase (2.5 units); the four deoxynucleotide triphosphates (0.5 m*M* each); and oligonucleotide primers (10 pmol each) upstream (5′-CTCCCACCAGTCCAGCGTCT-3′; sense) and downstream (5′-GTCATGTTCAGCATTGCGGC-3′; antisense) that specify amplicons of 354 and 288 bp corresponding to NR1b and NR1a, respectively. NR1b contains a 21-amino acid sequence, designated as the N1 cassette, that is encoded by exon 5, whereas exon 5 is spliced out of the NR1a variant. Relative proportions of NR mRNAs were estimated by adding a trace amount of [^{32}P]dCTP (2 μCi; 0.66 pmol) to each PCR assay tube, replacing water. Samples were resolved on 1.5% ethidium bromide-stained agarose gels to separate and visualize amplicons and photographed while illuminated with UV light. Gel pieces corresponding to NR1a and NR1b were excised, and radioactivity was quantified in a liquid scintillation system using the tritium window and Cerenkov radiation.

PCR of NR2 was performed essentially as described above for NR1 and previously published (Vallano et al., 1996) using primers that amplify a common region in NR2A, NR2B, and NR2C. After PCR, equal aliquots were digested with restriction enzymes to distinguish NR2A, NR2B, and NR2C using *Bpm*I, *Bfa*I, or *Sca*I, respectively. Cerebellar granule cells do not express NR2D (Audinat et al., 1994). Digested samples were resolved on 1.5% ethidium bromide-stained agarose gels to separate and visualize amplicons and photographed while illuminated with UV light. The units of restriction enzymes used in the digestion reaction were determined to be saturating for the range of amplicons tested. The fragments produced after digestion were shown by Southern blotting analysis to be derived from the appropriate NR2 subunit-specific cDNAs (Audinat et al., 1994). All three NR2 cDNAs have equivalent efficiencies of amplification under the conditions of assay (Vallano et al., 1996).

Measurement of intracellular Ca^{2+} levels

Cerebellar granule cells (7–10 DIV) grown as a monolayer on glass coverslips were loaded with 2 μM fura-2 acetoxymethyl ester for 45 min in buffer containing 125 m*M* NaCl, 5 m*M* KCl, 1.5 m*M* $CaCl_2$, 5.6 m*M* glucose, and 25 m*M* sodium HEPES (pH 7.4) plus 0.1% bovine serum albumin (Parks et al., 1991). After rinsing, the NR response was measured in the same buffer as follows: The coverslip was placed in a holder that was inserted into a 1-cm^2 cuvette with a stir bar. A baseline response was recorded. NMDA and glycine (or glutamate and glycine) were added in a volume not exceeding 0.5% of the total volume in the cuvette. The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was calculated from the ratio of fluorescent intensities obtained with excitation at 340 and 380 nm as described by Grynkiewicz et al. (1985), where $[Ca^{2+}]_i = K_D[(R - R_{min})/(R_{max} - R)] \times (F380_{min}/F380_{max})$, using a K_D of 224 n*M* for fura-2/Ca^{2+}. Emission was measured at 510 nm. R_{min} is the ratio obtained under nominally Ca^{2+}-free conditions where Ca^{2+} is displaced with 25 m*M* EGTA. R_{max} represents the Ca^{2+}-saturated condition, which was obtained in the presence of 20 μM ionomycin. $F380_{max}$ and $F380_{min}$ represent

the fluorescent values obtained at 380 nm in the presence of ionomycin and EGTA, respectively.

The resting level of $[Ca^{2+}]_i$ in astrocyte-poor cultures was 35.8 ± 2.8 n*M* (n = 23), and that in astrocyte-rich cultures was 33.2 ± 2.8 n*M* (n = 10). The NMDA (100 μM plus 10 μM glycine)-mediated increase over resting $[Ca^{2+}]_i$ levels was significantly reduced in the presence of 2 m*M* extracellular Mg^{2+} (268.1 ± 48.5 vs. 3.8 ± 1.8 n*M*, n = 7) or 150 μM 2-amino-5-phosphonovalerate (AP-5) (235.5 ± 30.6 vs. 8.3 ± 4.4 n*M*, n = 8). In the absence of external Ca^{2+} there was a significant decrease in the NMDA response, which was recovered by addition of Ca^{2+} (12.0 ± 2.8 vs. 296.7 ± 50.0 n*M*, n = 4). NMDA- or glutamate-mediated increases in Ca^{2+} level were decreased by 35.4 ± 8.2 and 28.5 ± 5.8%, respectively, when tetrodotoxin (TTX; 1 μM; to block Na^+ channels) and nifedipine (5 μM; to block voltage-sensitive Ca^{2+} channels) were included in the cuvette. Inclusion of dantrolene (30 μM; to interfere with release of Ca^{2+} from intracellular stores) in addition to TTX and nifedipine did not further reduce either NMDA- or glutamate-induced responses (25.4 ± 3.2 and 27.2 ± 2.3%, respectively). On this basis, the fura-2 experiments described in Results were performed in the presence of TTX and nifedipine.

Statistical analysis

Results stated in the text are mean ± SEM values and have been analyzed for their statistical significance using an ANOVA, followed by Tukey's test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Glia protect against glutamate- or NMDA-mediated neurotoxicity

Cultures were initially grown for 8–10 DIV in the presence (designated astrocyte-poor) or absence (designated astrocyte-rich) of AraC, a mitotic inhibitor of glial proliferation. After 8 DIV, numerous GFAP-positive astrocytes and microglia are present in cultures incubated without AraC, and neurons grown in the presence of AraC show vulnerability to neurotoxic concentrations of excitatory amino acids. Figure 1 is a photomicrograph demonstrating the neurotoxic effects of glutamate in astrocyte-poor cultures and protection in astrocyte-rich cultures. FDA or PI was used to label living or dead cells, respectively, 24 h after transient exposure (15 min) of cultures to glutamate (100 μM, no exogenous glycine). In a previous report we demonstrated that AP-5, an NR antagonist, protected neurons against glutamate-mediated toxicity (Leahy et al., 1994).

To test whether the response of cerebellar granule cells to NMDA was similarly affected by coculture with astrocytes, NMDA-mediated toxicity was examined. Figure 2 is a photomicrograph showing the neurotoxic effects of 500 μM NMDA in astrocyte-rich and astrocyte-poor cultures. In astrocyte-poor cultures, significant toxicity was observed in response to NMDA and was inhibited by AP-5. Astrocyte-rich cultures exposed to NMDA demonstrated significantly less toxicity.

Figure 3 includes a summary of glutamate-mediated toxicity data derived from three of three paired astrocyte-rich or astrocyte-poor cell preparations. Glutamate-mediated toxicity was significant in astrocyte-poor cultures, as assessed by comparing the percentage of viable neurons in control versus glutamate (100 μM)-treated cultures: 93.8 ± 0.8 versus 56.5 ± 1.6%, respectively. In astrocyte-rich cultures, the percentage of viable neurons in control and glutamate-treated cultures was not significantly different: 97.9 ± 1.1 versus 87.9 ± 4.7%, respectively. These data are in good agreement with previous reports demonstrating a protective effect of glia against glutamate-mediated neurotoxicity in cerebral cortical cultures (Rosenberg et al., 1992), where it was further demonstrated that protection was due to the presence of a sodium-coupled glutamate transport activity in glia. Figure 3 also summarizes cell viability data obtained from five of six paired astrocyte-rich or astrocyte-poor cell preparations after treatment with vehicle or 500 μM NMDA. In astrocyte-poor cultures, there was a significant difference in the percentage of viable neurons in control and NMDA-treated cultures: 93.4 ± 0.7 versus 62.1 ± 5.6%, respectively. In contrast, the difference was not significant in astrocyte-rich cultures: percent viability, control = 98.0 ± 0.6% versus 500 μM NMDA = 87.3 ± 3.7%. In one of six paired preparations, a comparable degree of toxicity was observed.

Growing granule neurons in an atmosphere of 10% CO_2 improves survival relative to the standard atmosphere of 5% CO_2, possibly by delaying the expression of NRs (Leahy et al., 1994). In a parallel series of experiments performed using neurons grown in 10% CO_2 (medium pH 7.1) instead of 5% CO_2 (medium pH 7.4), cell viability was significantly reduced in five of five astrocyte-poor (percent viability, control = 84.3 ± 2.8% vs. 500 μM NMDA = 56.6 ± 5.3%) but not astrocyte-rich (percent viability, control = 98.0 ± 0.5% vs. 500 μM NMDA = 92.2 ± 2.6%) preparations treated with NMDA. Collectively, the data suggest that glia protect against NMDA-mediated neurotoxicity by a mechanism that is distinct from protection against glutamate-mediated neurotoxicity, because glia do not contain a transport system for NMDA. We next explored the basis for this protective effect.

Glycine is a coagonist at NRs. Typically, sufficient amounts of glycine are produced by neuronal cultures such that addition of exogenous glycine is not necessary to produce a glutamate response or glutamate-mediated toxicity (see, e.g., Parks et al., 1991; Rosenberg et al., 1992; Leahy et al., 1994). However, this response may be submaximal, and because glia contain at least one type of glycine transporter (Zafra et al., 1995), a possible explanation for the observed protection in astrocyte-rich cultures is decreased glycine availability owing to uptake into glia. When exogenous glycine (10 μM) [EC_{50} of ~150 n*M* (see McBain and Mayer, 1994); in cerebellar granule neurons, maximal responses to NMDA were observed with 1–3 μM exogenous glycine (Parks et al., 1991)] was added to-

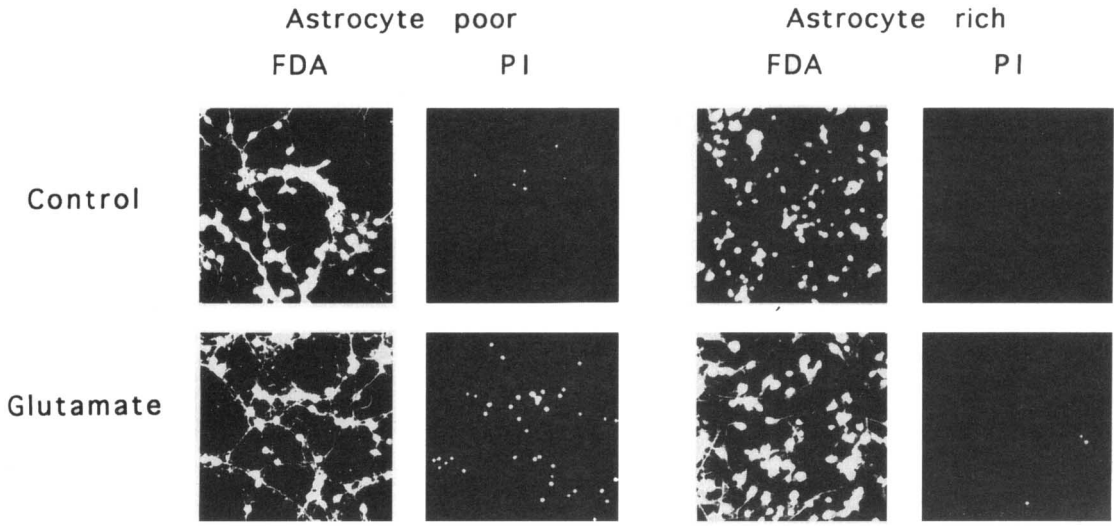

FIG. 1. Glia protect against glutamate-mediated neurotoxicity in dissociated cultures of cerebellar granule cells. Cells were grown for 9 DIV in the presence (astrocyte-poor; **left panels**) or absence (astrocyte-rich; **right panels**) of AraC, a mitotic inhibitor that is typically included in granule cell preparations to prevent astrocyte proliferation. The neurotoxic effects of 100 μM L-glutamate or control (no glutamate) were examined using FDA to stain living cells and PI to stain the nuclei of dead cells (see Materials and Methods). Fluorescent photomicrographs are shown. Note that a significant number of dead cells (PI-stained) were observed in astrocyte-poor, but not astrocyte-rich, cultures treated with glutamate.

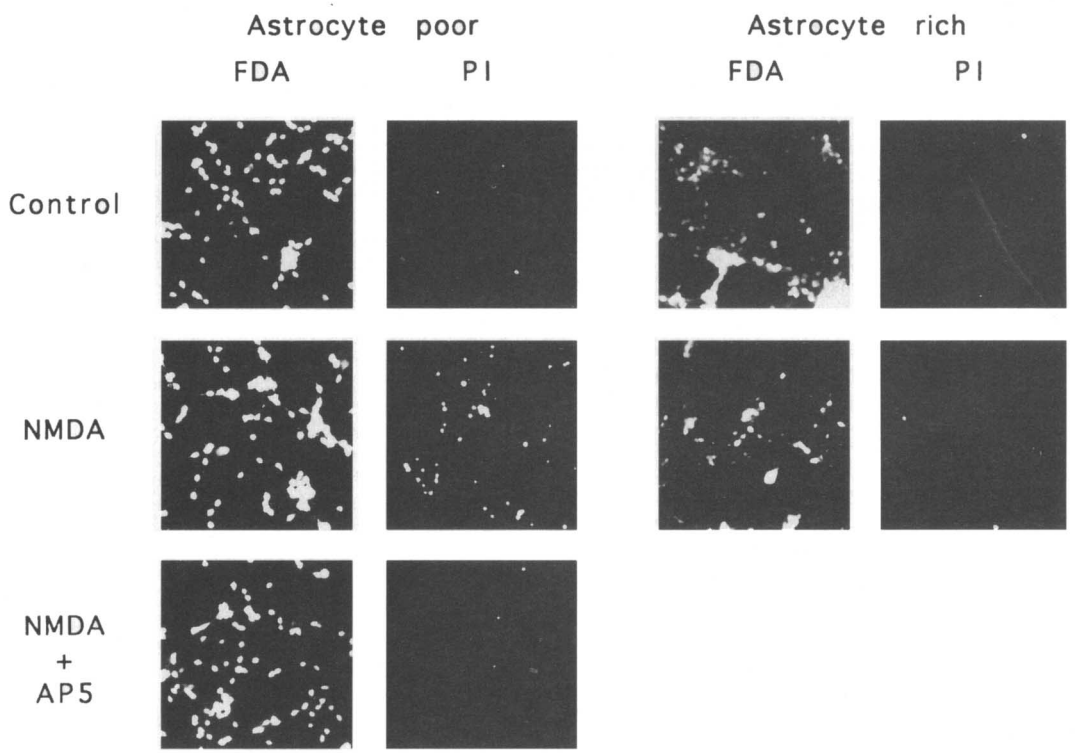

FIG. 2. Glia or AP-5 protects against NMDA-mediated neurotoxicity in dissociated cultures of cerebellar granule cells. Cells were grown for 9 DIV in the presence (astrocyte-poor; **left panels**) or absence (astrocyte-rich; **right panels**) of AraC. The neurotoxic effects of 500 μM NMDA (with or without 100 μM AP-5, an NR antagonist) or control (no NMDA) were examined using FDA to stain living cells and PI to stain the nuclei of dead cells (see Materials and Methods). Fluorescent photomicrographs are shown. Note that a significant number of dead cells (PI-stained) were observed in astrocyte-poor, but not astrocyte-rich, cultures treated with NMDA. Also shown is protection against NMDA-mediated toxicity in astrocyte-poor cultures by coincubation with AP-5 (left panel, **bottom**).

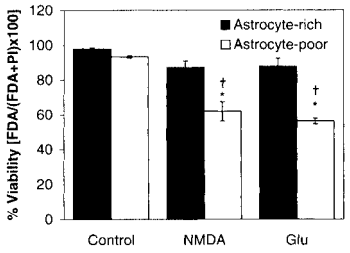

FIG. 3. Summary of glutamate (Glu)- and NMDA-mediated neurotoxicity in astrocyte-rich or astrocyte-poor cultures. Cerebellar granule cells were grown for 8–10 DIV in the presence (open columns) or absence (solid columns) of AraC. Neurotoxic effects of 500 μM NMDA or 100 μM Glu were quantified as detailed in Materials and Methods. The average FDA/FDA + PI ratio was measured from two separate fields per well in duplicate wells. Results are expressed as mean ± SEM (bars) values of three paired cell preparations challenged with Glu and five paired cell preparations challenged with NMDA. Symbols indicate statistically significant differences at $p < 0.05$ where * is a comparison between each treated group and its corresponding control group and † is a comparison between paired groups grown in the presence or absence of AraC. After correcting for minor differences in control viability, percent viability after NMDA treatment in astrocyte-poor cultures was 66.6 ± 6.2 versus 89.1 ± 4.0% in astrocyte-rich cultures, which is significantly different.

gether with increasing concentrations of NMDA (0–500 μM), astrocyte-rich cultures showed no significant toxicity, whereas astrocyte-poor cultures showed significant toxicity at 100, 250, and 500 μM NMDA, relative to the no-drug control (Fig. 4A). These data suggest that the protection observed in astrocyte-rich cultures is not due to enhanced glial glycine transport.

KA, a nondesensitizing agonist of AMPA and KA receptors, mediates a distinct form of neurotoxicity with rapid and delayed components, in which Cl^- and not Ca^{2+} has a central role (Kato et al., 1991; Teichberg, 1992). Neurotoxic responses to NMDA versus KA were compared to determine if protection was observed for the KA-mediated form of toxicity. In this case, the method used to quantify cell viability was based on a comparison of FDA staining of living cells in control or drug-treated samples, instead of calculating the number of living cells as a percentage of the total number of living plus dead cells. This was done because very few or no dead neurons (PI-stained) were detectable in the cultures lacking AraC 24 h after challenge with KA, despite the fact that there was a significant decrease in neuronal number, and substantial neuronal deterioration was apparent by phase-contrast microscopy within hours of KA exposure. A plausible interpretation is phagocytosis of dead cells by glia. In contrast, in the experiments presented in Fig. 4A, there was no significant difference in total neuronal number between untreated and NMDA-treated cultures lacking AraC. Although exposure of neurons to NMDA did not typically result in a reduction in total neuronal number, the same method of quantifying cell viability was used under the two conditions of challenge shown in Fig. 4B. The percentage of viable neurons was sig-

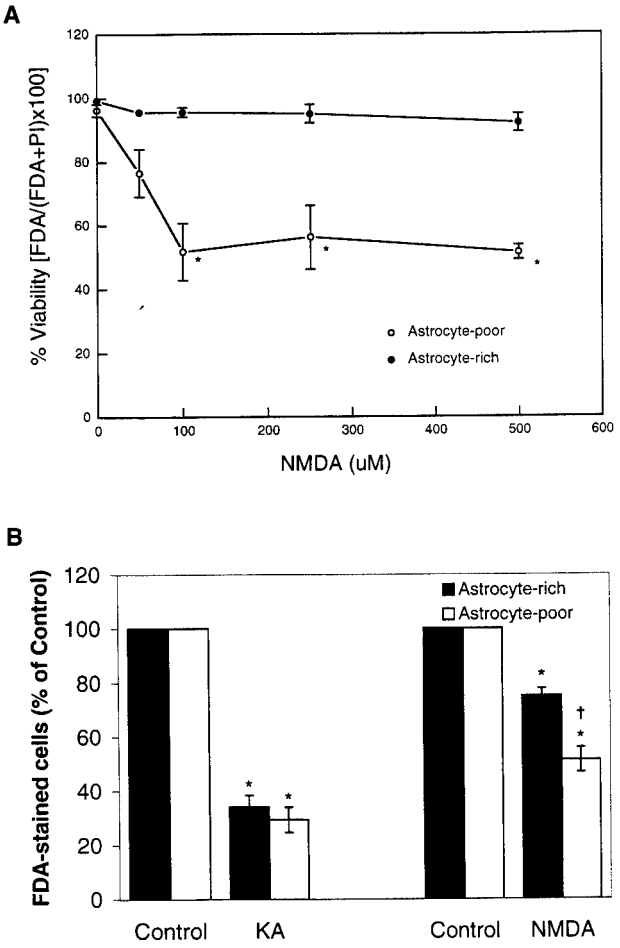

FIG. 4. NMDA concentration–response curve and comparison of NMDA and KA effects on cell viability. **A:** Granule cells were grown for 8 DIV in the absence (astrocyte-rich; ●) or presence (astrocyte-poor; ○) of AraC and then exposed to various concentrations of NMDA plus 10 μM glycine. Neurotoxicity was quantified using FDA and PI as described in Materials and Methods. Data are mean ± SEM (bars) FDA/FDA + PI ratio taken from two to four separate fields in the same well. Results are from duplicate wells in two separate cell preparations. There is a significant difference in percent neuronal viability when comparing astrocyte-rich and astrocyte-poor cultures treated with 100, 250, or 500 μM NMDA. Note that use of higher concentrations of glycine (100 μM and 1 mM) did not influence the degree of protection observed in astrocyte-rich cultures, as assessed by phase-contrast microscopic examination of two separate cell preparations. **B:** Cerebellar granule cells were grown for 8–10 DIV in the presence (open columns) or absence (solid columns) of AraC. Cell viability in response to no drug (control), 100 μM NMDA plus 10 μM glycine (NMDA), or 500 μM KA was quantified by FDA staining (see Materials and Methods). The average number of FDA-stained cells was measured in two separate fields per well in duplicate wells. Results are corrected to percentages of control and expressed as mean ± SEM (bars) values (n = 4 different paired cell preparations). Symbols indicate statistically significant differences at $p < 0.05$ where * is a comparison between each treated group and its corresponding control group and † is a comparison between paired groups grown in the presence or absence of AraC. Note that there is a significant difference in the number of FDA-stained cells between astrocyte-rich and astrocyte-poor cultures treated with NMDA but not with KA.

nificantly reduced in both astrocyte-rich and astrocyte-poor cultures after exposure to 100 μM NMDA/10 μM glycine. Nevertheless, there was a significantly greater proportion of viable neurons in astrocyte-rich versus astrocyte-poor cultures: 75.1 ± 2.6 versus 51.2 ± 4.6%, respectively. In contrast, the degree of neurotoxicity in response to 500 μM KA was significant and equivalent in both astrocyte-rich and astrocyte-poor cultures: 34.1 ± 4.3 and 29 ± 4.6%, respectively. These data indicate that protection by glia is selective for the form of toxicity mediated by NMDA. The absence of PI-stained neurons in cultures lacking AraC, challenged with KA, and examined after 24 h is likely due to phagocytosis, possibly by microglia. Consistent with this, cell viability, as measured by dividing the number of living FDA-stained neurons by the total number of neurons (FDA- plus PI-stained neurons), was significantly greater in astrocyte-rich versus astrocyte-poor cultures: 98.8 ± 0.3 versus 88.8 ± 1.5% (n = 21), respectively. However, it is also possible that survival of neurons is enhanced in the presence of glia or that a combination of phagocytosis as well as enhanced survival accounts for the observed difference.

The next series of experiments was designed to evaluate whether the degree of toxicity is related to the relative abundance of glia. To test this, a type of experiment similar to that described in Fig. 4 was conducted, except that cells were grown in defined medium instead of serum-containing medium. Under these conditions, there is a marked reduction in astrocyte and microglial proliferation even in the absence of AraC, relative to cultures grown in medium containing serum (Wang et al., 1994; Sasagasako et al., 1996). Also, the morphology of the type 1 astrocytes that predominate in these cultures is altered (Raff et al., 1983*a,b*). Figure 5 compares GFAP-positive astrocytes under the four conditions of culture. The highest proportion of astrocytes is present in serum-containing medium lacking AraC (~10% of cells are GFAP-positive), followed by defined medium lacking AraC (~3–5% of cells are GFAP-positive), serum-containing medium plus AraC (~2–3% of cells are GFAP-positive), and then defined medium plus AraC (<1% of cells are GFAP-positive). Consistent with this, there was a greater increase in metabolism of the mitochondrial substrate MTT in serum-containing medium compared with defined medium lacking AraC (184.5 ± 18.8 vs. 146.2 ± 12.9%, respectively) when each of these was compared with their corresponding AraC-containing control (Fig. 6A).

Figure 6B shows that neuronal viability was significantly reduced in both astrocyte-rich and astrocyte-poor cultures in response to 100 μM NMDA/10 μM glycine (astrocyte-rich percent viability, control = 93.6 ± 1.1 vs. NMDA = 52.2 ± 6.4%; astrocyte-poor percent viability, control = 91.0 ± 2.0 vs. NMDA = 29.2 ± 6.0%). Nevertheless, there was a significantly greater proportion of viable neurons in astrocyte-rich versus astrocyte-poor cultures: 55.9 ± 7.2 versus 31.8 ± 6.1%, respectively. In contrast, a significant and equivalent degree of toxicity was observed in both types of cultures in response to KA (astrocyte-rich percent viability, control = 94.4 ± 1.4 vs. KA = 45.8 ± 3.9%; astrocyte-poor percent viability, control = 91.0 ± 2.0% vs. KA = 42.9 ± 5.4%). There was no evidence of phagocytosis after exposure to KA in any of the cultures grown in defined medium, presumably due to lower proportions of microglia and/or astrocytes. Accordingly, cell viabilities in control cultures were comparable. These data suggest that the number or morphology of glia can influence the degree of protection against NMDA-induced neurotoxicity and that protection by glia is selective for the form of toxicity mediated by NMDA.

Glia do not affect NR1 or NR2 subunit mRNA expression in cerebellar granule cells

In cerebellum, in situ hybridization evidence supports the gradual induction of an alternatively spliced form of NR1, designated as NR1b, containing an amino-terminal sequence encoded by exon 5 (the N1 cassette) that alters receptor sensitivity to glutamate, NMDA, and several neuromodulators (Durand et al., 1992; Nakanishi et al., 1992). Thus, a possible explanation for the reduced NMDA-mediated toxicity in astrocyte-rich cultures of granule cells is altered expression of NR1b, relative to NR1a. To test this, a RT-PCR assay that uses primers flanking the sequence encoded by exon 5 to produce different-size amplicons corresponding to NR1b or NR1a mRNAs was used (Vallano et al., 1996). Figure 7 is a photograph of an ethidium bromide-stained gel showing the relative proportions of amplicons corresponding to NR1a and NR1b mRNAs in astrocyte-rich and astrocyte-poor cultures at 8 DIV. For comparison, the developmental increase in NR1b content in whole rat cerebellum is shown, as well as the pattern obtained in adult rat hippocampus. Note that NR1a migrates at a lower molecular mass than NR1b, which is consistent with the absence of an additional 63 bp in the primary transcript. In the different cell preparations examined, NR1a made up 87.3 ± 4.4 (n = 8) and 87.5 ± 7.1% (n = 5) of the total NR1 amplicon in astrocyte-poor and astrocyte-rich cultures, respectively. These data indicate that the predominant variant of NR1 mRNA in cells under both culture conditions is NR1a, which lacks the N1 cassette encoded by exon 5.

During granule cell development in situ (Monyer et al., 1994; Watanabe et al., 1994) and in vitro (Vallano et al., 1996), there is a developmental switch from expression of NR2B subunit mRNA to NR2A subunit mRNA followed by a gradual increase in levels of NR2C mRNA, which predominates in the adult NRs. Consistent with this, a developmental switch in the predicted electrophysiological responses of granule neurons composed of these subunits is observed (Audinat et al., 1994; Farrant et al., 1994). Because the functional properties of NRs are influenced by the individual NR2 subunit composition, a second RT-PCR

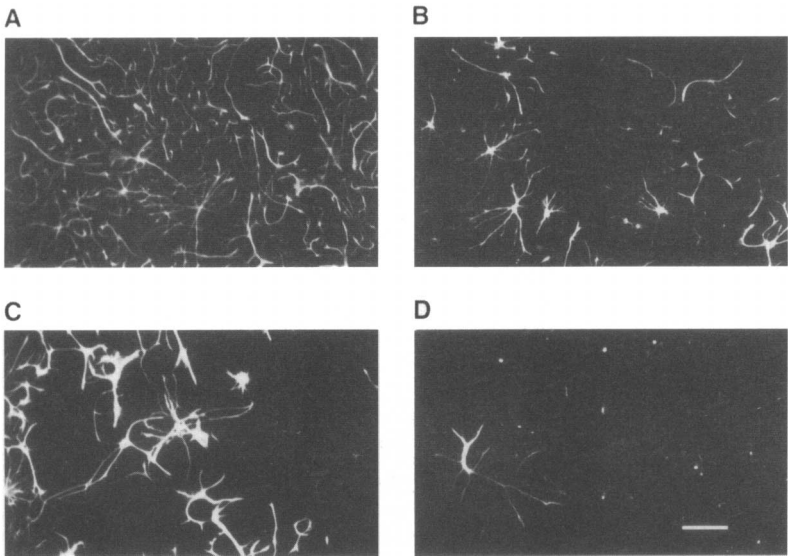

FIG. 5. Comparison of immunoreactive GFAP in granule cell preparations. Cells were grown in the absence (**A** and **C**) or presence (**B** and **D**) of AraC in serum-containing medium (A and B) or defined medium (C and D). After 8 DIV, cells were fixed and incubated with antibody against GFAP followed by a fluorescein isothiocyanate-conjugated secondary antibody. Bar = 100 μm. To estimate the proportion of GFAP-positive cells in the preparations, double labeling with GFAP to label astrocytes and then PI to stain all of the cells was performed. Cell numbers were counted by two independent observers.

analysis was performed for NR2A, B, and C subunit mRNAs. No significant differences in the relative proportions of the NR2 subunit mRNAs were observed in three separate preparations of astrocyte-rich and astrocyte-poor granule cell cultures (Fig. 8). In both culture conditions examined at 8 DIV, the predominant NR2 subunit mRNA was NR2A, with low levels of NR2B and NR2C mRNAs. Collectively, these data indicate that the NR1 and NR2 subunit compositions are similar in the two types of cultures. However, this does not rule out the possibility that glial-associated alterations in receptor density or sensitivity account for the observed differences in neurotoxic responses to NMDA. It was not possible to assess the absolute levels of NR subunit mRNAs in the two types of cultures, as an indicator of receptor density, because whole-cell RNA extracted from astrocyte-rich cultures represents an indeterminate mixture of both glial and neuronal RNAs.

Glia do not affect NR-mediated increases in $[Ca^{2+}]_i$

To evaluate the possibility that glia reduce NMDA-mediated toxicity by reducing the sensitivity of NRs, NMDA-mediated increases in $[Ca^{2+}]_i$ were measured under the different conditions of culture. This method uses a cuvette/spectrofluorometer system and therefore measures the response in the entire population of cells on the coverslip. Figure 9A is the concentration–response curve for 10–60 μM NMDA plus 10 μM glycine using astrocyte-rich or astrocyte-poor cultures. The NMDA-induced increase in $[Ca^{2+}]_i$ is not signifi-

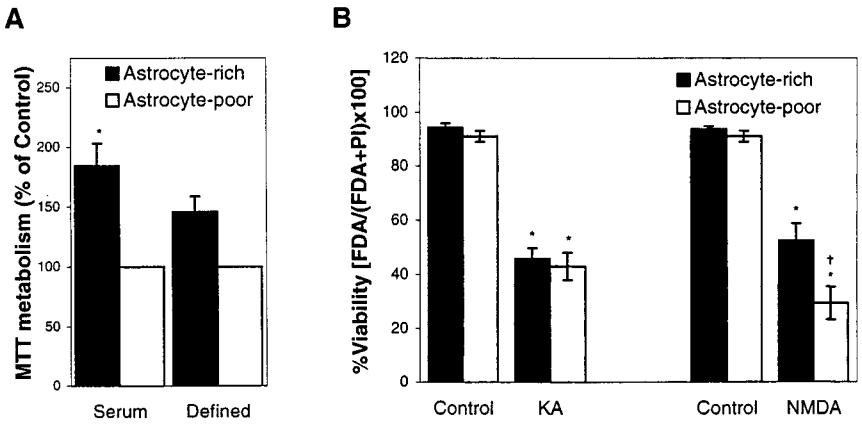

FIG. 6. Metabolism of MTT and neurotoxic effects of NMDA or KA. **A:** Cells were grown for 8 DIV in the presence (open columns) or absence (closed columns) of AraC in serum-containing or defined medium. Metabolism of the mitochondrial substrate, MTT, was compared under the four conditions of cell culture. Symbol indicates statistically significant differences at $p < 0.05$ where * is a comparison between each treated group and its corresponding control group (set at 100%). **B:** Cerebellar granule cells were grown for 8–10 DIV in the presence (open columns) or absence (solid columns) of AraC. Neurotoxic effects of 100 μM NMDA plus 10 μM glycine (NMDA) or 500 μM KA were quantified as detailed in Materials and Methods. The average FDA/FDA + PI ratio was measured in two separate fields per well. Duplicate wells were assayed for each condition. Results are expressed as (bars) mean ± SEM values of four different paired cell preparations. Symbols indicate statistically significant differences at $p < 0.05$ where * is a comparison between each treated group and its corresponding control group and † is a comparison between paired groups grown in the presence or absence of AraC. Note that there is a significant difference in percent viability between astrocyte-rich and astrocyte-poor cultures treated with NMDA but not with KA.

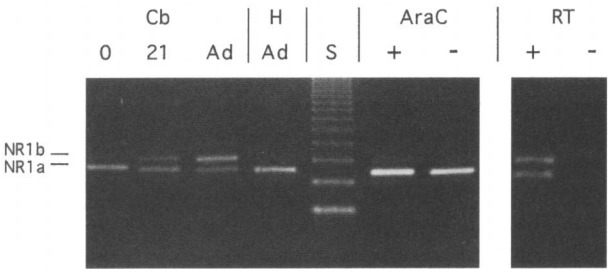

FIG. 7. RT-PCR analysis of NR1a and NR1b in granule cells, cerebellum (Cb), and hippocampus (H). Granule cells were grown for 8 DIV in the presence (+) or absence (−) of AraC, and RNA was extracted and processed for NR1 RT-PCR as detailed in Materials and Methods. Amplified product was resolved using 1.5% agarose gel electrophoresis, stained with ethidium bromide, and photographed under UV illumination. Amplicons corresponding to NR1a (288 bp) and NR1b (354 bp) were the major products observed. For comparison, RNA was extracted from Cb at postnatal days 0 and 21 and adult (Ad) and also from adult H and processed for NR1 RT-PCR. The lane marked S is the molecular weight marker that was used as a standard (123-bp ladder). No difference in the relative proportion of NR1a or NR1b was observed when comparing astrocyte-rich and astrocyte-poor cultures. To demonstrate the absence of contaminating DNA, several samples were subjected to RT-PCR in the presence (+) and absence (−) of reverse transcriptase enzyme (RT). An example is shown.

cantly different in the two culture conditions at any of the concentrations tested. Figure 9B is the concentration–response curve for 2–30 μM glutamate plus 10 μM glycine using both astrocyte-rich and astrocyte-poor cultures. The glutamate concentration–response curve for astrocyte-rich cultures is shifted to the right, compared with astrocyte-poor cultures (2, 5, and 10 μM glutamate are significantly different). Such a shift would be predicted if a glial transport system was effectively reducing the amount of glutamate available for NR activation. These data indicate that astrocytes do not affect Ca^{2+} influx in response to NMDA and must therefore influence a downstream signaling pathway that mediates the neurotoxic response.

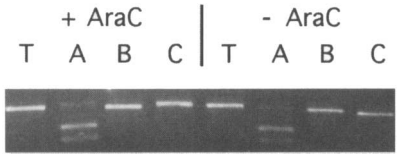

FIG. 8. RT-PCR analysis of NR2 subunits in granule cells. Granule cells were grown for 8 DIV in the presence (+) or absence (−) of AraC, and RNA was extracted and processed for NR2 RT-PCR as detailed in Materials and Methods. Equal aliquots of amplified product were digested in the absence (lanes T) or presence of appropriate restriction enzyme to cleave product corresponding to NR2A (lanes A), NR2B (lanes B), or NR2C (lanes C), resolved using 1.5% agarose gel electrophoresis, stained with ethidium bromide, and photographed under UV illumination. Amplicon corresponding to NR2A was the major product detected in both conditions. Note that minor differences among the lanes is due to minor differences in the initial position of the wells into which the samples were loaded.

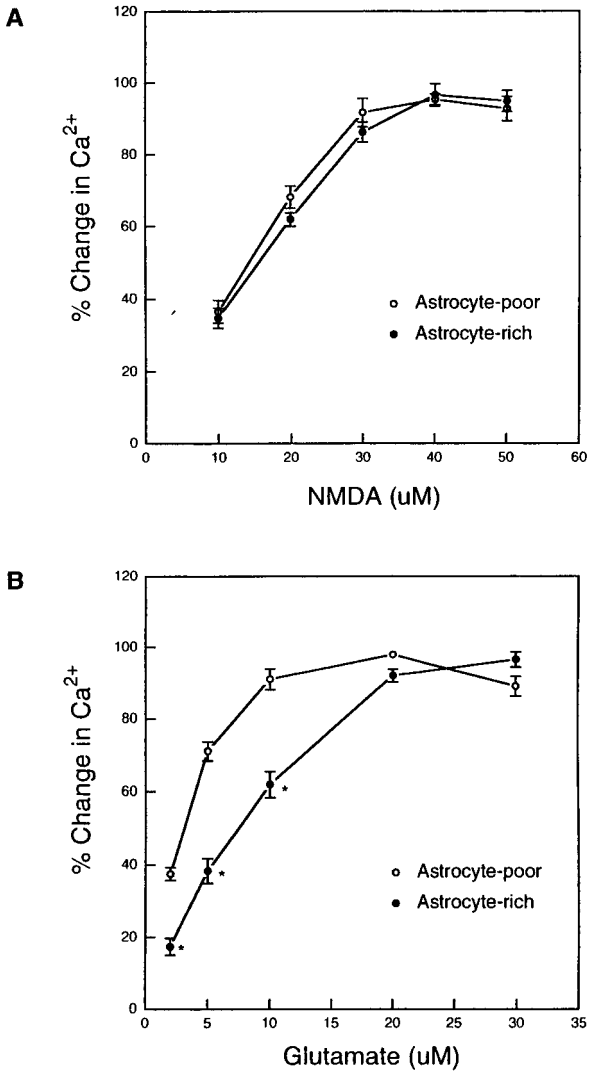

FIG. 9. Changes in $[Ca^{2+}]_i$ levels in response to NMDA or glutamate in astrocyte-rich or astrocyte-poor cultures. Granule cells were grown for 7–9 DIV in the absence (astrocyte-rich; ●) or presence (astrocyte-poor; ○) of AraC, loaded with fura-2 acetoxymethyl ester, and analyzed for alteration in $[Ca^{2+}]_i$ in response to NMDA/glycine or glutamate/glycine (see Materials and Methods). Data are changes, relative to no treatment, in $[Ca^{2+}]_i$ expressed as a percentage of the maximal response for each coverslip and are mean ± SEM (bars) values from four cell preparations treated with NMDA (**A**) or five cell preparations treated with glutamate (**B**). There is no significant difference between astrocyte-rich and astrocyte-poor cultures treated with NMDA, whereas cultures treated with 2, 5, or 10 μM glutamate were significantly different. $^*p < 0.05$ for comparison between cells grown in the presence or absence of AraC. Note that Ca^{2+} levels are expressed as percentages of the maximal response to NMDA to reduce variation between preparations. Similarly, when absolute increases in Ca^{2+} levels in response to NMDA in astrocyte-rich versus astrocyte-poor preparations were compared, no significant differences were observed (data not shown).

Arachidonate metabolites mediate NMDA-induced toxicity in cerebellar granule neurons

To gain understanding into the mechanism of astrocyte protection against NMDA-mediated toxicity

TABLE 1. *Effects of pharmacologic agents on NMDA-mediated toxicity in cerebellar granule neurons*

NMDA +	MTT metabolism (%)
None	53.8 ± 5.8
NDGA	95.6 ± 2.6
INDO	73.2 ± 0.3
FK506	71.6 ± 6.3
CYCLO	61.0 ± 0.4
W-7	46.6 ± 4.5

Cerebellar granule cells were grown in the presence of AraC for 9 DIV. MTT assay was performed 24 h after exposure of cells to 100 μM NMDA/10 μM glycine in the presence or absence of 10 μM NDGA, 10 μM indomethacin (INDO), 100 nM FK506, 100 nM cyclosporin A (CYCLO), or 20 μM W-7. MTT metabolism, an index of mitochrondrial viability, was measured in duplicate wells for each condition, and the results are expressed as a percentage of the control (100%; no NMDA or pharmacologic agent). Data are mean ± SEM values (n = 2 different cell preparations). Note that complete protection with NDGA, partial protection with cyclosporin A, and no protection with W-7 were also observed in a separate cell preparation in which 50 μM glutamate/10 μM glycine, instead of NMDA, was used to induce toxicity. The other compounds were not tested in the glutamate experiment.

in cerebellar granule cells, it is first necessary to determine the relevant pathways that are activated subsequent to ligand binding to and Ca^{2+} influx through NRs. To test this, a series of pharmacological agents that have been shown to be partially or fully effective as inhibitors of glutamate-mediated neurotoxicity in various types of neuronal cultures were examined using astrocyte-poor granule cell cultures, where significant toxicity is reliably observed. Table 1 shows the results of these experiments. Complete protection was observed with NDGA (10 μM), which interferes with the production of arachidonic acid metabolites via the lipoxygenase pathway. Partial protection was observed with indomethacin (10 μM), which interferes with the production of arachidonic acid metabolites via the cyclooxygenase pathway. Cyclosporin A (100 nM) or FK506 (100 nM), inhibitors of calcineurin, also showed partial protection. No protection was observed with W-7 (20 μM), a calmodulin inhibitor that interferes with activation of nitric oxide (NO) synthase (NOS).

DISCUSSION

Cerebellar granule cells contain all known classes of excitatory amino acid receptors, and those cultured in the continued presence of 25 mM KCl, which improves survival (Gallo et al., 1987), have been used extensively to study the contributions of different classes of glutamate receptors to neurotoxicity (Lysko et al., 1989; Cox et al., 1990; Schramm et al., 1990). Owing to their unique postnatal pattern of migration, relative to other neuronal types, highly enriched preparations of granule cells (>95%) can be obtained for study using as a tissue source 7–9-day postnatal rodent cerebellum (Levi et al., 1989). Similar to elevated KCl levels, NMDA is trophic in immature cultures (Balazs et al., 1988) but after 8 DIV brief exposure to glutamate or NMDA induces cell death (Lysko et al., 1989; Schramm et al., 1990) by a combination of necrosis and apoptosis (Ankarcrona et al., 1995). Although sensitivity to the neurotoxic effects of glutamate is not observed until 8–9 DIV, NR-mediated Ca^{2+} influx is maximal by 6 DIV (Bessho et al., 1994), indicating that NRs are necessary but not sufficient effectors of neurotoxicity in cerebellar granule cell cultures. Cerebellar granule cells also develop neurotoxic sensitivity to agents that activate the AMPA and KA types of ionotropic glutamate receptors. However, a nondesensitizing agonist such as KA rather than glutamate is required (Kato et al., 1991). Moreover, KA-mediated toxicity in cerebellar granule neurons is dependent on the presence of extracellular Cl^- and Na^+, not Ca^{2+} (Kato et al., 1991). Downstream pathways that activate xanthine oxidase, but not NOS, leading to the generation of superoxide radicals appear to be critical (Dykens et al., 1987; Puttfarcken et al., 1992, 1993).

Cultures used in the present study developed vulnerability to neurotoxic concentrations of glutamate or NMDA after ~8 DIV, and both Ca^{2+} influx and neurotoxicity were inhibited by inclusion of the NR antagonist AP-5 in the incubation medium. These observations confirm previous studies demonstrating that activation of NRs mediates glutamate neurotoxicity in cerebellar granule cells (Lysko et al., 1989; Cox et al., 1990; Schramm et al., 1990). Our observation that glutamate-mediated toxicity was significantly reduced in astrocyte-rich cultures compared with astrocyte-poor cultures is consistent with earlier studies in cortical neurons demonstrating the presence of a glutamate transport system in astrocytes (Rosenberg et al., 1992; see also Sugiyama et al., 1989) and suggests that cortical neurons and cerebellar neurons are alike in this respect. However, unlike cortical neurons, NMDA-mediated toxicity was also significantly reduced in astrocyte-rich cultures. This effect cannot be attributed to transport of NMDA into glia because they lack NRs or an NMDA transport system (Cull-Candy, 1995). RT-PCR, Ca^{2+} uptake, and glycine availability studies indicated that the composition and sensitivity of NRs, which mediate the initial phase of the neurotoxic response, were not altered under the two conditions of culture. Furthermore, glia did not influence KA-mediated neurotoxicity. These data are consistent with several reports showing that KA and NMDA differ in their mechanism of inducing cell death (Kato et al., 1991; DiStasi et al., 1991; Teichberg, 1992) and further indicate that glia are selective in protecting against the form of neurotoxicity that is mediated by NR activation.

In the present study, the possibility that the relative proportion of astrocytes affects the degree of protection was tested by growing cells in defined medium, which reduces astrocyte proliferation (Wang et al., 1994; Sa-

sagasako et al., 1996) but does not eliminate it altogether. Under these conditions, protection in astrocyte-rich cultures was less than complete, relative to cells grown in serum-containing medium, but was nonetheless significant, relative to astrocyte-poor cultures. As other factors such as astrocyte differentiation are also influenced by growing cells in defined medium (Raff et al., 1983*a,b*), this evidence supports the theory, but does not unequivocally demonstrate, that protection is positively correlated with astrocyte number. In this respect, it is also important to note that the role of microglia needs to be evaluated. In cultures grown in serum-containing, but not defined, medium lacking AraC and challenged with KA, the total number of neurons was consistently reduced. However, dead neurons were undetectable, presumably owing to microglial-mediated phagocytosis, which may also account for a significant increase in the apparent viability of control neurons grown in serum-containing medium lacking AraC, relative to those containing AraC, or grown in defined medium.

As an initial step in understanding the basis for neuroprotection in astrocyte-rich cultures, we attempted to define the critical pathways leading to toxicity in astrocyte-poor cultures with the understanding that some of these pathways may converge and that pharmacological agents are not completely selective. Several mechanisms have been proposed as downstream effectors of NR-mediated neurotoxicity. Elevated levels of calcium could increase or activate (a) calpain I, leading to degradation of neuronal structural proteins (Siman and Noszek; 1988; Siman et al., 1989). However, DiStasi et al. (1991) demonstrated that calpain-dependent proteolysis of fodrin is selectively associated with NR activation but does not have a causative role in NR-mediated toxicity in granule cells (see also Manev et al., 1991). (b) The agent increased or activated could be NOS, leading to the production of NO and the formation of peroxynitrite radicals. In cortical neurons, NOS inhibitors protect against NMDA-induced toxicity, and cortical cultures from NOS-deficient mice have a marked attenuation in NMDA-induced neurotoxicity (Dawson et al., 1991, 1993*a*, 1996). However, using hippocampal or cerebellar slices (Garthwaite and Garthwaite, 1994), cultured neurons from fetal rat forebrain (Reynolds and Hastings, 1995), or cultured cerebellar granule cells (Kiedrowski et al., 1991; Puttfarcken et al., 1992; Ankarcrona et al., 1996; Malcolm et al., 1996; present study), it was concluded that NMDA-induced toxicity is not mediated by NO. As NO can be protective or destructive depending on the stage of the ischemic process and on the cellular source of NO (for review, see Iadecola, 1997), the time course of administration and specificity of various NOS inhibitors/activators may be important in determining their effects on glutamate-induced toxicity. (c) Ca^{2+}/calmodulin-dependent phosphatase (calcineurin) and mitochondrial deenergization (Dawson et al., 1993*b*; Ankarcrona et al., 1996) could be increased or activated. Cyclosporin A protects the cell against loss of mitochondrial membrane potential. Both cyclosporin A and FK506 are inhibitors of calcineurin, and they partially protect cerebellar granule cells against glutamate-induced toxicity (Ankarcrona et al., 1996; present study). Note that studies in cerebral cortical neurons suggest that cyclosporin A and FK506 are neuroprotective because they prevent calcineurin-mediated dephosphorylation and subsequent activation of NOS (Dawson et al., 1993*b*). However, the lack of evidence for NO as a mediator of glutamate-induced cell death in granule cell cultures points to another site of action. (d) The entity that is up-regulated could be phospholipase, leading to liberation of arachidonic acid and the production of reactive oxygen species. Arachidonic acid is released in response to NR activation (Dumuis et al., 1988), and its metabolites represent a source of toxic reactive oxygen species in neurons. In hippocampal neurons, low concentrations of arachidonic acid support survival, whereas higher concentrations are toxic in a manner that is blocked by NDGA but not indomethacin (Okuda et al., 1994). Consistent with this, NDGA was the most effective agent tested in our study using cerebellar granule neurons. Other investigators observed partial protection against NR-mediated toxicity in hippocampal (Rothman et al., 1993) and cortical (Reynolds and Hastings, 1995) cultures using similar concentrations of NDGA. In the hippocampal neuron study, no effect of NDGA on NMDA-induced inward currents or elevation of $[Ca^{2+}]_i$ was observed, suggesting that inhibition of a downstream pathway, namely, lipoxygenase, was involved. Thus, a possible explanation for the protective effect of astrocytes on NMDA-mediated neurotoxicity is that they accumulate highly diffusible toxic arachidonic acid metabolites that are generated in response to NMDA receptor activation, thereby reducing the concentration of these compounds in neurons. An alternative possibility is that glia modulate the development or phenotype of neurons in a manner that selectively reduces their susceptibility to the NR-mediated form of toxicity. Efforts to distinguish between these possibilities will be the focus of future studies.

Acknowledgment: This work was supported by U.S. Public Health Service grant NS 27603 and New York Heart grant 970181. We gratefully acknowledge the excellent technical assistance of Mr. Dan Marshall.

REFERENCES

Ankarcrona M., Dypbukt J. M., Bonfoco E., Zhivotovsky B., Orrenius S., Lipton S. A., and Nicotera P. (1995) Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. *Neuron* **15,** 961–973.

Ankarcrona M., Dypbukt J. M., Orrenius S., and Nicotera P. (1996) Calcineurin and mitochondrial function in glutamate-induced neuronal cell death. *FEBS Lett.* **394,** 321–324.

Audinat E., Lambolez B., Rossier J., and Crepel F. (1994) Activity-dependent regulation of *N*-methyl-D-aspartate receptor subunits

expression in cerebellar granule cells. *Eur. J. Neurosci.* **6,** 1792–1800.

Balazs R., Jorgensen O. S., and Hack N. (1988) *N*-Methyl-D-aspartate promotes the survival of cerebellar granule cells in culture. *Neuroscience* **27,** 437–451.

Banati R. B. and Graeber M. B. (1994) Surveillance, intervention and cytotoxicity: is there a protective role of microglia? *Dev. Neurosci.* **16,** 114–127.

Barres B. A. (1991) New roles for glia. *J. Neurosci.* **11,** 3685–3694.

Beal M. F. (1994) Molecular mechanisms of excitotoxicity, in *Recent Advances in the Treatment of Neurodegenerative Disorders and Cognitive Dysfunction* (Racagni G., Brunello N., and Langer S. Z., eds), pp. 102–111. Karger, Basel.

Bessho Y., Nawa H., and Nakanishi S. (1994) Selective up-regulation of an NMDA receptor subunit mRNA in cultured cerebellar granule cells by K^+-induced depolarization and NMDA treatment. *Neuron* **12,** 87–95.

Brierly J. B. and Graham D. I. (1984) Hypoxia and vascular disorders of the central nervous system, in *Greenfield's Neuropathology* (Adams J. H., Corsellis J. A. N., and Duchen L. W., eds), pp. 125–156. John Wiley & Sons, New York.

Carmichael J., DeGraff W. G., Gazdar A. F., Minna J. D., and Mitchell J. B. (1987) Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res.* **47,** 936–942.

Choi D. W. (1994) Glutamate receptors and the induction of excitotoxic neuronal death. *Prog. Brain Res.* **100,** 47–51.

Choi D. W. (1996) Ischemia-induced neuronal apoptosis. *Curr. Opin. Neurobiol.* **6,** 667–672.

Choi D. W. and Rothman S. M. (1990) The role of glutamate neurotoxicity in hypoxic–ischemic neuronal death. *Annu. Rev. Neurosci.* **13,** 171–182.

Chomczynski P. and Sacchi N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162,** 156–159.

Collingridge G. L. and Bliss T. V. (1995) Memories of NMDA receptors and LTP. *Trends Neurosci.* **18,** 54–56.

Cox J. A., Felder C. C., and Henneberry R. C. (1990) Differential expression of excitatory amino acid receptor subtypes in cultured cerebellar neurons. *Neuron* **4,** 941–947.

Cull-Candy S. (1995) NMDA receptors: do glia hold the key? *Curr. Biol.* **5,** 841–843.

Dawson V. L. (1995) Nitric oxide: role in neurotoxicity. *Clin. Exp. Pharmacol. Physiol.* **22,** 305–308.

Dawson V. L., Dawson T. M., London E. D., Bredt D. S., and Snyder S. H. (1991) Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. *Proc. Natl. Acad. Sci. USA* **88,** 6368–6371.

Dawson V. L., Dawson T. M., Bartley D. A., Uhl G. R., and Snyder S. H. (1993*a*) Mechanisms of nitric oxide-mediated neurotoxicity in primary brain cultures. *J. Neurosci.* **13,** 2651–2661.

Dawson T. M., Steiner J. P., Dawson V. L., Dinerman J. L., Uhl G. R., and Snyder S. H. (1993*b*) Immunosuppressant FK506 enhances phosphorylation of nitric oxide synthase and protects against glutamate nuerotoxicity. *Proc. Natl. Acad. Sci. USA* **90,** 9808–9812.

Dawson V. L., Kizushi V. M., Huang P. L., Snyder S. H., and Dawson T. M. (1996) Resistance to neurotoxicity in cortical cultures from neuronal nitric oxide synthase-deficient mice. *J. Neurosci.* **16,** 2479–2487.

Delaney C. L., Brenner M., and Messing A. (1996) Conditional ablation of cerebellar astrocytes in postnatal transgenic mice. *J. Neurosci.* **16,** 6908–6918.

Desagher S., Glowinski J., and Premont J. (1996) Astrocytes protect neurons from hydrogen peroxide toxicity. *J. Neurosci.* **16,** 2553–2562.

DiStasi A. M. M., Gallo V., Ceccarini M., and Petrucci T. C. (1991) Neuronal fodrin proteolysis occurs independently of excitatory amino acid-induced neurotoxicity. *Neuron* **6,** 445–454.

Dugan L. L., Bruno V. M. G., Amagasu S. M., and Giffard R. G. (1995) Glia modulate the response of murine cortical neurons to excitotoxicity: glia exacerbate AMPA neurotoxicity. *J. Neurosci.* **15,** 4545–4555.

Dumuis A., Sebben M., Haynes L., Pin J.-P., and Bockaert J. (1988) NMDA receptors activate the arachidonic acid cascade system in striatal neurons. *Nature* **336,** 68–70.

Durand G. M., Gregor P., Zheng X., Bennett M. V. L., Uhl G. R., and Zukin R. S. (1992) Cloning of an apparent splice variant of the *N*-methyl-D-aspartate receptor NMDAR1 with altered sensitivity to polyamines and activators of protein kinase C. *Proc. Natl. Acad. Sci. USA* **89,** 9359–9363.

Dykens J. A., Stern A., and Trenkner E. (1987) Mechanism of kainate toxicity to cerebellar neurons in vitro is analogous to reperfusion tissue injury. *J. Neurochem.* **49,** 1222–1228.

Farrant M., Feldmeyer D., Takahashi T., and Cull-Candy S. G. (1994) NMDA-receptor channel diversity in the developing cerebellum. *Nature* **368,** 335–339.

Gallo V., Kingsbury A., Balazs R., and Jorgensen O. S. (1987) The role of depolarization in the survival and differentiation of cerebellar granule cells in culture. *J. Neurosci.* **7,** 2203–2213.

Garthwaite G. and Garthwaite J. (1994) Nitric oxide does not mediate acute glutamate neurotoxicity, nor is it neuroprotective, in rat brain slices. *Neuropharmacology* **33,** 1431–1438.

Grynkiewicz G. M., Poenie M., and Tsien R. Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260,** 3440–3450.

Hollmann M. and Heinemann S. (1994) Cloned glutamate receptors. *Annu. Rev. Neurosci.* **17,** 31–108.

Iadecola C. (1997) Bright and dark sides of nitric oxide in ischemic brain injury. *Trends Neurosci.* **20,** 132–139.

Jones K. H. and Senft J. A. (1985) An improved method to determine cell viability by simultaneous staining with fluorescein diacetate–propidium iodide. *J. Histochem. Cytochem.* **33,** 77–79.

Kato K., Puttfarcken P. S., Lyons W. E., and Coyle J. T. (1991) Developmental time course and ionic dependence of kainate-mediated toxicity in rat cerebellar granule cell cultures. *J. Pharmacol. Exp. Ther.* **256,** 402–411.

Kiedrowski L., Manev H., Costa E., and Wroblewski J. T. (1991) Inhibition of glutamate-induced cell death by sodium nitroprusside is not mediated by nitric oxide. *Neuropharmacology* **30,** 1241–1243.

Laurie D. J., Putzke J., Zieglgänsberger W., Seeburg P. H., and Tölle T. R. (1995) The distribution of splice variants of the NMDAR1 subunit mRNA in adult rat brain. *Mol. Brain Res.* **32,** 94–108.

Leahy J. C., Chen Q., and Vallano M. L. (1994) Chronic mild acidosis reduces functional expression of NMDA receptors and enhances long-term survival in primary cultures of cerebellar granule cells. *Neuroscience* **63,** 457–470.

Levi G., Aloisi F., Ciotti M. T., Thangnipon W., Kingsbury A., and Balazs R. (1989) Preparation of 98% pure granule cell cultures, in *A Dissection and Tissue Culture Manual of the Nervous System* (Shahar A., de Vellis J., Vernadakis A., and Haber B., eds), pp. 211–214. Alan R. Liss, New York.

Lysko P. G., Cox J. A., Vigano A., and Henneberry R. C. (1989) Excitatory amino acid neurotoxicity at the *N*-methyl-D-aspartate receptor in cultured neurons: pharmacological characterization. *Brain Res.* **499,** 258–266.

Malcolm C. S., Ritchie L., Grieve A., and Griffiths R. (1996) Glutamate toxicity in primary cerebellar cultures from mouse brain is unaffected by changes in cGMP levels. *Neuroreport* **7,** 1650–1654.

Manev H., Favaron M., Siman R., Guidotti A., and Costa E. (1991) Glutamate neurotoxicity is independent of calpain I inhibition in primary cultures of cerebellar granule cells. *J. Neurochem.* **57,** 1288–1295.

Marcaida G., Minana M. D., Grisolia S., and Felipo V. (1995) Lack of correlation between glutamate-induced depletion of ATP and neuronal death in primary cultures of cerebellum. *Brain Res.* **695,** 146–150.

McBain C. J. and Mayer M. L. (1994) *N*-Methyl-D-aspartic acid receptor structure and function. *Physiol. Rev.* **74,** 723–760.

Meldrum B. and Garthwaite J. (1990) Excitatory amino acid neuro-

toxicity and neurodegenerative disease. *Trends Pharmacol. Sci.* **11,** 379–387.

Monyer H., Sprengel R., Schoepfer R., Herb A., Higuchi M., Lomeli H., Burnashev N., Sakmann B., and Seeburg P. H. (1992) Heteromeric NMDA receptors: molecular and functional distinction of subtypes. *Science* **256,** 1217–1221.

Monyer H., Burnashev N., Laurie D. J., Sakmann B., and Seeburg P. H. (1994) Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* **12,** 529–540.

Nakanishi N., Axel R., and Shneider N. A. (1992) Alternative splicing generates functionally distinct *N*-methyl-D-aspartate receptors. *Proc. Natl. Acad. Sci. USA* **89,** 8552–8556.

Okuda S., Saito H., and Katsuki H. (1994) Arachidonic acid: toxic and trophic effects on cultured hippocampal neurons. *Neuroscience* **63,** 691–699.

Parks T. N., Artman L. D., Alasti N., and Nemeth E. F. (1991) Modulation of *N*-methyl-D-aspartate receptor-mediated increases in cytosolic calcium in cultured rat cerebellar granule cells. *Brain Res.* **552,** 13–22.

Pulsinelli W. A. (1985) Selective neuronal vulnerability: morphological and molecular characteristics. *Prog. Brain Res.* **63,** 29–37.

Puttfarcken P. S., Lyons W. E., and Coyle J. T. (1992) Dissociation of nitric oxide generation and kainate-mediated neuronal degeneration in primary cultures of rat cerebellar granule cells. *Neuropharmacology* **31,** 565–575.

Puttfarcken P. S., Lyons W. E., and Coyle J. T. (1993) Kainic acid-induced lipid peroxidation: protection with butylated hydroxytoluene and U78517F in primary cultures of cerebellar granule cells. *Brain Res.* **624,** 223–232.

Raff M. C., Abney E. R., Cohen J., Lindsay R., and Noble M. (1983*a*) Two types of astrocytes in cultures of developing rat white matter: differences in morphology, surface gangliosides, and growth characteristics. *J. Neurosci.* **3,** 1289–1300.

Raff M. C., Miller R. H., and Noble M. (1983*b*) A glial progenitor cell that develops in vitro into an astrocyte or an oligodendrocyte depending on culture medium. *Nature* **303,** 390–396.

Reynolds I. J. and Hastings T. G. (1995) Glutamate induces the production of reactive oxygen species in cultured forebrain neurons following NMDA receptor activation. *J. Neurochem.* **15,** 3318–3327.

Rosenberg P. A. and Aizenman E. (1989) Hundred-fold increase in neuronal vulnerability to glutamate toxicity in astrocyte-poor cultures of rat cerebral cortex. *Neurosci. Lett.* **103,** 162–168.

Rosenberg P. A., Amin S., and Leitner M. (1992) Glutamate disguises neurotoxic potency of glutamate agonists in cerebral cortex in dissociated cell culture. *J. Neurosci.* **12,** 56–61.

Rothman S. M., Yamada K. A., and Lancaster N. (1993) Nordihydroguaiaretic acid attenuates NMDA neurotoxicity—action beyond the receptor. *Neuropharmacology* **32,** 1279–1288.

Sasagasako N., Toda K., Hollis M., and Quarles R. H. (1996) Myelin gene expression in immortalized Schwann cells: relationship to cell density and proliferation. *J. Neurochem.* **66,** 1432–1439.

Scheetz A. J. and Constantine-Paton M. (1994) Modulation of NMDA receptor function: implications for vertebrate neural development. *FASEB J.* **8,** 745–752.

Schramm M., Eimerl S., and Costa E. (1990) Serum and depolarizing agents cause acute neurotoxicity in cultured cerebellar granule cells: role of the glutamate receptor responsive to *N*-methyl-D-aspartate. *Proc. Natl. Acad. Sci. USA* **87,** 1193–1197.

Schousboe A. and Frandsen A. (1995) Glutamate receptors and neurotoxicity, in *CNS Neurotransmitters and Neuromodulators: Glutamate* (Stone T. W., ed), pp. 239–251. CRC Press, Boca Raton, Florida.

Siman R. and Noszek J. C. (1988) Excitatory amino acids activate calpain I and induce structural protein breakdown *in vivo*. *Neuron* **1,** 279–287.

Siman R., Noszek J. C., and Kegerise C. (1989) Calpain I activation is specifically related to excitatory amino acid induction of hippocampal damage. *J. Neurosci.* **9,** 1579–1590.

Sucher N. J., Awobuluyi M., Yun-Beom C., and Lipton S. A. (1996) NMDA receptors: from genes to channels. *Trends Pharmacol. Sci.* **17,** 348–355.

Sugiyama K., Brunori A., and Mayer M. L. (1989) Glial uptake of excitatory amino acids influences neuronal survival in cultures of mouse hippocampus. *Neuroscience* **32,** 779–791.

Szatkowski M. and Attwell D. (1994) Triggering and execution of neuronal death in brain ischaemia: two phases of glutamate release by different mechanisms. *Trends Neurosci.* **17,** 359–365.

Teichberg V. I. (1992) Excitotoxins, glutamate receptors, and excitotoxicity, in *Handbook of Experimental Pharmacology* (Herken H. and Hucho F., eds), pp. 483–506. Springer-Verlag, New York.

Traynelis S. F., Hartley M., and Heinemann S. F. (1995) Control of proton sensitivity of the NMDA receptor by RNA splicing and polyamines. *Science* **268,** 873–876.

Vallano M. L., Lambolez B., Audinat E., and Rossier J. (1996) Neuronal activity differentially regulates NMDA receptor subunit expression in cerebellar granule cells. *J. Neurosci.* **16,** 631–639.

Vernadakis A. (1996) Glia–neuron intercommunications and synaptic plasticity. *Prog. Neurobiol.* **49,** 185–214.

Wang L.-C., Baird D. H., Hatten M. E., and Mason C. A. (1994) Astroglial differentiation is required for support of neurite outgrowth. *J. Neurosci.* **14,** 3195–3207.

Watanabe M., Inoue Y., Sakimura K., and Mishina M. (1992) Developmental changes in distribution of NMDA receptor channel subunit mRNAs. *Neuroreport* **3,** 1138–1140.

Watanabe M., Mishina M., and Inoue Y. (1994) Distinct spatiotemporal expressions of five NMDA receptor channel subunit mRNAs in the cerebellum. *J. Comp. Neurol.* **343,** 513–519.

Westergaard N., Fosmark H., and Schousboe A. (1991) Metabolism and release of glutamate in cerebellar granule cells cocultured with astrocytes from cerebellum or cerebral cortex. *J. Neurochem.* **56,** 59–66.

Zafra F., Aragon C., Olivares L., Danbolt N. C., Gimenez C., and Storm-Mathisen J. (1995) Glycine transporters are differentially expressed among CNS cells. *J. Neurosci.* **15,** 3952–3969.

Zukin R. S. and Bennett M. V. L. (1995) Alternatively spliced isoforms of the NMDAR1 receptor subunit. *Trends Neurosci.* **18,** 306–313.