Glutamate Receptor-Mediated Currents and Toxicity in Embryonal Carcinoma Cells

D. M. Turetsky,[1,4] J. E. Huettner,[2,4] D. I. Gottlieb,[3,4] M. P. Goldberg,[1,4] and D. W. Choi[1,4,*]

Departments of [1]Neurology, [2]Cell Biology and Physiology, and [3]Anatomy and Neurobiology, and the [4]Center for the Study of Nervous System Injury, Washington University School of Medicine, St. Louis, Missouri 63110

SUMMARY

While primary neuronal cell cultures have been used to investigate excitotoxicity, development of cell lines exhibiting glutamate receptor-mediated death is desirable. P19 mouse embryonal carcinoma cells, exposed to retinoic acid and plated onto a layer of cultured mouse cortical glial cells, differentiated into neuron-like elements immunoreactive for neurofilaments and neuron-specific enolase. Whole-cell recordings revealed inward currents in response to extracellular application of either NMDA or kainate. The NMDA-induced currents exhibited a voltage-dependent blockade by magnesium, required glycine for maximal activation, and were blocked by the NMDA antagonist dizocilpine. Kainate-induced currents were blocked by the AMPA/kainate receptor antagonist CNQX. Exposure to 500 μM NMDA for 24 h destroyed most P19 cells (EC_{50} approximately 70 μM); death was prevented by dizocilpine or D-APV. Exposure to 500 μM kainate also resulted in widespread death reduced by CNQX. Thus differentiated P19 cells exhibited both excitatory amino acid responses and vulnerability to excitotoxicity, characteristic of CNS neurons. These cells may provide a genetically open system useful for studying glutamate receptor-mediated phenomena at a molecular level. © 1993 John Wiley & Sons, Inc.

Keywords: **excitotoxicity, NMDA, AMPA, kainate, P19, cell death.**

INTRODUCTION

Glutamate is the major excitatory neurotransmitter of the mammalian central nervous system. Excessive exposure to glutamate can kill central neurons (Lucas and Newhouse, 1957; Olney, 1969) and such neurotoxicity, or "excitotoxicity," likely contributes to the neuronal loss associated with several neurological diseases. These include stroke, trauma, hypoglycemia, and epilepsy, and may possibly include certain neurodegenerative diseases (Meldrum, 1985; Rothman and Olney, 1987; Choi, 1988).

The potential clinical significance of glutamate neurotoxicity has encouraged efforts to delineate underlying mechanisms. Study of these mechanisms *in vivo* has been hampered by the complexity of the intact nervous system as well as by the widespread existence of cellular glutamate uptake. In order to avoid these complicating factors much of the work on excitotoxicity has been carried out in several *in vitro* systems, including dissociated primary neuronal cultures (Choi, 1985; Choi, Maulucci-Gedde, and Kriegstein, 1987; Rothman, 1985; Rothman, Thurston and Hauhart, 1987; Garthwaite and Garthwaite, 1986a; Hahn, Aizenman, and Lipton, 1988; Ogura, Miyamoto, and Kudo, 1988; Marcoux et al., 1988; Manev et al., 1989; Frandsen, Drejer, and Schousboe, 1989), acute brain slice preparations (Garthwaite and Garthwaite, 1986b; Ellren and Lehmann, 1989), and isolated retina (Olney et al., 1986). These and other *in vitro* studies have supported the idea that glutamate neurotoxicity is triggered by the activation of membrane cation channels gated by *N*-methyl-D-aspartate (NMDA) or alpha-amino-3 -

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* To whom correspondence should be addressed.

hydroxy - 5 - methyl - 4 - isoxazolepropionic acid (AMPA)/kainate type glutamate receptors (Watkins and Olverman, 1987; Watkins, Krogsgaard-Larson, and Honore, 1990), leading to Ca^{2+} influx and a resultant cell Ca^{2+} overload (Choi, 1992). However, the difficulty of manipulating the genetic composition of postmitotic neurons has been an important experimental limitation. This limitation might be overcome in a neuronal cell line, if one could be identified that expressed ion channel-linked glutamate receptors and exhibited glutamate toxicity analogous to that observed in neurons.

Although glutamate at high concentrations has been previously found to be toxic to several cell lines, such toxicity has fundamental differences from glutamate toxicity on primary neurons. Murphy et al. (1989) observed that exposure to millimolar concentrations of glutamate can destroy N18-RE-105 neuroblastoma-primary retina hybrid cells, but unlike primary neurons, N18-RE-105 cells were not injured by exposure to the selective glutamate receptor agonists NMDA, kainate, or AMPA. Since cell death was proportional to inhibition of cystine uptake via a cystine/glutamate antiporter, Murphy and colleagues proposed that death resulted from cellular cystine deprivation, leading to glutathione depletion, and vulnerability to free radicals. More recently, Chovanes et al. (1993) have proposed that metabotropic glutamate receptors may play a role in glutamate-induced injury of N18-RE-105 cells, as well as in the injury of NH2 cells (N18TG-2 neuroblastoma-mouse hypothalamic hybrid cells).

Prolonged exposure to millimolar concentrations of glutamate has been also reported to kill a neuronal cell line derived from rat cerebellum, SC9, but this cytotoxicity, unlike glutamate neurotoxicity in primary cortical culture (Choi et al., 1987), requires the presence of extracellular glutamine (Simantov, 1989). At low glutamine concentrations, glutamate actually had a growth-promoting effect; furthermore the NMDA receptor agonist, aspartate, was not toxic even at a concentration of 6.25 m*M* and an exposure duration of 4 days. Another line, NCB-20 mouse neuroblastoma-Chinese hamster brain hybrid cells, has been reported to have mRNA capable of inducing functional NMDA receptors in oocytes, but have not been found to themselves express functional NMDA receptors (Lerma, Kushner, Spray, Bennett, and Zukin, 1989), or exhibit NMDA-induced cytotoxicity (unpublished observations).

One cell line with potential for exhibiting glutamate receptor-mediated death was the P19 embryonal carcinoma line, a pluripotent line derived from a teratocarcinoma in C3H/He mice (McBurney and Rogers, 1982). This euploid line is maintained as undifferentiated cells, but upon treatment with high doses of retinoic acid differentiates into neurons, astrocytes, and fibroblast-like cells (Jones-Villeneuve, McBurney, Rogers and Kalnins, 1982). Conversely, induction with either dimethyl sulfoxide or low doses of retinoic acid results in the formation of cardiac and skeletal muscle (McBurney, Jones-Villeneuve, Edwards, and Anderson, 1982). The ability of P19 cells to generate a range of cell phenotypes has made them a valuable system for studying the early events that subserve cellular commitment and terminal differentiation. P19-derived neurons extend processes typical of axons and dendrites at the EM level (McBurney et al., 1988). They are immunoreactive for neuron-specific markers, such as the 68 and 160 kD neurofilament proteins, as well as markers suggestive of neuronal maturity, such as neuron-specific enolase (McBurney et al., 1988; Sharma and Notter, 1988). Well-developed chemical synapses have been observed by EM, and biosynthetic enzymes for cholinergic and adrenergic neurotransmitter pathways have been described (Sharma and Notter, 1988; McBurney et al., 1988). P19 neuron-like cells exhibit high-affinity GABA uptake (Bell, Jardine, and McBurney, 1986; McBurney et al., 1988), and have mRNA for glutamic acid decarboxylase (GAD) (Bain et al., 1993), although GAD activity has not been found.

The goal of the present study was to examine the specific possibility that P19-derived-neurons might express ion channel-linked glutamate receptors and test whether the cells could be killed by exposure to NMDA or kainate. An abstract has appeared (Turetsky et al., 1992).

Methods

Cell Culture

P19 cells were obtained from the American Type Culture Collection. Undifferentiated P19 cells were maintained in alpha minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). For differentiation, near-confluent cultures were trypsinized and dissociated to single cells. 1×10^6 cells were then suspended in 10 ml alpha-MEM, containing 5% FBS and

500 n*M* retinoic acid, and seeded onto a 10-cm bacteriological culture dish. The cells do not adhere to the plastic and form large aggregates in suspension. Cells were fed after 2 days by transferring the suspension to a 15-ml tube, allowing the aggregates to settle, and replacing the old medium with fresh medium containing retinoic acid. After the fourth day of retinoic acid treatment, aggregates were trypsinized, dissociated, resuspended in alpha-MEM + 10% FBS, and plated onto confluent primary glial cultures at a density of approximately 3.6 $\times 10^6$ cells per 24-well plate. For electrophysiology experiments, cells were plated at 0.38 $\times 10^6$ per 35-mm dish. On the second day after plating, cells were switched to Eagle's MEM (Earle's salts) supplemented with 20 m*M* glucose, 5% FBS, and 5% horse serum (plating medium), and cytosine arabinoside (Ara-C, final concentration 20 μM) was added to inhibit division of nonneuronal cells; the Ara-C was removed 2 days later by feeding cultures with plating medium. Cultures were subsequently maintained in this medium, and fed every 2–3 days. Toxicity experiments were performed between days 5 and 12 after the final plating.

For some physiology experiments, differentiated P19 cells were plated directly onto culture dishes, without a primary glial feeder layer. These were plated at 0.75 $\times 10^6$ cells per 35-mm dish, and were inhibited on days 2–5, when the self-generated glial monolayer approached confluency. Cultures were exposed to Ara-C for 24 h, and were then switched to low-glucose Dulbecco's MEM containing 10% horse serum, for maintenance. Otherwise, culture procedures were identical. Electrophysiology experiments were performed on differentiated neuron-like P19 cells plated both with and without a primary glial feeder layer, with similar results.

Primary glial cultures were generated from postnatal days 1–3 rodents. For rat cultures, neocortices were dissociated and plated in Eagle's MEM supplemented with 20 m*M* glucose, 10% FBS, and 10% horse serum, at a density of 1 hemisphere per 24-well plate (Falcon, Primeria). Glial monolayers were used after 2–4 weeks *in vitro*, and were not fed prior to the addition of P19 cells. Mouse glia were prepared as above, except that they were plated at a lower density (0.5 hemispheres/24-well plate), in plating medium additionally containing 10 ng/ml epidermal growth factor.

Electrophysiology

Boralex pipettes were coated with Sylgard, fire-polished, and filled with 140 m*M* $CsCH_3SO_3$, 5 m*M* CsCl, 10 m*M* EGTA, and 10 m*M* Hepes, pH adjusted to 7.4 with CsOH. The culture dish was perfused at a rate of 1–2 ml/min with Tyrode's solution: 150 m*M* NaCl, 4 m*M* KCl, 2 m*M* $CaCl_2$, 2 m*M* $MgCl_2$, 10 m*M* glucose, and 10 m*M* Hepes, pH adjusted to 7.4 with NaOH. Excitatory amino acids were dissolved in 160 NaCl, 2 m*M* $CaCl_2$, 10 m*M* Hepes, pH adjusted to 7.4 with NaOH. In addition, 1 m*M* $MgCl_2$ and 10 μM dizocilpine were added for experiments on AMPA/kainate receptors. Control and agonist solutions were applied to the cells by local perfusion from a linear array of microcapillaries (2 μl Drummond microcaps). The external solution was exchanged with a time constant of 30–50 ms. Whole-cell and single-channel currents were recorded with an Axopatch 200 amplifier, filtered at 1–2 KHz (−3 db, 4 pole Bessel), and digitized at 5–10 kHz. For storage and off-line analysis, digitized whole-cell currents were compressed by averaging 3 ms of data at 0.1- to 1-s intervals. Membrane potentials have been corrected for a junction potential of −10 mV between the internal solution and the Tyrode's solution in which seals were formed.

Immunocytochemistry

P19 cultures were fixed for 10 min with 4% paraformaldehyde in phosphate-buffered saline (PBS) and blocked twice, for 30 min each time, in 10% normal goat serum (NGS) in PBS. For the neuron-specific enolase or neurofilament antibodies, 0.25% Triton X-100 was included in the blocking solution to permeabilize the cells. Cultures were incubated in primary antibody for two h, at room temperature. The rat monoclonal anti-M6 (courtesy of C. Lagenaur) was used diluted 1:25 in 2% NGS in PBS. Polyclonal anti-neurofilament 200 was diluted 1:200, and neuron-specific enolase antiserum 1:10, in 2% NGS, 0.25% Triton X-100 in PBS. Cells were then incubated in biotinylated secondary antibodies (both diluted 1:200) for 30 min, followed by 30 min in avidin–biotin–peroxidase reagent (Vector). Staining was visualized with the chromagen 3-amino-9-ethylcarbazole (AEC).

Excitatory Amino Acid Exposure and Assessment of Injury

For toxicity experiments, cultures were washed three times in MEM (with 20 m*M* glucose) to remove serum. Excitatory amino acid agonists and/or antagonists were then added to the bathing medium, and the cells were returned to a 37°C, humidified 5% CO_2 incubator. For experiments with NMDA, 10 μM glycine was added to all conditions, including controls. Dizocilpine (10 μM) was included in all kainate exposures to block potential activation of NMDA receptors by endogenous glutamate release.

Neuronal loss was assessed by direct counts of identified microscopic fields in sister cultures. A few hours prior to toxicity experiments, cells were observed under phase-contrast optics at 200×, and videomicrographs were recorded using a charge-coupled device camera (Javelin) and optical memory disk recorder (Panasonic). For each 15-mm culture well, two separate 200× fields were identified using an objective marker, and all

neurons included in the videomicrograph were counted. Care was taken to choose fields of similar density, with evenly spread-out cells. Videomicrograph fields contained an average of 35–40 cells (range: 15–70). Neuronal death was assessed after 24 h of drug exposure by relocating, rerecording, and recounting the original fields. In several experiments, viability of the remaining cells was confirmed by subsequent staining with 0.4% trypan blue for 10 min.

Reagents

D-Aminophosophonovalerate (D-APV) was purchased from Tocris Neuramin. 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), and dizocilpine (MK-801, Merck, Sharp & Dohme) were obtained from Research Biochemicals, Inc. Neuron-specific enolase antiserum was supplied by Incstar. Most other drugs and antibodies were obtained from Sigma.

RESULTS

P19/Glial Co-Culture System

Published P19 protocols involve plating retinoic acid-treated aggregates (McBurney et al., 1988) or dissociated cells (Levine and Flynn, 1986) directly onto plastic dishes or coated glass coverslips. We found this protocol produced large clusters of neuron-like cells on a monolayer of flat cells, with relatively few isolated cells between the clusters. In addition to the difficulty of determining viable cell number in the large clusters, we found that the cultures had high levels of ongoing cell death, and were extremely vulnerable to wash-induced damage (not shown). To improve the viability and stability of differentiated P19 cells prior to toxic exposure, we examined the feasibility of a glial co-culture system.

As previously described (McBurney et al., 1988), undifferentiated P19 cells formed a confluent monolayer mostly consisting of phase-dark, polygonal cells [Fig. 1(A)]. After exposure to 500 nM retinoic acid for 4 days, the resulting aggregates were mechanically and enzymatically dissociated, and the cells were plated on either bare polystyrene or a previously established confluent monolayer of mouse cortical glia (>95% astrocytes). In both culture conditions some cells began elaborating processes within the first 24 h after plating. P19 cells plated on mouse glial monolayers generated approximately the same number of process-bearing cells as sister cultures plated directly onto polystyrene, but those in the co-cultures were markedly less prone to clustering, remaining mostly isolated for the first few days, and thereafter migrating into small clumps. These process-bearing cells exhibited typical neuronal morphology under phase-contrast optics [Fig. 1(B)], and were stained with antibodies to both neuron-specific enolase [Fig. 1(E)] and the 200 kD neurofilament protein [Fig. 1(D)].

In co-cultures, most of these neuron-like cells remained viable for up to 12 days. Ongoing neuronal loss was typically 10% per day between days 5 and 10. Since dead cells left little cellular debris on the glial monolayer, >95% of neuron-like cells were viable (by trypan blue exclusion) prior to use. A few small, isolated islands of fibroblast-like cells were also present in the mature co-cultures.

No neurons were present in primary glial cultures prior to addition of P19 cells. To ensure that neuron-like cells in the co-cultures were derived exclusively from P19 cells, we plated some differentiated P19 cells on rat, rather than on mouse, glial monolayers and immunoperoxidase-stained them for M6, a cell surface antigen specific for mouse cells (Lund et al., 1985), present in CNS neurons (Lagenaur et al., 1992), and known to be expressed by differentiated P19s (D. Gottlieb, unpublished observations). In co-cultures generated from several differentiations and tested at various ages between 6 and 12 days after plating, >90% of neurons were M6 positive [Fig. 1(C)]. Glial monolayers showed no reactivity with neurofilament-200 or neuron-specific enolase antibodies.

Electrophysiology

Excitatory amino acids activated inward whole-cell currents in nearly all P19 cells studied between 8 and 12 days after induction; uninduced cells lacked such responses. NMDA (20 μM) plus 300 nM glycine (Johnson and Ascher, 1987) evoked currents in 26/29 cells (Fig. 2). Nearly complete inhibition was achieved with 1 mM $MgCl_2$ (Nowak et al., 1984; Mayer and Westbrook, 1985), the competitive NMDA antagonist, 50 μM DL-APV (Davies et al., 1981), or with the glycine site antagonist, 1 μM 5,7-dichlorokynurenic acid (Baron et al., 1990; Leeson et al., 1991) [Fig. 2(A)]. In seven cells tested with 20 μM NMDA and 300 nM glycine, 50 μM DL-APV blocked 94 ± 2% (S.E.M.) of the current, 5,7-dichlorokynurenic acid inhibited 94% ± 3% of the current, and $MgCl_2$ produced 97% ± 2% block at holding potentials of −80 to −90

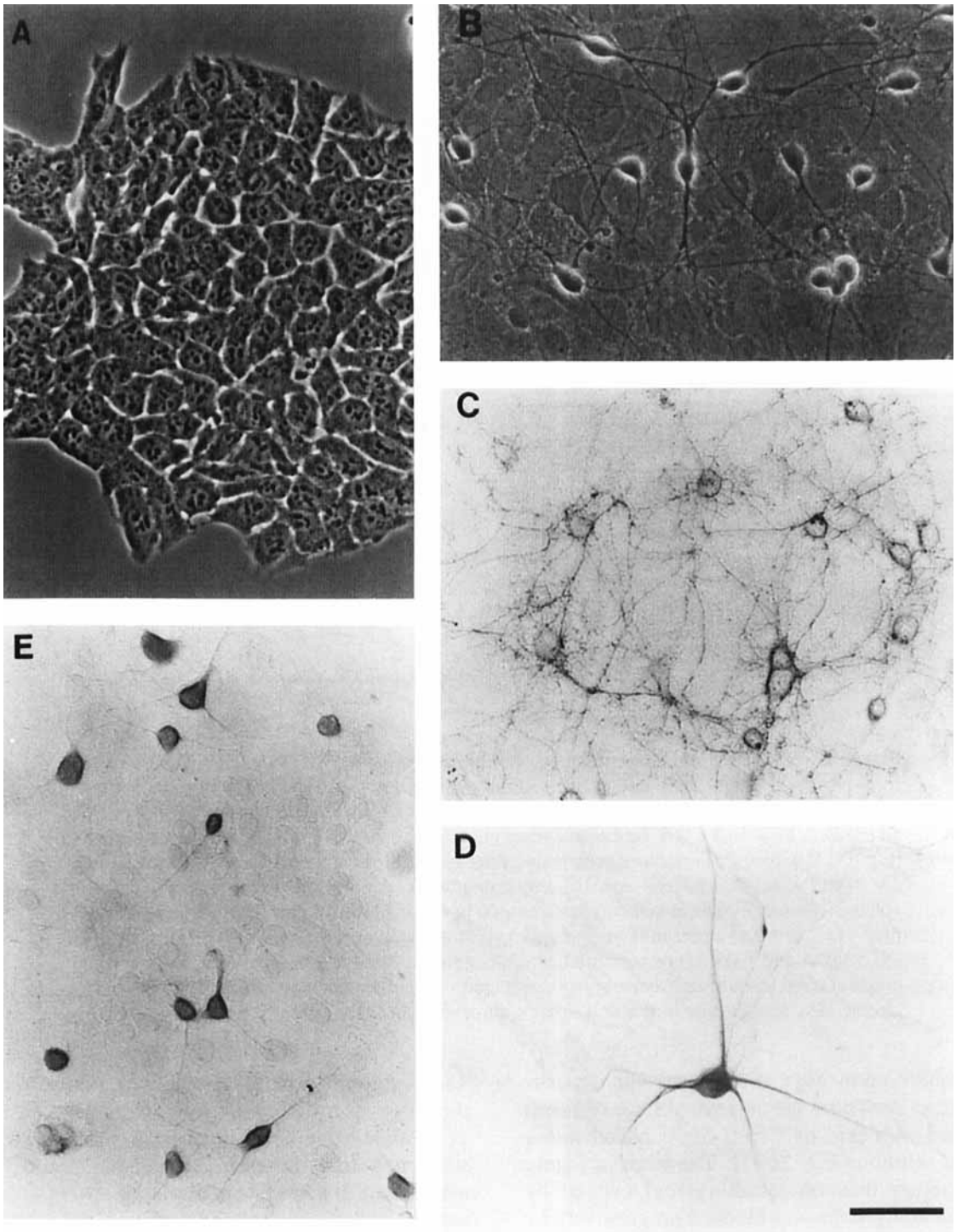

Figure 1 Neuronal differentiation of P19 cells after retinoic acid treatment. (A) Phase-contrast photomicrograph of undifferentiated P19 cells. (B) Phase-contrast photomicrograph of differentiated P19 cells (phase-bright, process-bearing) plated on a glial monolayer. (C) Immunoperoxidase staining for M6, a mouse-specific central nervous system antigen. (D) Immunostaining with polyclonal anti-NF-200. (E) Immunostaining for neuron specific enolase. Original magnification 400×; scale bar = 50 μm (A, C, D, E) or 66 μm (B).

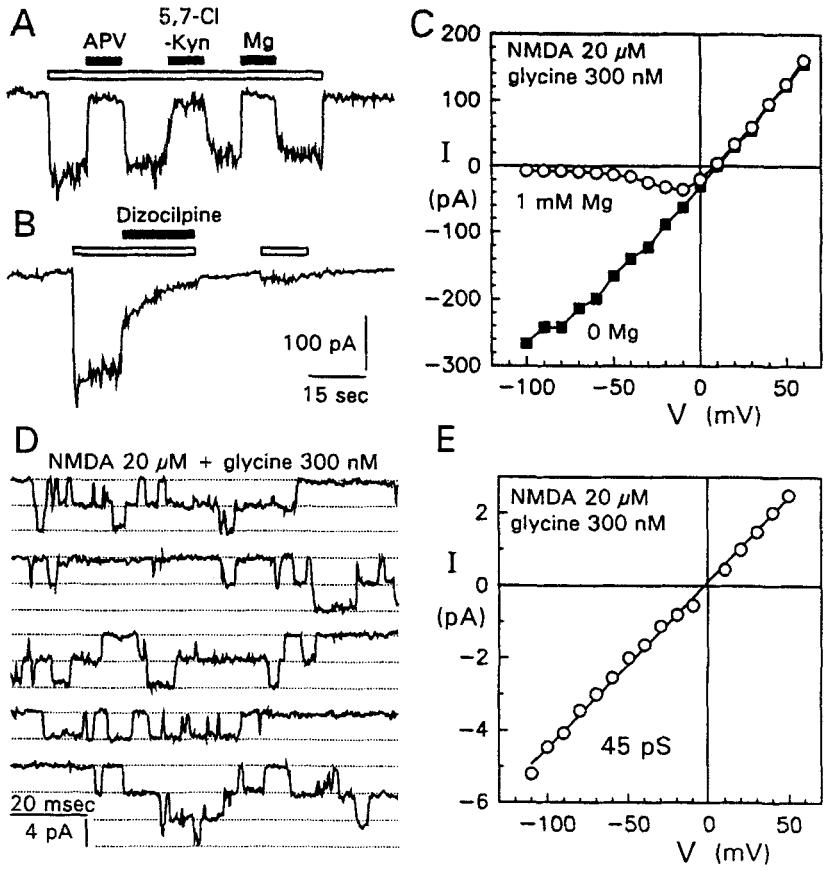

Figure 2 Whole-cell and single-channel currents activated by NMDA in P19-derived neurons. (A) NMDA (20 μM) and glycine (300 nM) were applied to the cell during the period indicated by the open bar. The whole-cell current evoked by NMDA was blocked reversibly by 50 μM D,L-APV, by 1 μM 5,7-dichlorokynurenic acid, and by 1 mM $MgCl_2$. (B) In a different cell, 10 μM dizocilpine produced progressive, long-lasting block when applied together with 20 μM NMDA plus 300 nM glycine. Holding potential in (A and B), −90 mV. (C) Current–voltage relations for whole-cell current activated by 20 μM NMDA and 300 nM glycine in 1 mM Mg^{2+} (circles) and 0 mM Mg^{2+} (squares). (D) Single-channel currents activated by 20 μM NMDA and 300 nM glycine in an outside-out patch. Holding potential, −90 mV. (E) In a different outside-out patch, current–voltage relation for unitary currents gated by NMDA plus glycine. The straight line indicates a single channel conductance of 45 pS.

mV. Inhibition by Mg^{2+} diminished with depolarization, as shown by the current–voltage relations recorded with zero or 1 mM Mg^{2+} added to the agonist solution [Fig. 2(C)]. The trace in Figure 2(B) shows that whole-cell current evoked by NMDA and glycine was blocked progressively by the noncompetitive NMDA channel blocker, 10 μM dizocilpine (Wong et al., 1986). As is the case with primary mammalian neurons (Huettner and Bean, 1988), a brief wash with control solution failed to remove the inhibition by dizocilpine.

In outside-out patches from four P19 neuron-like cells, NMDA and glycine induced channel activity with a predominant unitary conductance of 45 pS. Agonist-dependent openings of lower conductance occurred with low frequency. Figure 2(D) shows five contiguous traces from an outside-out patch exposed to 20 μM NMDA and 300 nM glycine at a holding potential of −90 mV. The current voltage relation for unitary events shown in Figure 2(E) was constructed with data from a different patch. Unitary current reversed near zero mV, and the slope indicates a conductance of 45 pS.

Inward currents were also activated by kainate (43/47 cells), glutamate (19/23 cells), and quisqualate (6/7 cells), all tested in the presence of 1 mM $MgCl_2$ and 10 μM dizocilpine [Fig. 3(A,B)].

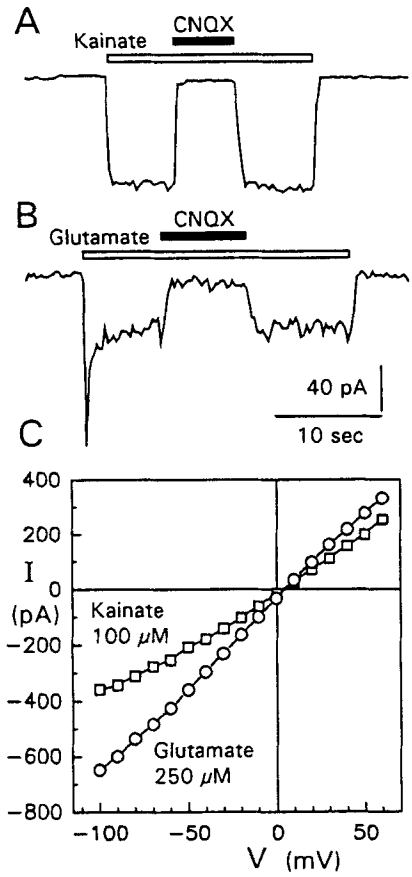

Figure 3 Whole-cell currents evoked by kainate and glutamate at non-NMDA receptors. (A) Kainate, 100 μM, was applied during the open bar. (B) In a different cell, 100 μM glutamate activated an initial transient current and a sustained current. CNQX, at 5 μM (closed bar), produced strong inhibition of the currents evoked by kainate A and glutamate B. In both A and B, 10 μM dizocilpine was included in the agonist and control solutions to block NMDA receptor channels. Holding potential in (A and B), −90 mV. (C) Current–voltage relations for whole-cell currents evoked by 100 μM kainate (squares) and 250 μM glutamate (circles) in two different cells.

In most cells, glutamate or quisqualate evoked an initial peak of current, which desensitized to a lower steady-state level (cf. Kiskin, Krishtal, and Tsyndrenko, 1986) [Fig. 3(B)]. Current activated by kainate did not desensitize in any of the cells studied. The selective antagonist CNQX (Honore et al., 1988), at 5 μM, blocked 96% ± 1% of the current evoked by 100 μM kainate in 17 cells. When tested against 100 μM glutamate on seven cells, 5 μM CNQX inhibited 75% ± 4% of the whole-cell current. Figure 3(C) shows typical current voltage relations for kainate and glutamate recorded in two different cells. In all but one of 27 cells tested with kainate or glutamate the I–V relationship was nearly linear and reversed between −5 and +5 mV. The single exception was a cell that showed strong inward rectification of current evoked by 200 μM kainate.

In spite of the widespread expression of functional NMDA and AMPA/kainate receptors, there was little evidence for spontaneous excitatory synaptic transmission. Spontaneous inward currents suggestive of excitatory synaptic activity were observed in only two of 57 P19 neuron-like cells.

Excitatory Amino Acid Neurotoxicity

A 24-h exposure to either NMDA or kainate resulted in widespread loss of P19 neuron-like cells (Fig. 4); no gross injury was apparent in the glial monolayer by phase-contrast morphology or trypan blue exclusion. In most cases, cell debris staining with trypan blue remained behind after excitatory amino acid exposure, indicating that cell death rather than simple detachment had occurred.

NMDA exposure produced only limited cytotoxicity before the sixth day after plating (two experiments, data not shown). Subsequently (7–11 days after plating), exposure to 500 μM NMDA and 10 μM glycine resulted in an average loss of 92.9% of the cells (±3.0 S.E.M., $n = 8$ experiments, 3–5 cultures per experiment) [Fig. 4(A,C)]. In contrast, exposure to sham wash conditions resulted in 32.9% neuronal loss (±2.1 S.E.M., $n = 17$ experiments). NMDA toxicity was concentration-dependent in the range of 10–300 μM, with half-maximal toxicity around 70 μM (Fig. 5). NMDA toxicity was completely blocked by 1 mM D-APV or 10 μM dizocilpine [Fig. 4(C)].

Exposure to 500 μM kainate for 24 h resulted in 72.0% cell death (±4.9 S.E.M., $n = 7$ experiments, 3–5 cultures per experiment). Compared to NMDA toxicity, the effect of kainate was less consistently observed, with neuronal loss ranging between 44.7% and 82.3% (7 experiments). The competitive AMPA/kainate receptor antagonist, CNQX (200 μM) substantially reduced kainate toxicity [Fig. 4(C)].

DISCUSSION

Whole-cell recordings revealed that induction of a neuronal phenotype in P19 cells led to the expres-

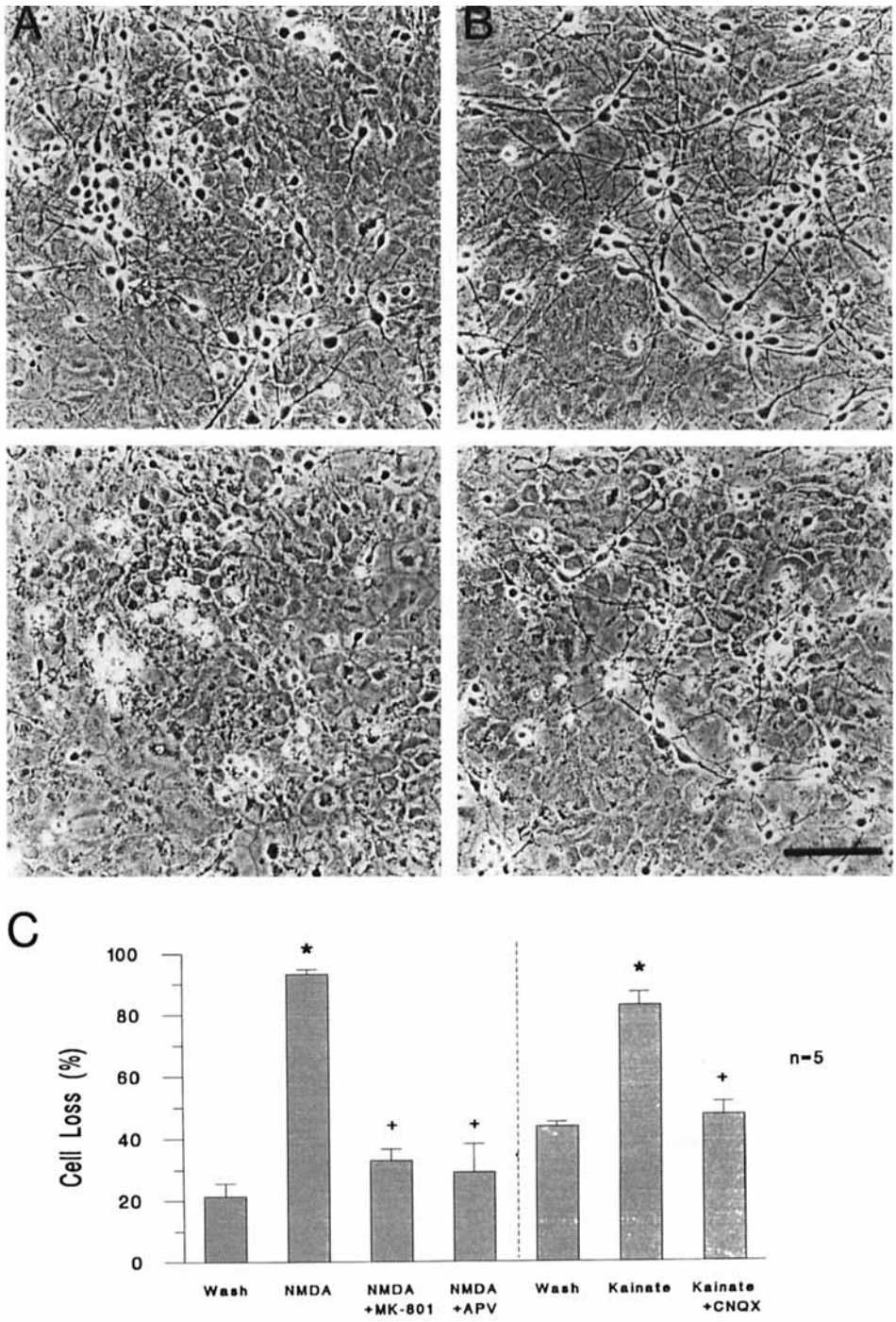

Figure 4 Excitatory amino acid-induced death of P19-derived neurons. (A, B) Morphological damage produced by exposure to NMDA and kainate. Phase-contrast photomicrographs of identified fields from sister cultures taken before (top) and after (bottom) 24-h exposure to 500 μM NMDA + 10 μM glycine (A), or 500 μM kainate (B). Scale bar = 100 μm. (C) Pharmacology of NMDA and kainate induced toxicity. Cell loss after 24 h exposure to 500 μM NMDA was reduced to baseline by inclusion of 10 μM dizocilpine (MK-801) or 1 mM D-APV in the bathing medium. Similarly, 200 μM CNQX attenuated the toxicity resulting from 24 h exposure to 500 μM kainate. Values represent mean (+ S.E.M.) loss of P19-derived neurons determined by cell counts of marked 200× microscopic fields before and after the indicated exposure (n = 5 cultures, 2 fields per culture). This experiment is representative of more than 5

sion of both NMDA and AMPA/kainate receptors similar to those described in other mammalian central neurons. NMDA-evoked inward currents were inhibited by both competitive and noncompetitive selective antagonists, exhibited voltage-dependent blockade by Mg^{2+} (Nowak et al., 1984; Mayer and Westbrook, 1985), and demonstrated progressive inhibition by the channel-blocking drug dizocilpine (Huettner and Bean, 1988). In outside-out patches from P19 cells, NMDA gated single-channel openings with a unitary conductance of 45 pS, which is similar in magnitude to native counterparts (Cull-Candy and Usowicz, 1989; Jahr and Stevens, 1987; Nowak et al., 1984). Our preliminary experiments indicate that the NMDA receptor channels are permeable to monovalent cations and Ca^{2+} (J. Huettner, unpublished observations). Furthermore, the currents activated by kainate in P19 cells were similar in pharmacology and desensitization kinetics to non-NMDA responses in central neurons (Kiskin et al., 1986; Patneau and Mayer, 1991). In future work, it will be important to determine which of the many glutamate receptor subunits (Nakanishi, 1992) are expressed by P19-derived neurons. The linear current–voltage relationship observed in the present study with AMPA/kainate receptor stimulation is consistent with the widespread expression of the edited form of the GluR2 (GluR-B) subunit (Sommer et al., 1991). We have recently learned that MacPherson and colleagues (1992) have also observed depolarizing responses to glutamate in differentiated P19 cells.

The finding that P19 cells expressed conventional NMDA and non-NMDA receptors led us to test whether prolonged exposure to excitatory amino acids would be directly toxic. A 24-h exposure to NMDA or kainate-induced cell death comparable to that seen in primary cortical cell cultures under similar experimental conditions (Choi et al., 1987; Koh and Choi, 1988). The minimum duration of exposure required to kill P19 cells was not investigated systematically, but in preliminary experiments, substantial death was also induced following a 1-h exposure to NMDA (D. Turetsky et al., unpublished observations). The pharmacology of excitotoxic injury of both P19 cells and cortical neurons suggests that the injury was likely mediated by ion channel-linked glutamate receptors. However, the P19 cells were somewhat less vulnerable: NMDA exposure destroyed virtually all P19 cells, but the toxic EC_{50} for NMDA on P19 cells was about 70 μM, versus about 20 μM on cortical neurons (Koh and Choi, 1988). Small differences in glutamate receptor number or characteristics, or in factors such as cell calcium handling or cellular antioxidant defenses, could account for the lower vulnerability of P19 cells to excitotoxic injury compared to primary cortical neurons.

The glutamate receptor-mediated nature of excitotoxicity observed in the present study on P19 cells thus differs from that previously described on several other cell lines. As discussed above, glutamate-induced injury of N18-RE-105 cells may be mediated by inhibition of cystine uptake, leading to reduced production of glutathione, and heightened vulnerability to free radical-induced injury (Murphy et al., 1989). The general applicability of this intriguing mechanism of excitotoxic injury remains to be determined. In preliminary experiments, we have found that cultured primary cortical neurons tolerate removal of extracellular cystine for 24–48 h without gross injury (Rose, Liu, and Choi, 1992).

It will be interesting to determine if other cell lines can be differentiated to neuron-like cells expressing functional ionotropic glutamate receptors. A recent independent study by Younkin et al. (1993) reports that NTera2 human embryonal carcinoma cells exposed to retinoic acid treatment also express glutamate receptors and vulnerability to excitotoxicity. Future comparison of glutamate receptor behavior and excitotoxicity on NTera2 and P19 neuron-like cells may yield insights into some basic principles governing glutamate receptor expression and function. From a practical standpoint, the shorter processing time (10 days) required to generate P19 neuron-like cells, compared to that required to generated NTera2 neuron-like cells (10 weeks, Pleasure, Page, and Lee, 1992; Younkin et al., 1993), may provide an experimental advantage.

We found that plating P19 cells on a previously established feeder layer of cortical glia enhanced

similar experiments. * indicates significant difference from corresponding sham wash condition; + indicates significant difference from corresponding agonist alone exposure condition, at $p < 0.01$ by two-tailed t test with Bonferroni correction. Dizocilpine (10 μM) was included in (B) and in the right part of (C) to block nonspecific activation of NMDA receptors.

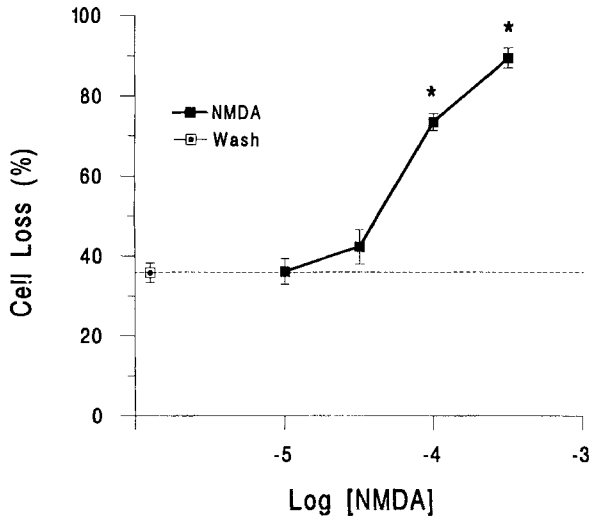

Figure 5 Concentration-dependence of NMDA toxicity. Differentiated P19 cultures were exposed to sham wash or to NMDA at the indicated concentrations for 24 h. Glycine (10 μM) was included in all conditions. Values represent mean (± S.E.M.) loss of P19-derived neurons, determined as described in legend to Figure 4 (n = 8 cultures, 2 fields per culture). * indicates significant difference from sham wash condition, at $p < 0.01$ by ANOVA and Student-Newman–Keul's test.

neuronal survival and the morphological assessment of cell death. We recognize that the presence of two distinct cell types may complicate subsequent neurochemical or molecular studies which require a purified cell population, and we are working to see if the benefit associated with co-culture can be achieved by other means. Another goal is to reduce the cell damage associated with sham wash, which sometimes reached 30%–40% of the total population. Approximately 50% of this cell loss could be prevented by including serum (5% fetal bovine and 5% horse) in the incubation medium (D. Turetsky et al., unpublished observations).

The P19 cell line is an attractive system for studying glutamate receptor-mediated behavior for several reasons. First, as discussed above, retinoic acid treatment induces multiple features of mature neurons. Second, undifferentiated P19 cells have a rapid doubling time (18 h), allowing ease of genetic manipulation. In general, rapid generation time in cell lines is rarely accompanied by the establishment of a stable, mature neuronal phenotype. Neuroblastomas and some retrovirally transformed lines fall at one end of the spectrum, with short doubling times, but a lack of mature, terminally differentiated neurons. On the other hand, the human megalencephaly-derived line, HCN-1, is an example of a cell line which has extensive neuronal differentiation, but also a long doubling time of 72 h (Ronnett et al., 1990). Finally, P19 cells are readily transfectable. They have been successfully used to explore the effects of gene overexpression [e.g., amyloid protein precursor (Yoshikawa, Aizawa, and Hayashi, 1992)], and antisense inhibition [e.g., MAP2 (Dinsmore and Solomon, 1991)] on neuronal differentiation and viability. As long as adequate caution is exercised in extrapolating results to other neuronal systems, the P19s should prove a valuable experimental tool.

Our finding that most of the P19 cells in our cultures express glutamate receptors contributes to the emerging picture of P19 neuronal differentiation pathways. It is interesting to note that key differences in neurochemical phenotype have been reported by different investigators. Sharma and Notter (1988) observed norepinephrine reuptake and catecholamine synthetic enzyme immunoreactivity in 70% of P19 neurons, but not choline acetyltransferase reactivity. McBurney and colleagues (McBurney et al., 1988; Jones-Villeneuve et al., McBurney, 1983) found choline acetyltransferase and AChE activity, but not catecholamine synthesis. It is possible that differences in transmitter phenotype reflect differences in the inductive effect of particular culture microenvironments; an alternative explanation might be a genetic change in the subclones of P19 cells utilized in different studies. It is likely that retinoic acid treatment activates genetic regulatory pathways in P19 cells that are normally activated in neurons of the developing CNS. One possibility is that retinoic acid broadly activates multiple neuronal genes. A second, more intriguing possibility is that retinoic acid exerts its action through a specific "master gene" whose normal function is to coordinate expression of phenotypes characteristic of central neuronal differentiation pathways. Further studies are necessary to distinguish between these possibilities.

The P19 embryonal carcinoma line provides an easily manipulated system that may offer advantages for the study of glutamate receptor-mediated neurotoxicity. In particular, the ability to up-regulate or down-regulate selected proteins in this genetically open system may facilitate investigation into the molecular mechanisms underlying excitotoxicity.

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REFERENCES

BAIN, G., RAMKUMAR, T. P., CHENG, J. M., and GOTTLIEB, D. I. (1993). Expression of the genes coding for glutamic acid decarboxylase in pluripotent cell lines. *Mol. Brain Res.* **17**:23–30.

BARON, B. M., HARRISON, B. L., MILLER, F. P., MCDONALD, I. A., SALITURO, G., SCHMIDT, C. J., SORENSEN, S. M., WHITE, H. S., and PALFREYMAN, M. G. (1990). Activity of 5,7-dichlorokynurenic acid, a potent agonist at the *N*-methyl-D-aspartate receptor-associated glycine binding site. *Mol. Pharmacol.* **38**:554–561.

BELL, J. C., JARDINE, K., and MCBURNEY, M. W. (1986). Lineage-specific transformation after differentiation of multipotential murine stem cells containing a human oncogene. *Mol. Cell Biol.* **6**:617–625.

CHOI, D. W. (1985). Glutamate neurotoxicity in cortical cell culture is calcium dependent. *Neurosci. Lett.* **58**:293–297.

CHOI, D. W. (1988). Glutamate neurotoxicity and diseases of the nervous system. *Neuron* **1**:623–634.

CHOI, D. W. (1992). Excitotoxic cell death. *J. Neurobiol.* **23**:1261–1276.

CHOI, D. W., MAULUCCI-GEDDE, M. A., and KRIEGSTEIN, A. R. (1987). Glutamate neurotoxicity in cortical cell culture. *J. Neurosci.* **7**:357–368.

CHOVANES, G. I., COLOGER, A., LU, S., TJAN, C., and SIMON, N. (1993). Excitatory amino acid-induced cytotoxicity in continuous neural cell lines. *J. Neurotrauma* (Abstract) **9**:383.

CULL-CANDY, S. G. and USOWICZ, M. M. (1989). On the multiple-conductance single channels activated by excitatory amino acids in large cerebellar neurones of the rat. *J. Physiol. (Lond.)* **415**:555–582.

DAVIES, J., EVANS, R. H., FRANCIS, A. A., JONES, A. W., and WATKINS, J. C. (1981). 2-Amino-5-phosphonovalerate (2APV), a potent and selective antagonist of amino acid-induced and synaptic excitation. *Neurosci. Lett.* **21**:77–81.

DINSMORE, J. H. and SOLOMON, F. (1991). Inhibition of MAP2 expression affects both morphological and cell division phenotypes of neuronal differentiation. *Cell* **64**:817–826.

ELLREN, K. and LEHMANN, A. (1989). Calcium dependency of *N*-methyl-D-aspartate toxicity in slices from the immature rat hippocampus. *Neuroscience* **132**:371–379.

FRANDSEN, A., DREJER, J., and SCHOUSBOE, A. (1989). Direct evidence that excitotoxicity in cultured neurons is mediated via *N*-methyl-D-aspartate (NMDA) as well as non-NMDA receptors. *J. Neurochem.* **53**:297–299.

GARTHWAITE, G. and GARTHWAITE, J. (1986a). Amino acid neurotoxicity: intracellular sites of calcium accumulation associated with the onset of irreversible damage to rat cerebellar neurones *in vitro*. *Neurosci. Lett.* **71**:53–58.

GARTHWAITE, G. and GARTHWAITE, J. (1986b). Neurotoxicity of excitatory amino acid receptor agonists in rat cerebellar slices: dependence on calcium concentration. *Neurosci. Lett.* **66**:193–198.

HAHN, J. S., AIZENMAN, E., and LIPTON, S. A. (1988). Central mammalian neurons normally resistant to glutamate toxicity are made sensitive by elevated extracellular Ca^{2+}: toxicity is blocked by the *N*-methyl-D-asparate antagonist MK-801. *Proc. Natl. Acad. Sci. USA* **85**:6556–6560.

HONORE, T., DAVIES, S. N., DREJER, J., FLETCHER, E. J., JACOBSEN, P., LODGE, D., and NIELSEN, F. E. (1988). Quinoxalinediones: potent competitive non-NMDA glutamate receptor antagonists. *Science* **241**:701–703.

HUETTNER, J. E. and BEAN, B. P. (1988). Block of *N*-methyl-D-aspartate-activated current by the anticonvulsant MK-801: selective binding to open channels. *Proc. Natl. Acad. Sci. USA* **85**:1307–1311.

JAHR, C. E. and STEVENS, C. F. (1987). Glutamate activates multiple single channel conductances in hippocampal neurons. *Nature* **325**:522–525.

JOHNSON, J. W. and ASCHER, P. (1987). Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature* **325**:529–531.

JONES-VILLENEUVE, E. M. V., MCBURNEY, M. W., ROGERS, K. A., and KALNINS, V. I. (1982). Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells. *J. Cell Biol.* **94**:253–262.

JONES-VILLENEUVE, E. M. V., RUDNICKI, M. A., HARRIS, J. F., and MCBURNEY, M. W. (1983). Retinoic acid-induced neural differentiation of embryonal carcinoma cells. *Mol. Cell Biol.* **3**:2271–2279.

KISKIN, N. I., KRISHTAL, O. A., and TSYNDRENKO, A. YA. (1986). Excitatory amino acid receptors in hippocampal neurons: kainate fails to desensitize them. *Neurosci. Lett.* **63**:225–230.

KOH, J. and CHOI, D. W. (1988). Vulnerability of cultured cortical neurons to damage by excitotoxins: differential susceptibility of neurons containing NADPH-diaphorase. *J. Neurosci.* **8**:2153–2163.

LAGENAUR, C., KUNEMUND, V., FISCHER, G., FUSHIKI, S., and SCHACHNER, M. (1992). Monoclonal M6 antibody interferes with neurite extension of cultured neurons. *J. Neurobiol.* **23**:71–88.

LEESON, P. D., BAKER, R., CARLING, R. W., CURTIS, N. R., MOORE, K. W., WILLIAMS, B. J., FOSTER, A. C., DONALD, A. E., KEMP, J. A., and MARSHALL, G. R. (1991). Kynurenic acid derivatives. Structure-activity relationships for excitatory amino acid antagonism and identification of potent and selective antagonists

at the glycine site on the *N*-methyl-D-aspartate receptor. *J. Med. Chem.* **34**:1243–1252.

LERMA, J., KUSHNER, L., SPRAY, D. C., BENNETT, M. V. L., and ZUKIN, R. S. (1989). mRNA from NCB-20 cells encodes the *N*-methyl-D-aspartate/phencyclidine receptor: A *Xenopus* oocyte expression study. *Proc. Natl. Acad. Sci. USA* **86**:1708–1711.

LEVINE, J. M. and FLYNN, P. (1986). Cell surface changes accompanying the neural differentiation of an embryonal carcinoma cell line. *J. Neurosci.* **6**:3374–3384.

LUCAS, D. R. and NEWHOUSE, J. P. (1957). The toxic effect of sodium L-glutamate on the inner layers of the retina. *Arch. Ophthalmol.* **58**:193–201.

LUND, R. D., CHANG, F. -L. F., HANKIN, M. H., and LAGENAUR, C. F. (1985). Use of a species specific antibody for demonstrating mouse neurons transplanted to rat brains. *Neurosci. Lett.* **61**:221–226.

MACPHERSON, P. A., MAGNUSON, D. S. K., MORASSUTTI, D. J., STAINES, W. A., MARSHALL, K. C., and MCBURNEY, M. W. (1992). Neurons derived from P19 cells contain L-glutamate, GABA, and their receptors. *Soc. Neurosci. Abstr.* **18**:612.

MANEV, H., FAVARON, M., GUIDOTTI, A., and COSTA, E. (1989). Delayed increase of Ca^{2+} influx elicited by glutamate: role in neuronal death. *Mol. Pharmacol.* **36**:106–112.

MARCOUX, F. W., GOODRICH, J. E., PROBERT, A. W., and DOMINICK, M. A. (1988). Ketamine prevents glutamate-induced calcium influx and ischemia nerve cell injury. In: *Sigma and Phencyclidine-Like Compounds as Molecular Probes in Biology*. E. F. Domino and J. Kamenka, Eds. NPP Books, Ann Arbor, MI, pp. 735–746.

MAYER, M. L. and WESTBROOK, G. L. (1985). The action of *N*-methyl-D-aspartic acid on mouse spinal neurones in culture. *J. Physiol. (Lond.)* **361**:65–90.

MCBURNEY, M. W., JONES-VILLENEUVE, E. M. V., EDWARDS, M. K. S., and ANDERSON, P. J. (1982). Control of muscle and neuronal differentiation in a cultured embryonal carcinoma cell line. *Nature* **299**:165–167.

MCBURNEY, M. W., REUHL, K. R., ALLY, A. I., NASPURI, S., BELL, J. C., and CRAIG, J. (1988). Differentiation and maturation of embryonal carcinoma-derived neurons in cell culture. *J. Neurosci.* **8**:1063–1073.

MCBURNEY, M. W. and ROGERS, B. J. (1982). Isolation of male embryonal carcinoma cells and their chromosomal replication patterns. *Dev. Biol.* **89**:503–508.

MELDRUM, B. (1985). Possible therapeutic applications of antagonists of excitatory amino acid neurotransmitters. *Clin. Sci.* **68**:113–122.

MURPHY, T. H., MIYAMOTO, M., SASTRE, A., SCHNAAR, R. L., and COYLE, J. T. (1989). Glutamate toxicity in a neuronal cell line involves inhibition of cystine transport leading to oxidative stress. *Neuron* **2**:1547–1558.

NAKANISHI, S. (1992). Molecular diversity of glutamate receptors and implications for brain function. *Science* **258**:597–603.

NOWAK, L., BREGESTOVSKI, P., ASCHER, P., HERBET, A., and PROCHIANTZ, A. (1984). Magnesium gates glutamate-activated channels in mouse central neurons. *Nature* **307**:462–465.

OGURA, A., MIYAMOTO, M., and KUDO, Y. (1988). Neuronal death *in vitro:* parallelism between survivability of hippocampal neurones and sustained elevation of cytosolic Ca^{2+} after exposure to glutamate receptor agonist. *Exp. Brain Res.* **73**:447–458.

OLNEY, J. W. (1969). Brain lesion, obesity and other disturbances in mice treated with monosodium glutamate. *Science* **164**:719–721.

OLNEY, J. W., PRICE, M. T., SAMSON, L., and LABRUYERE, J. (1986). The role of specific ions in glutamate neurotoxicity. *Neurosci. Lett.* **65**:65–71.

PATNEAU, D. K. and MAYER, M. L. (1991). Kinetic analysis of interactions between kainate and AMPA: evidence for activation of a single receptor in mouse hippocampal neurons. *Neuron* **6**:785–798.

PLEASURE, S. J., PAGE, C., and LEE, V. M. -Y. (1992). Pure, postmitotic, polarized human neurons derived from NTera 2 cells provide a system for expressing exogenous proteins in terminally differentiated neurons. *J. Neurosci.* **12**:1802–1815.

RONNETT, G. V., HESTER, L. D., NYE, J. S., CONNORS, K., and SNYDER, S. H. (1990). Human cortical neuronal cell line: establishment from a patient with unilateral megalencephaly. *Science* **248**:603–605.

ROSE, K., LIU, D., and CHOI, D. W. (1992). Nitric oxide synthase activation, or cystine depletion, may not be critical to NMDA receptor-mediated injury in murine cortical cultures. *Soc. Neurosci. Abstr.* **18**:645.

ROTHMAN, S. M. (1985). The neurotoxicity of excitatory amino acids is produced by passive chloride influx. *J. Neurosci.* **5**:1483–1489.

ROTHMAN, S. M. and OLNEY, J. W. (1987). Excitotoxicity and the NMDA receptor. *Trends Neurosci.* **10**:299–302.

ROTHMAN, S. M., THURSTON, J. H., and HAUHART, R. E. (1987). Delayed neurotoxicity of excitatory amino acids *in vitro*. *Neuroscience* **22**:471–480.

SHARMA, S. and NOTTER, M. F. D. (1988). Characterization of neurotransmitter phenotype during neuronal differentiation of embryonal carcinoma cells. *Dev. Biol.* **125**:246–254.

SIMANTOV, R. (1989). Glutamate neurotoxicity in culture depends on the presence of glutamine: implications for the role of glial cells in normal and pathological brain development. *J. Neurochem.* **52**:1694–1699.

SOMMER, B., KOHLER, M., SPRENGEL, R., and SEEBURG, P. H. (1991). RNA editing in brain controls a determinant of ion flow in glutamate-gated channels. *Cell* **67**:11–19.

TURETSKY, D. M., HUETTNER, J. E., GOTTLIEB, D. I.,

GOLDBERG, M. P., and CHOI, D. W. (1992). Excitotoxic damage to cells derived from an embryonal carcinoma cell line. *Second NIMH Conference on Molecular Neurobiology,* Keystone, CO (Abstract), NIMH, Bethesda, MD.

WATKINS, J. C., KROGSGAARD-LARSEN, P., and HONORE, T. (1990). Structure-activity relationships in the development of excitatory amino acid receptor agonists and competitive antagonists. *Trends Pharm. Sci.* **11**:25–33.

WATKINS, J. C. and OLVERMAN, H. J. (1987). Agonists and antagonists for excitatory amino acid receptors. *Trends Neurosci.* **10**:265–272.

WONG, E. H. F., KEMP, J. A., PRIESTLEY, T., KNIGHT, A. R., WOODRUFF, G. N., and IVERSEN, L. L. (1986). The anticonvulsant MK-801 is a potent *N*-methyl-D-aspartate antagonist. *Proc. Natl. Acad. Sci. USA* **83**:7104–7108.

YOSHIKAWA, K., AIZAWA, T., and HAYASHI, Y. (1992). Degeneration *in vitro* of post-mitotic neurons overexpressing the Alzheimer amyloid protein precursor. *Nature* **359**:64–67.

YOUNKIN, D. P., TANG, C. -M., HARDY, M., REDDY, U. R., SHI, Q. -Y., PLEASURE, S. J., LEE, V. M. -Y., and PLEASURE, D. (1993). Inducible expression of neuronal glutamate receptor channels in the NT2 human cell line. *Proc. Natl. Acad. Sci. USA* **90**:2174–2178.