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Glia Modulate the Response of Murine Cortical Neurons to Excitotoxicity: Glia Exacerbate AMPA Neurotoxicity

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We have developed "pure" neuronal cultures (< 1% astrocytes) from mouse neocortex to study the effect of glial cells on the response of neurons to injury. Cortical neurons were found to require glial-conditioned medium to survive. Immature neurons, 2–4 d *in vitro*, deprived of glial-conditioned medium, underwent apoptosis over 48 hr, as suggested by condensed nuclear morphology, DNA fragmentation, and protection by inhibition of macromolecular synthesis. Apoptosis induced by trophic factor deprivation has been described for other neuronal populations, such as superior cervical ganglion and dorsal root ganglion cells. Cortical neurons in pure culture provide another neuronal population for the study of apoptosis induced by trophic factor deprivation. We then studied the interaction of neurons and glia under excitotoxic conditions. Experiments on mature cultures showed that pure neuronal cultures were at least 10-fold more sensitive to acute glutamate exposure than were neuronal-glial ("mixed") cocultures. The difference in sensitivity between pure neurons and mixed cultures was reduced when mixed cultures were treated with the glutamate uptake inhibitor, L-*trans*-pyrrolidine-2,4-dicarboxylic acid (*trans*-PDC). In 24 hr exposure to *N*-methyl-D-aspartate (NMDA), or oxygen, glucose deprivation, pure neurons were more sensitive than mixed cultures; *trans*-PDC again increased the sensitivity of mixed cultures to nearly that of pure neuronal cultures. In contrast, mixed and pure neuronal cultures exposed to NMDA for 10 min, or to kainate for 24 hr, had similar injury dose–response curves, suggesting that glial glutamate uptake is a less important protective mechanism in these excitotoxic injuries. Surprisingly, pure neurons were less sensitive than mixed cultures to (*RS*)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) toxicity at concentrations up to 100 μM. This does not reflect astrocyte toxicity, as AMPA at concentrations to 1 mM did not injure astrocyte cultures. Glial cultures showed increased levels of glutamate in the extracellular medium in response to exposure to AMPA, but not NMDA or kainate. However, pure neuronal and mixed cultures exposed to the same concentration of AMPA did not have elevated levels of glutamate in the media. We found that glia were generally neuroprotective under excitotoxic conditions, likely through their ability to clear extracellular glutamate. However, the presence of glia exacerbated AMPA neurotoxicity.

[Key words: astrocytes, glutamate, ischemia, NMDA, kainate, cell culture, neocortex, AMPA]

Glutamate receptor-mediated excitotoxicity is believed to contribute to neuronal injury in many disease states, including CNS trauma and hemorrhage, neurodegenerative diseases, and stroke (Choi and Rothman, 1990; Meldrum and Garthwaite, 1990). Non-neuronal cells, in particular, astrocytes, may modify neuronal responses to excitotoxicity (Rosenberg and Aizenman, 1989; Sugiyama et al., 1989; Hewett et al., 1994) and anoxia (Vibulsreth et al., 1987). Glia may provide substrates for neuronal energy metabolism (Quach et al., 1978; Fox et al., 1988; Swanson et al., 1990), and are important in removing glutamate from the extracellular space. Rosenberg and Aizenman (1989) have shown that a reduction of the number of glia in neuronal glial cocultures (from 90% to 30%) increases the sensitivity of neurons to glutamate toxicity 100-fold, and glia protect neurons from death due to glutamine in culture medium by uptake of nonenzymatically generated glutamate (Rosenberg, 1991). Neurons, in turn, may modify the homeostatic function of glia. Volterra et al. (1992), reported decreased reuptake of glutamate by astrocytes in response to arachidonic acid, which can be released from neurons (Sanfeliu et al., 1990). Interactions between glia and neurons may be critical in determining the outcome of injury. To address questions about glial–neuronal interactions during injury, studies of neurons and glia separately complement studies carried out in mixed cultures.

Although there are several published methods for growing cortical neuronal cell cultures from mouse or rat brain (Yu et al., 1984; Borg et al., 1985; Rosenberg and Aizenman, 1989), most of these pure cultures still contained a significant percentage of astrocytes (10–60%), or were maintained for only short durations, though highly purified neuronal cultures from rat brain have been reported (Aizenman and deVellis, 1987). Instead of focusing on using a chemically defined medium, we used glial-conditioned medium to support the culture of essentially pure mouse cortical neurons to complement the relatively pure astrocyte and mixed neuronal–glial cultures already in use in our laboratory. Glial-conditioned medium was essential for survival

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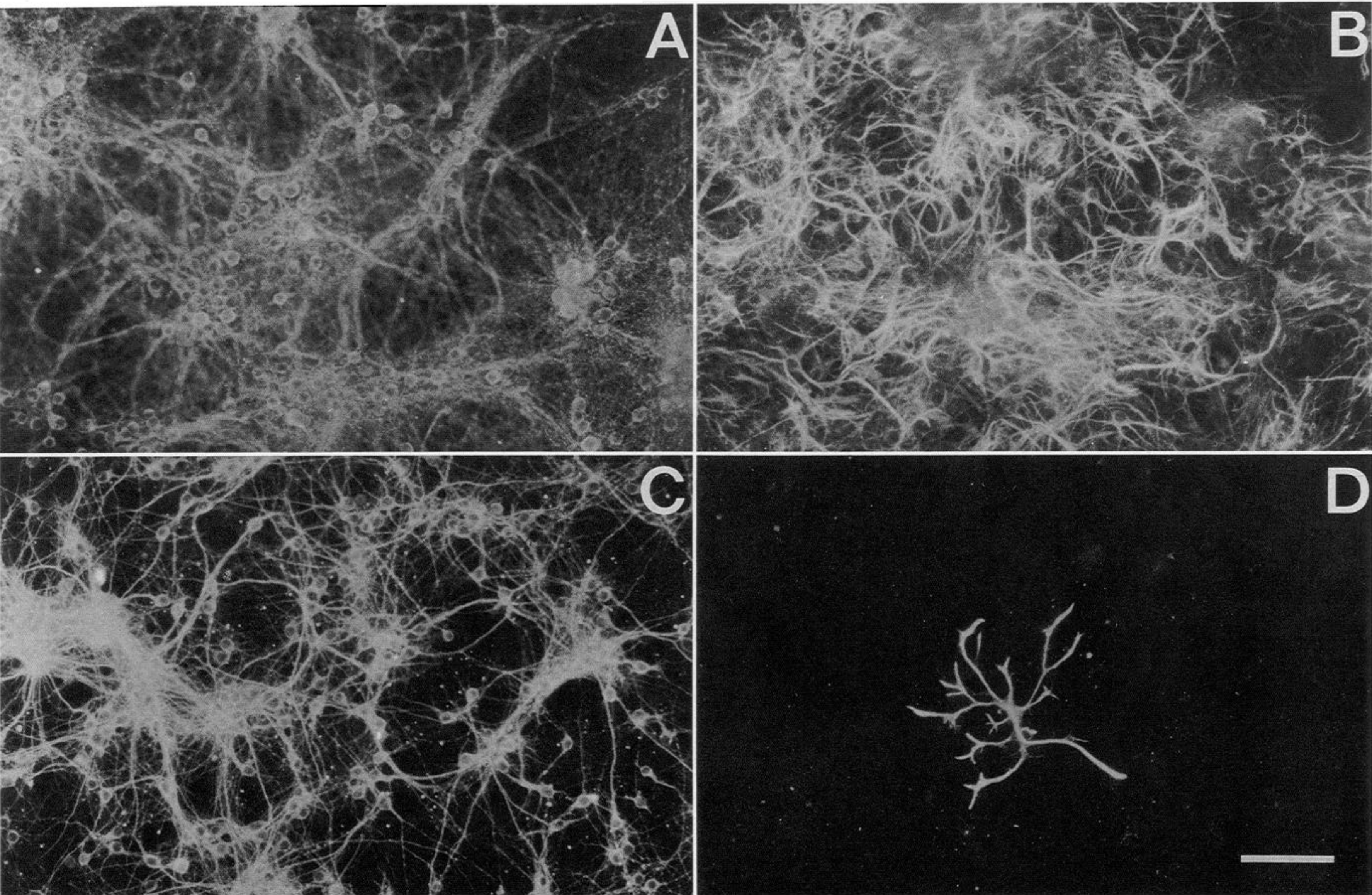

Figure 1. Immunohistochemical staining of mixed and pure neuronal cultures for MAP 2 and GFAP. Mixed (*A* and *B*) and pure neuronal (*C* and *D*) cultures at DIV 12 showing MAP 2 (*A* and *C*), and GFAP (*B* and *D*) immunopositive cells. The MAP 2 panels show neuronal morphology that is similar in mixed and pure neuronal cultures. GFAP staining reveals the confluent layer of astrocytes in mixed cultures, but less than 1% GFAP-positive cells in the pure neuronal cultures. Magnification, 20×; scale bar, 40 μm.

Table 1. Cell counts of neurons and glia in mixed and pure cortical neuronal cultures

	NSE-positive cells		GFAP-positive cells	
	DIV 2	DIV 12	DIV 2	DIV 12
Mixed cultures	3450	4250	10,050	9870
Neuronal cultures	3020	2460	22	11

Mixed cortical cultures and pure neuronal cultures were stained for neuron-specific enolase (NSE) and glial fibrillary acidic protein (GFAP) at DIV 2 and DIV 12. Cells counted in five microscope fields are shown for each condition.

Table 2. Development of sensitivity to 10 min NMDA exposure

Age in culture (d)	% Maximum LDH release
8	14.7 ± 2.8
11	86.6 ± 8.9*
14	100 ± 18.8*

Values shown are mean ± SEM (n = 4). LDH release from pure neuronal cultures of the indicated ages, 24 hr after 10 min exposure to 300 μM NMDA, expressed as percentage of LDH released by freeze–thaw of the same cultures.
* Different from day 8 value by ANOVA and Student–Newman–Keuls test, $p < 0.05$.

of immature cultures. These cultures were characterized histologically and developmentally, and their response to injury was assessed.

An abstract has appeared (Giffard et al., 1992).

Materials and Methods

Cell cultures. Pure neuronal cell cultures were prepared from fetal mice at 14–15 d gestation (Dichter, 1978; Choi et al., 1987) with the following modifications. The brains were removed from fetal mice, freed of meninges, and the cortical hemispheres removed from the rest of the brain. Neocortices were minced and treated with 0.09% trypsin for 20 min at 37°C. After centrifugation at 4000 rpm for 3 min, the pellet was resuspended in 2 ml PM by gentle trituration through a flame-polished pasteur pipette. The cell suspension was diluted with PM and plated at 400 μl per well, 3.5 hemispheres (approximately 2×10^7 cells) per 24-well culture plate; these polystyrene culture plates had been previously coated with poly-D-lysine (100 ng/ml) and laminin (4 ng/ml, in water) overnight, washed with water, then MEM, and stored at 4°C until use. Twenty-four to 48 hr after plating, half of the culture medium was replaced with GCM, and cytosine arabinoside (Ara-C, 3 μM) was added to each well. Cultures were kept at 37°C in a humidified incubator with 5% CO_2 atmosphere without further medium changes. Except as noted, cells were used on days *in vitro* (DIV) 11–15.

Mixed cortical cultures were prepared in identical fashion through the trituration step. The cell suspension was then plated at 3.5 hemispheres per 24-well plate on plates containing a confluent monolayer of astrocytes. Astrocyte cultures were prepared by plating cortical cell suspensions from 1–2-d-old mouse pups, 1 cortical hemisphere/24 multiwell Primaria plate (Rose et al., 1993). The mixed cortical cultures were fed twice weekly with MEMglc with 10% horse serum (first three feedings), and then MEMglc. The astrocyte cultures were fed once per week, and were generally confluent within 2 weeks. Mixed cultures were used for experiments at DIV 13–15.

Immunohistochemistry. For photomicrography, cultures were stained for microtubule-associated protein 2 (MAP 2), and glial fibrillary acidic protein (GFAP). Cells were washed twice with PBS (phosphate-buffered saline), fixed for 30 min with 4% paraformaldehyde, washed three times with PBS, then permeabilized with 0.25% Triton X-100 in PBS for 10 min. Cells were washed, blocked with serum, then incubated with the primary antibody for 24 hr at 4°C, at 1:300 for anti-MAP 2, or 1:10 for anti-GFAP. After washing three times, secondary antibody, labeled with either BODIPY or Cy3, was added for 30 min at room temperature in the dark. After three final washes the cells were photographed using a Nikon Diaphot microscope equipped with a mercury lamp.

For cell counts, cultures were stained for neuron-specific enolase (NSE 1:100) and glial fibrillary acidic protein (GFAP 1:10) at DIV 2 and DIV 12. Cells were washed, then incubated in biotinylated anti-mouse IgG at 1:100 dilution for 30 min. After reaction with avidin and biotin-horseradish peroxidase (ABC kit, Vector Labs, Burlingame, CA) staining was developed by exposure to 0.05% diaminobenzidine/0.01% H_2O_2 (2–10 min).

Staining for NADPH-diaphorase was performed as described by Koh and Choi (1988), based on the original protocol of Scherer-Singler et al. (1983). Microglia were identified using the lectin, *Banderiera simplicifolia* I, coupled to horseradish peroxidase. After 30 min incubation at room temperature with lectin at 1:50 dilution, lectin binding was identified using 0.05% DAB/0.01% H_2O_2 (Colton et al., 1992). Cobalt staining for calcium-conducting AMPA/kainate receptors was done as previously described (Pruss et al., 1991).

DNA fragmentation and nuclear morphology. DNA fragmentation was assessed by 3′ end labeling as described previously (Tilly and Hsueh, 1993). In brief, 24 or 48 hr after being washed free of GCM, the overlying medium was removed while rapidly freezing the cell layer on dry ice. DNA was extracted, 3′ end labeled (500 ng DNA per reaction) with α^{32}P-dideoxy-ATP using terminal deoxynucleotidyl transferase. The labeled DNA was separated on 2% agarose gels and visualized by autoradiography using Kodak X-OMAT AR film (Rochester, NY).

Nuclear morphology was assessed at the level of light microscopy on live cultures using the nuclear stain Hoechst dye 33258 (Wallen et al., 1983; Belizario et al., 1993) at 5 μg/ml final concentration added for 10–15 min at 37°C to allow uptake before observation with a Nikon Diaphot microscope equipped for epifluorescence with UV filterblock. Brightly fluorescent staining nuclei either showing a single highly condensed mass of chromatin or lobulated condensed chromatin were scored as apoptotic, compared to dimmer, diffusely fluorescent normal nuclei.

Deprivation of glial-conditioned medium. Pure neuronal cultures on DIV 2 or 3 were washed free of GCM by four medium exchanges using MEMglc. Control cultures were washed an equal number of times with glial-conditioned medium, and additional control sister cultures were observed without any medium exchange. Cultures were assayed for LDH release into the medium to quantitate injury (Koh and Choi, 1987) using a UVmax platereader (Molecular Devices, Menlo Park, CA), stained with Hoechst dye or solubilized for analysis of DNA fragmentation, 24 and 48 hr later. Cultures deprived of glial-conditioned medium in the presence of actinomycin D, 0.3 μM, or cycloheximide, 0.3 μM, had the drugs added in the last medium exchange.

Brief exposure to NMDA or glutamate. The culture medium was exchanged twice with HBSS, then glutamate or NMDA was added in the final wash. In experiments with the glutamate uptake inhibitor, *trans*-PDC (Bridges et al., 1991), 100 μM drug was added at the beginning of the 10 min exposure to glutamate or NMDA, and was present both during the exposure and during the subsequent 24 hr. Agonist exposure was performed at room temperature in room air for 10 min. After 10 min, the agonists were washed out by triple exchange of the medium with HBSS (with 5.5 mM glucose), followed by double exchange with MEMglc. The cultures were kept at 37°C in a 5% CO_2 incubator; injury was assessed after 24 hr by microscopic examination, and quantified by measuring lactate dehydrogenase (LDH) efflux. LDH efflux has been shown to be proportional to the number of dead cells assessed by Trypan blue staining (Koh and Choi, 1987). The amount of LDH released by sham-washed controls was subtracted to give the LDH signal specific to the insult, and 24 hr LDH values are expressed as a fraction of the

Table 3. $^{45}Ca^{2+}$ Accumulation during 10 min exposure to NMDA or glutamate

Condition	$^{45}Ca^{2+}$ accumulation (cpm)
Control	84 ± 13
NMDA 0.5 mM	545 ± 44*
Glutamate 0.5 mM	373 ± 45*
Kainate 1 mM	121 ± 5

Values shown are mean ± SEM (n = 4).
*Significant difference from control by ANOVA and Student–Newman–Keuls test, $p < 0.05$.

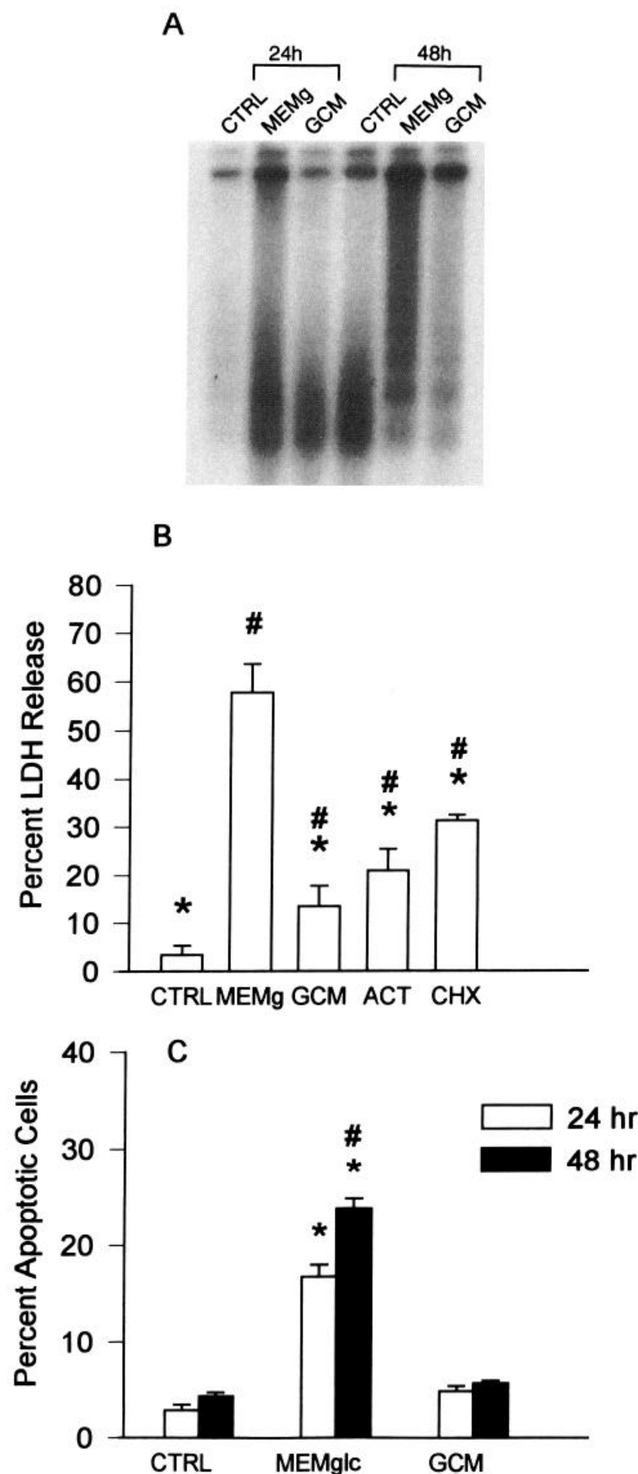

Figure 2. Effects of deprivation of glial-conditioned medium on young neuronal cultures. *A,* Autoradiogram showing DNA fragmentation in cultures deprived of glial-conditioned medium. Equal amounts of DNA from sister DIV 3 cultures were 3′ end labeled after 24 or 48 hr in the indicated condition. Cultures were left undisturbed (*CTRL*), or were washed into MEMglc (*MEMg*), or glial-conditioned medium (*GCM*). End-labeled DNA was separated on a 2% agarose gel. *B,* The effect of actinomycin and cycloheximide on neurons deprived of glial-conditioned medium. Sister cultures were left undisturbed (*CTRL*), or washed into MEMglc (*MEMg*), glial-conditioned medium (*GCM*), or MEMglc plus actinomycin D (*ACT*) or cycloheximide (*CHX*). After 48 hr, LDH release by the cultures was measured. Values shown are mean ± SD for $n = 4$. *Asterisk* (*) indicates statistically significant difference from MEMglc by ANOVA and Student–Newman–Keuls test, $p < 0.05$, *number sign* (#) indicates significant difference from CTRL. *C,* The percentage of apoptotic cells increased over 48 hr after removing glial-conditioned medium. Neuronal cultures were stained with Hoechst dye 33258 and counted 24 and 48 hr after medium change into MEMglc, GCM, or while left undisturbed (*CTRL*). Cells were scored as apoptotic or diffusely fluorescent, according to nuclear morphology. Numbers shown are apoptotic cells as a percentage of the total cells counted per field, mean ± SEM for counts of five or six 40× microscope fields, containing 100–200 cells each, for each condition. *Asterisk* (*) indicates statistically significant difference, $p < 0.05$ by ANOVA and Student–Newman–Keuls test, compared to control for the same time. *Number sign* (#) Indicates significant difference from the same condition at 24 hr.

maximal neuronal LDH released by 24 hr exposure to 500 μM NMDA for mixed cultures, or freeze–thaw for pure cultures.

Twenty-four hour exposure to AMPA, kainate, or NMDA. Cultures were washed three times with MEMglc, and receptor agonists (NMDA, 3-300 μM; AMPA, 1–100 μM; kainate, 3–300 μM) were added for 24 hr; neuronal death was then quantitated by LDH efflux. The NMDA receptor antagonist, MK-801 (10 μM), was included with AMPA or kainate to allow assessment of non-NMDA receptor toxicity without secondary activation of NMDA receptors by release of endogenous glutamate.

Oxygen, glucose-deprivation injury. Cells were placed in an anoxic chamber (Forma, Marietta, OH) in an atmosphere of 85% N_2, 10% H_2, and 5% CO_2, and were deprived of oxygen and glucose by triple exchange of the medium with deoxygenated BSS containing no glucose. Oxygen- and 5.5 mM glucose-containing BSS was used for control cultures. Cultures were incubated in a 37°C, humidified incubator in the anoxic chamber for specified periods of time, and then rescued with oxygenated MEMglc. Oxygen in the chamber was ≤2 mm Hg measured with an oxygen electrode (Microelectrodes, Londonderry, NH). At the end of the period of oxygen, glucose-deprivation, cultures were transferred to the 37°C, normoxic, 5% CO_2 incubator for 24 hr, and cell death was determined after 24 hr.

$^{45}Ca^{2+}$ accumulation. Cultures were washed twice with HBSS (with 5.5 mM D-glucose), and then exposed to NMDA (300 μM) or kainate in HBSS containing tracer $^{45}Ca^{2+}$ (0.5 μCi/culture well; New England Nuclear, Boston, MA) (Hartley et al., 1993). The exposure was terminated after 10 min by exchanging the medium four times with unlabeled HBSS, followed by lysis of the cells by addition of 0.2% sodium dodecyl sulfate (SDS). The cells were left in SDS for 2 hr, and the lysate was then transferred to scintillation vials. An additional wash of each culture well with SDS was added to the vial, which then underwent β-counting in duplicate. For kainate exposure, MK-801 was present to prevent secondary activation of NMDA receptors.

Measurement of extracellular glutamate. To determine the concentration of extracellular glutamate, an aliquot of medium was removed from the cultures, and amino acids were derivatized with *o*-phthaldialdehyde/2-mercaptoethanol reagent (1:1, vol/vol). Samples were injected onto a Waters Resolve C18 column, and amino acids separated using a gradient, beginning 4 min after injection, from 100% 0.1 M potassium acetate, pH 7.35/methanol 20% (80:20) to 100% methanol/0.1 M potassium acetate pH 7.35 (80:20) over 7 min, a modification of the technique described by Lindroth and Mopper (1979). Amino acids were detected with a fluorescence detector, and commercial standards were injected to identify peaks and allow quantitation based on peak area.

Reagents and solutions. Endotoxin-free water, glutamine, and Minimal Essential Medium (MEM, 330-1430) were obtained from GIBCO (Grand Island, NY). Fetal bovine serum and horse serum came from Hyclone Laboratories (Logan, UT). Laminin was purchased from Collaborative Research, Inc. (Bedford, MA). Epidermal growth factor (EGF), Hoechst dye 33258, propidium iodide, *Banderiera simplicifolia* I lectin linked to horseradish peroxidase and all other chemicals for tissue culture were purchased from Sigma (St. Louis, MO); *trans*-PDC was from Tocris Neuramin (Bristol, England). Falcon plasticware came from Becton Dickinson (Lincoln, NJ). MK-801 was from Merck, Sharp and Dohm (Essex, PA). NSE and GFAP antibodies were obtained from Incstar (Stillwater, MN); antibodies to MAP 2 and terminal deoxynucleotidyl transferase were from Boehringer–Mannheim (Indianapolis, IN). Fluorescent secondary antibodies were tagged with BODIPY (Mo-

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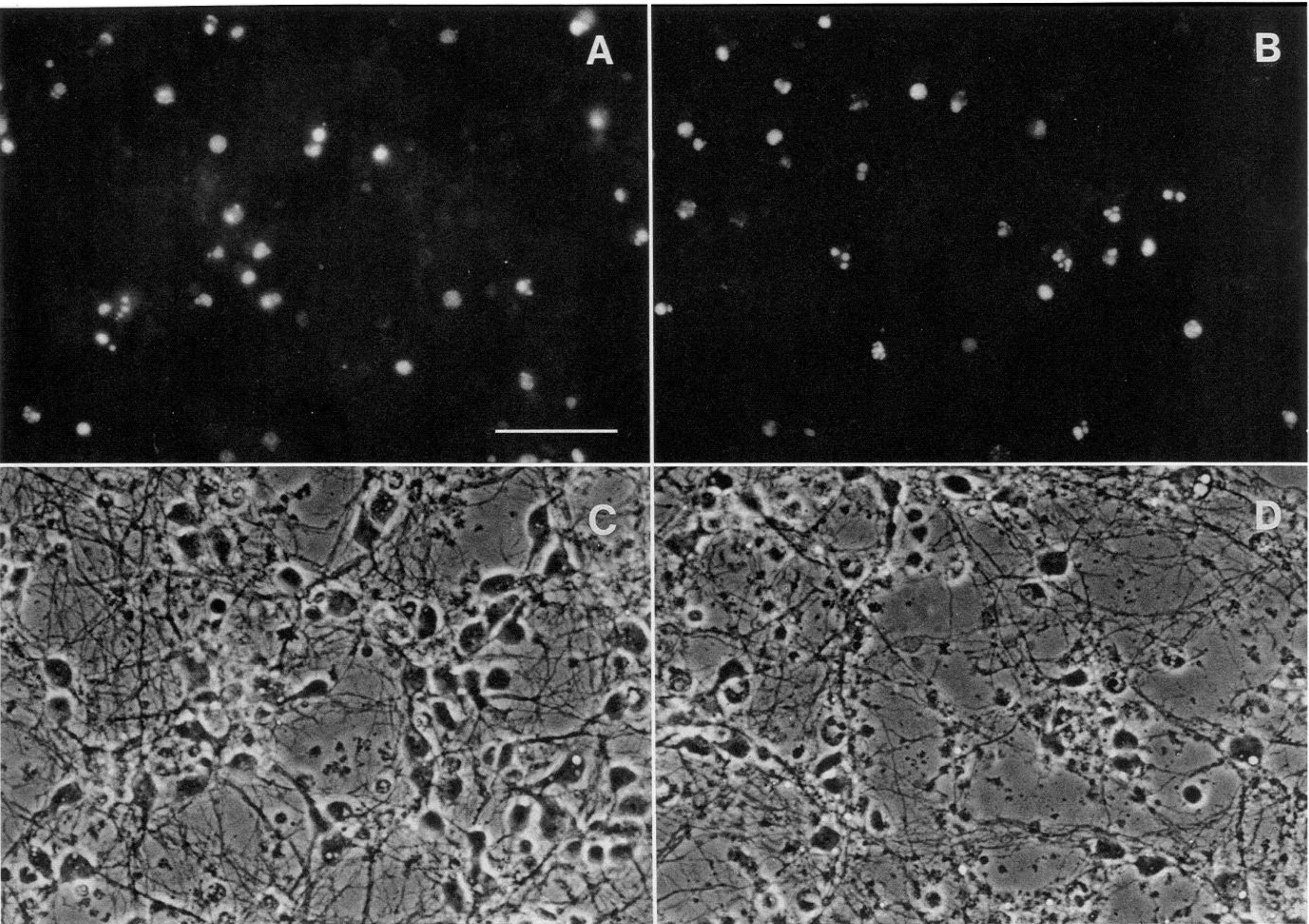

Figure 3. Cells were washed into either glial-conditioned medium (*A* and *C*) or MEMglc (*B* and *D*). After 24 hr cultures were stained with Hoechst dye and the same fields photographed for fluorescence (*A* and *B*) or with phase contrast optics (*C* and *D*). Photos were taken at 20×. Scale bar, 50 μm.

lecular Probes, Eugene, OR) or Cy3 (Jackson ImmunoResearch, West Grove, PA).

Balanced salt solution (BSS) consisted of (in mM) NaCl, 116; KCl, 5.4; $MgSO_4$, 0.8; NaH_2PO_4, 1.0; $CaCl_2$, 1.8; $NaHCO_3$, 26; with or without 5.5 mM D-glucose. HEPES-buffered BSS (HBSS) was prepared similarly, except that 10 mM HEPES (free acid) replaced the $NaHCO_3$, and NaCl was increased to 132 mM. The medium was brought to pH 7.4 using NaOH. Plating medium (PM) was MEMglc (MEM with 23 mM glucose) supplemented with glutamine to 2 mM, 5% fetal bovine serum, and 5% horse serum for neuronal and mixed cultures. Astrocyte cultures were plated in medium containing 10% fetal bovine serum, 10% horse serum, and 10 ng/ml EGF. Glial-conditioned medium (GCM) was prepared by incubating MEMglc with confluent astrocyte cultures grown in 75 cm^2 flasks for 2 weeks. After harvest, the medium was filtered for sterility and stored at 4°C.

Results

Development of neurons is similar with or without glia

The morphology of neurons grown in pure culture was essentially the same as neurons grown on a bed of astrocytes (Fig. 1*A,C*), as demonstrated by MAP 2 or NSE (data not shown) staining at the level of light microscopy. GFAP-immunoreactive cells constituted < 1% of the cells in the pure neuronal cultures (Fig. 1*D*, Table 1), in contrast to the confluent monolayer of cells seen in mixed cultures (Fig. 1*B*, Table 1). Cell counts for both types of culture are given in Table I at day *in vitro* (DIV) 2 and DIV 12. Staining with *Banderiera simplicifolia* I lectin for microglia revealed only 10–20 microglia per well in mixed cultures, and no microglia in the pure neuronal cultures examined (data not shown). Mixed and neuronal cultures had similar numbers of NADPH-diaphorase positive cells (< 1% of neurons), in agreement with earlier results (Koh and Choi, 1988). Both types of cultures had 10–15% neurons with calcium-conducting AMPA/kainate receptors, as demonstrated by cobalt staining (data not shown), consistent with a previous report by Turetsky et al. (1994).

The development of functional glutamate receptors, as defined by vulnerability to excitotoxic injury and LDH efflux, is essentially complete by DIV 11 in pure neuronal cultures. Cultures exposed to NMDA for 10 min showed little toxicity at DIV 8. By DIV 11, however, the percentage of neurons killed by exposure to NMDA was not statistically different from that seen in DIV 14 cultures (Table 2). In addition, DIV 11 neuronal cultures demonstrated $^{45}Ca^{2+}$ accumulation during 10 min exposure to NMDA or glutamate (Table 3) that was similar to that reported in a previous study (Hartley et al., 1993). Kainate and AMPA receptor-mediated neurotoxicity in neuronal cultures also was essentially maximal by DIV 11 (not shown). The development of vulnerability to excitotoxic neuronal injury paralleled that seen in sister mixed cultures.

Glial-conditioned medium suppresses apoptosis

Immature cultures, DIV 2–4, showed an absolute requirement for glial-conditioned medium to survive (Fig. 2*B*). Since the number of contaminating glial cells in these cultures is quite low, the glial-conditioned medium likely supplies an essential growth or survival factor(s). Astrocytes are known to release factors that promote neuronal survival (Giulian et al., 1993), including brain-derived neurotrophic factor (BDNF; Zafra et al., 1992). Furthermore, several types of neurons and glia (reviews Oppenheim, 1991; Raff et al., 1993; Johnson and Deckwerth, 1993) are known to undergo apoptosis on the removal of a growth or survival factor. We observed that withdrawal of glial-conditioned medium from pure cortical neuronal cultures induced apoptosis. In DIV 2 cultures, DNA fragmentation was detected by 3′ end labeling 24 and 48 hr after exchange of the glial-conditioned medium with MEMglc (Fig. 2*A*). Increased numbers of cells with brightly fluorescent, condensed, lobulated nuclei typical of apoptosis (Belizario et al., 1993) were present when cultures were stained with Hoechst dye beginning at 24 hr of deprivation, and clearly increasing in number at 48 hr (Figs. 2*C*, 3*B*). Degenerative morphological changes, especially affecting cell processes, are apparent with phase contrast microscopy by 24 hr (Fig. 3*D*). The survival of cells at 48 hr was substantially increased by inhibition of macromolecular synthesis with either cycloheximide or actinomycin D (Fig. 2*B*).

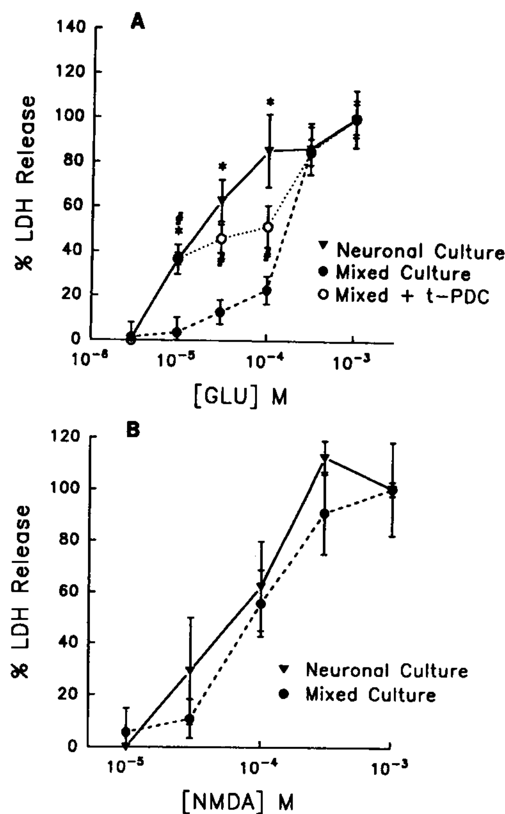

Figure 4. Dose response for 10 min glutamate or NMDA toxicity. *A*, Neuronal cultures are approximately 10 times more sensitive to glutamate than mixed cultures. Addition of the glutamate uptake inhibitor, *trans*-PDC to mixed cultures shifted the dose–response curve back towards that of pure neurons. Values shown are mean ± SEM for n = 4 for mixed and *trans*-PDC, n = 16 for pure neuronal cultures, results representative of three experiments. Significant difference at $p < 0.05$ between mixed cultures and pure neuronal cultures at the same concentration of glutamate by ANOVA and Student–Newman–Keuls test is shown by an *asterisk* (*). Difference between mixed cultures and mixed plus t-PDC is shown by a *number sign* (#). *B*, Dose response for 10 min NMDA toxicity. Pure and mixed cultures show similar responses to brief exposure NMDA toxicity, with no statistically significant difference. Values shown are mean ± SEM for n = 4, representative of three similar experiments.

Comparative sensitivity to excitotoxicity

Pure neurons (> DIV 11) were about 10-fold more sensitive to brief glutamate exposure than were mixed cultures, which contain approximately 70% glial cells (Fig. 4*A*). This differential sensitivity could be reduced by adding the specific glutamate uptake inhibitor, *trans*-PDC, to mixed cultures to block glial uptake of glutamate. Although *trans*-PDC may also reduce neuronal uptake of glutamate, as has been shown in hippocampal

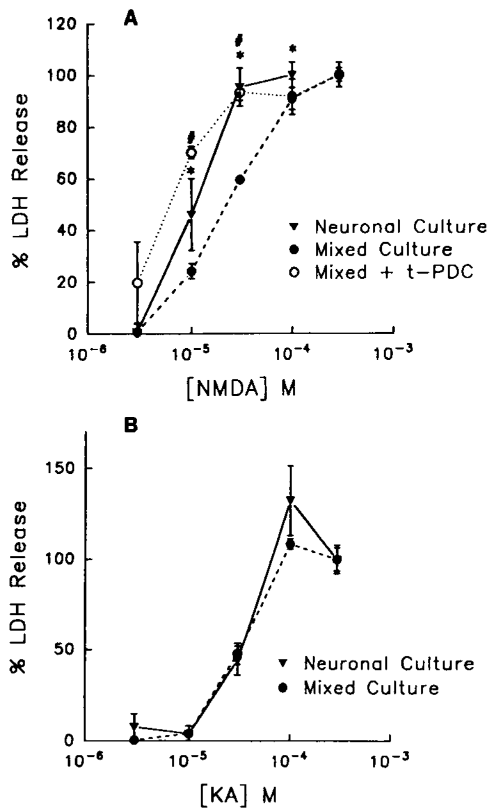

Figure 5. Comparative dose–response curves for 24 hr exposure to NMDA or kainate. *A*, The dose–response curve for NMDA in neuronal cultures is shifted somewhat to the left, possibly reflecting secondary release of glutamate, which is blunted by glial uptake in mixed cultures. Co-incubation with 100 μM *trans*-PDC shifted the dose–response curve of mixed cultures towards that of pure neurons. Values shown are mean ± SEM for $n = 4$, results are representative of three such experiments. *Asterisk* (*) indicates significant difference between pure and mixed cultures at the same concentration of NMDA by ANOVA and Student–Newman–Keuls test. *Number sign* (#) indicates difference between mixed cultures with and without *trans*-PDC. *B*, The EC_{50} of kainate in the presence of 10 μM MK801 in neuronal cultures is the same as in mixed cultures. Values shown are mean ± SEM for $n = 4$, representative of three such experiments.

slice (Isaacson and Nicoll, 1993), pure neuronal cultures incubated with *trans*-PDC showed no shift in their dose–response curve (data not shown), suggesting that this is a minor uptake mechanism, or that there is little bath glutamate present in neuronal cultures. The sensitivity of pure neuronal cultures and mixed cultures (Fig. 4*B*) to 10 min NMDA toxicity, however, was similar and was not significantly modified by treatment of the cultures with the glutamate uptake inhibitor (not shown).

Cortical neurons require prolonged (> 1 hr) exposure to non-NMDA receptor agonists to induce neuronal cell death (Koh et al., 1990). Non-NMDA receptor-mediated injury, and the injury produced by prolonged exposure to low concentrations of NMDA, result in insults with characteristics that are somewhat similar to each other, but are different from injury produced by 10 min exposure to high concentrations of NMDA or glutamate (Rose et al., 1991). We therefore determined the sensitivity of pure neurons to 24 hr exposure to NMDA, as well as kainate and AMPA. Pure neurons were more sensitive to injury produced by prolonged exposure to NMDA, and the sensitivity of mixed cultures is significantly increased by *trans*-PDC (Fig. 5*A*). The EC_{50} values for kainate in neuronal and mixed cultures were

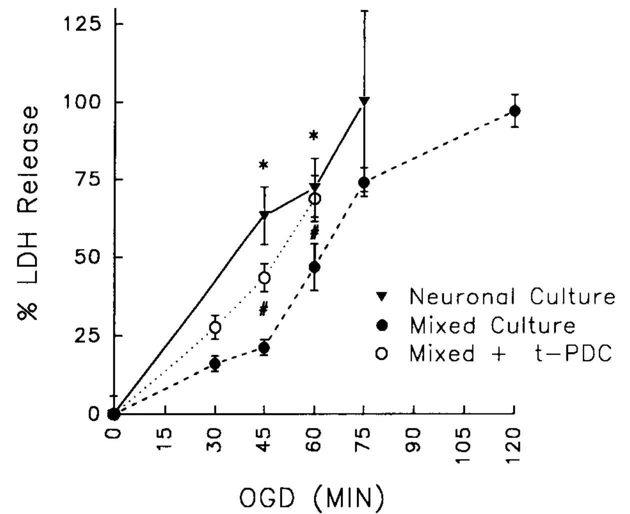

Figure 6. Oxygen, glucose deprivation (OGD) injury of pure neuronal cultures and mixed cultures. Pure neuronal cultures are more sensitive to injury than those containing glia. Addition of *trans*-PDC increases the sensitivity of mixed cultures. Values shown are mean ± SEM for $n = 4$–8, representative of data from three experiments. *Asterisk* (*) indicates significant difference between pure and mixed cultures for the same duration of oxygen, glucose deprivation by ANOVA and Student–Newman–Keuls test, $p < 0.05$. *Number sign* (#) indicates difference between mixed cultures with and without *trans*-PDC.

indistinguishable (Fig. 5*B*). The EC_{50} concentrations for mixed cultures exposed to NMDA for 10 min or 24 hr, glutamate for 10 min, or kainate for 24 hr were similar to those reported previously in mixed cultures (Koh and Choi, 1988).

In mixed cortical cell cultures, combined oxygen, glucose-deprivation injury has been shown to be mediated, in large part, by NMDA receptors (Kaku et al., 1991; Goldberg and Choi, 1993). In keeping with the greater vulnerability of pure neuronal cultures to NMDA toxicity, a shorter duration of combined oxygen, glucose deprivation was required to produce injury in pure neurons than was necessary for mixed cultures (Fig. 6). When mixed cultures were deprived of oxygen and glucose in the presence of *trans*-PDC, the curve shifted towards that of neurons alone. However, for neuronal cultures, as for mixed cultures, the oxygen, glucose-deprivation dose response varies more from plating to plating than do the glutamate agonist injury paradigms. In subsequent experiments, we have determined that the density of neuronal cultures is a critical determinant of vulnerability to oxygen, glucose-deprivation injury, and neuronal cultures that are less dense than those used for the reported experiments have been somewhat more resistant to oxygen, glucose-deprivation insult.

AMPA toxicity is exacerbated by glia

Surprisingly, mixed cultures were more sensitive to AMPA-induced injury than were pure neurons (Fig. 7). To determine whether extracellular glutamate levels might account for this finding, levels of glutamate in the media of cultures exposed to AMPA were measured. Glutamate levels in glial cultures were found to be elevated 2 hr after AMPA (Table 4). This was not due to glial cell death, as exposure to concentrations of AMPA up to 1 mM for 24 hr or 48 hr did not produce injury, as determined by LDH efflux or light microscopic morphology (data not shown). Others have reported a lack of gliotoxicity after 48 hr exposure to 2 mM AMPA (Bridges et al., 1992). Media from

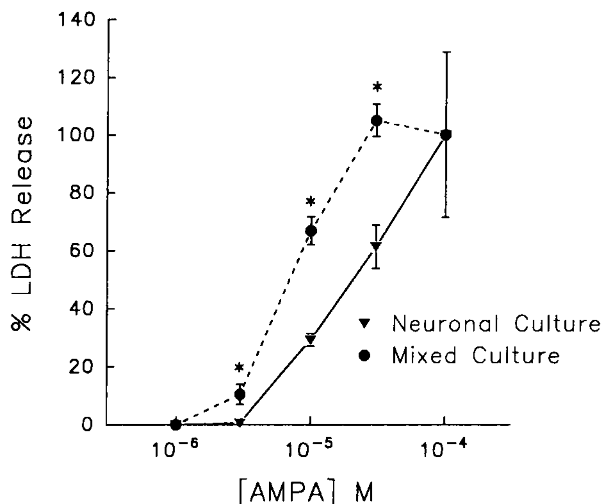

Figure 7. Neurons in the presence of astrocytes are approximately threefold more sensitive to AMPA toxicity than are neurons alone. Values shown are mean ± SEM for $n = 4$, representative of four experiments. *Asterisk* (*) indicates statistically significant difference between pure and mixed cultures at the same concentration of AMPA with $p < 0.05$ by ANOVA and Student–Newman–Keuls test. By taking the ratio of the EC_{50} (estimated graphically) for the four pairs of neuronal and mixed cultures, mixed cultures were found to be 3.08 ± 0.99 times more sensitive to AMPA than pure neuronal cultures (mean ± SD; $p < 0.05$ by *t*-test; 95% CI were 1.5 and 4.65).

pure neuronal cultures exposed to AMPA also showed a small increase in glutamate (Table 4). However, glutamate levels in mixed cultures exposed to AMPA were not elevated after 2 hr. Exposure of glial cultures to NMDA or kainate failed to increase glutamate in the medium. No glutamate determinations were done for AMPA exposures longer than 2 hr, when neuronal death begins to occur, since the amount of glutamate present would be increasingly confounded by glutamate from dead cells, and would no longer reflect glutamate released by astrocytes and neurons in response to AMPA.

Table 4. Concentration of glutamate in the media of cultures exposed to glutamate receptor agonists

Culture type	Condition	[Glu] (μM) in medium (mean ± SEM)	*n*
Glial	Control	0.39 ± 0.16*	12
	AMPA 3 μM	1.36 ± 0.64	8
	10 μM	2.38 ± 1.06	7
	30 μM	4.53 ± 1.69*	8
	100 μM	4.19 ± 1.34*	12
	Kainate 300 μM	0.37 ± 0.09	7
	NMDA 100 μM	0.12 ± 0.06	4
	300 μM	0.07 ± 0.05	4
Neuronal	Control	0.38 ± 0.09	7
	AMPA 30 μM	0.72 ± 0.13†	7
Mixed	Control	0.23 ± 0.11	8
	AMPA 30 μM	0.50 ± 0.26	7
	100 μM	0.45 ± 0.13	8

Glutamate was measured in the medium of cultures exposed to the indicated conditions for 2 hr. This time point precedes the onset of cell death, as measured by release of LDH into the medium, or loss of trypan blue exclusion. We routinely included MK-801 (10 μM) with either AMPA or kainate exposure in mixed cultures to eliminate secondary activation of NMDA receptors by endogenously released glutamate. Therefore, MK-801 was included with AMPA or kainate for all experiments.

* Different from glial control, $p < 0.05$ by ANOVA and Student–Newman–Keuls test.

† Different from pure neuronal control, $p < 0.05$ by ANOVA and Student–Newman–Keuls test.

Discussion

In establishing the conditions for the growth of murine cortical neurons without supporting glial cells, we observed that removal of glial-conditioned medium from immature cultures led to their death. This was likely due to apoptosis, as it was accompanied by internucleosomal cleavage of DNA, showed condensed nuclear morphology when stained with Hoechst 33258 dye, and was inhibited by macromolecular synthesis inhibitors. The time course of this induction was consistent with the findings of others studying sympathetic neurons acutely deprived of NGF (Martin et al., 1988). Cerebral astrocytes can be stimulated to produce BDNF by glutamate in combination with a β-adrenergic agonist (Zafra et al., 1992), and BDNF has been shown to improve survival of cortical neurons under certain conditions (Ghosh et al., 1994). Basic fibroblast growth factor (bFGF), another trophic factor that acts on central neurons (Morrison et al., 1986), can be induced in cultured rat astrocytes by exposure to glutamate (Pechan et al., 1993). What factors are present in glial-conditioned medium is not yet known.

Murine neuronal cultures lacking glia were more sensitive to glutamate toxicity than neurons in the presence of glia, in agreement with previous work in rat cultures (Rosenberg and Aizenman, 1989; Rosenberg et al., 1992; Robinson et al., 1993). Addition of a glutamate uptake inhibitor (*trans*-PDC) to mixed cultures increased their vulnerability to glutamate, consistent with the hypothesis that the differential sensitivity of pure neuronal and mixed cultures is due, in part, to glutamate uptake by glia. Pure neurons were also somewhat more sensitive to oxygen, glucose deprivation injury, in agreement with a prior report (Vibulsreth et al., 1987), and inhibition of glial glutamate uptake by *trans*-PDC increased the sensitivity of mixed, but not pure neuronal cultures, to oxygen, glucose deprivation injury.

Toxicity from 10 min exposure to NMDA was not significantly altered by the presence of astrocytes. NMDA, unlike glutamate, is not taken up by glia to an appreciable extent (Drejer et al., 1982; Brew and Attwell, 1987). In contrast, NMDA exposure for 24 hr was somewhat greater in pure neuronal cultures compared to mixed cultures. This excess vulnerability was reproduced in mixed cultures by the addition of *trans*-PDC, suggesting that endogenous release of glutamate may contribute to this slower form of NMDA injury. Injury produced by brief exposure to high concentrations of NMDA may differ from that produced by chronic exposure to lower concentrations of NMDA (Rose et al., 1991). The importance of endogenous glutamate release to injury may be one way in which these two insults diverge. Toxicity from prolonged (24 hr) exposure to kainate was not affected by the presence of astrocytes. This observation can be explained by lack of substantial accumulation of bath glutamate in our cultures after addition of kainate (Dugan, unpublished observations).

In contrast to the neuroprotective role of astrocytes in many excitotoxic injuries, the presence of astrocytes actually enhanced AMPA-induced neurotoxicity, suggesting that the interaction between neurons and glia in excitotoxicity is complex. That there is cross talk between neurons and glial is now well established.

Rapid inward currents in astrocytes have been observed with electrical activity in neighboring neurons (Murphy et al., 1993). Using Fura-2 photomicrography, multiple glial calcium spikes were observed with continuous stimulation of retinal nerve axons (Kriegler and Chiu, 1993); these calcium spikes are similar to those reported in astrocytes in response to glutamate (Cornell-Bell et al., 1990). A recent report of the effects of glia on neurotransmission in hippocampal culture demonstrated neuronal currents after direct glial depolarization (Mennerick and Zorumski, 1994), possibly reflecting glial glutamate release. Increased neuronal calcium in response to astrocyte stimulation was reported by both Nedergaard (1994) and Parpura et al. (1994). The latter study directly demonstrated glutamate release by glia exposed to bradykinin, and suggested that the concentration of released glutamate from glia was sufficient to stimulate neuronal glutamate receptors, specifically NMDA receptors, as the neuronal calcium increase was blocked by the NMDA receptor antagonist, AP-5. AMPA activation of astrocytes might result in similar glutamate release.

We postulated that reversal of astrocyte glutamate transport might explain the unexpected observation that pure neuronal cultures were less susceptible to AMPA toxicity than cultures containing a high proportion of astrocytes. The neuroprotective function of the glutamate transporter to remove excess extracellular glutamate might fail under conditions of energy failure or astrocyte depolarization (Nicholls and Attwell, 1990; Huang et al., 1993), resulting in loss of the transmembrane sodium gradient, and reversal of the glutamate transporter (Kaupinnen et al., 1988). Glutamate might also be released from astrocytes by an exocytotic process analogous to that demonstrated for acetylcholine release from muscle (Dan and Poo, 1992), or by astrocyte swelling (Kimelberg et al., 1990). Thus, extracellular glutamate in mixed cultures exposed to AMPA might derive not only from neurons, but from astrocytes, compounding the neurotoxic action of AMPA on the neurons. We found that AMPA induced release of glutamate from astrocyte cultures, consistent with a previous report of glutamate release from type-2 astrocytes in response to kainate or quisqualate (Levi and Patrizio, 1992). However, glutamate levels in mixed cultures exposed to AMPA were as low as levels in pure neuronal cultures, arguing against astrocytic glutamate release as an explanation for the enhanced vulnerability of mixed cultures to AMPA.

AMPA-induced glutamate release by astrocytes in pure glial cultures, but not in mixed cultures, might be explained by neuronal modulation of glial glutamate receptors. Cultured cortical astrocytes possess AMPA/kainate receptors (Sontheimer et al., 1988; Glaum et al., 1990; Jensen and Chiu, 1990; Condorelli et al., 1993; Holzwarth et al., 1994), and the complement of these receptors on astrocytes can vary by brain region and can be affected by culture conditions and stage of development (Hatten, 1985; Barres et al., 1990; Pearse, 1993). There is precedent for neurons determining expression of glial ion channels. The expression of calcium channels on astrocytes, for example, is affected by the presence of neurons in the culture (reviewed by Sontheimer, 1994). Further, it is possible that an astrocytic response to AMPA other than glutamate release might feed back on neurons and be deleterious.

An alternative explanation for enhanced AMPA vulnerability of mixed cultures is at the level of neuronal AMPA receptor number or composition. Modulation of the number, or subunit composition, of neuronal AMPA receptors by astrocytes might produce a more toxic complement of neuronal receptors. To our knowledge, there are, to date, no specific reports of astrocytic modification of glutamate receptor expression. However, this intriguing possibility provides a viable explanation of our results, and is a hypothesis that we are currently exploring.

The results reported here suggest that glia modify AMPA-mediated injury in mixed cultures by unexpectedly worsening injury. Whatever the mechanism whereby this occurs, these findings provide a novel additional mechanism for the protection seen with non-NMDA agonists in animal models of ischemia (Sheardown et al., 1990; Buchan et al., 1991), in that they may have important actions on glial glutamate receptors in addition to their effects on neuronal receptors.

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