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Characterization and mechanism of glutamate neurotoxicity in primary striatal cultures

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Excitatory amino acids may play a role in the pathogenesis of cell death in neurodegenerative diseases, including Huntington's disease (HD). In an attempt to develop a tissue culture model for HD, the toxicity of glutamate was examined in primary striatal cultures derived from newborn rats. Morphological criteria were used to determine the toxic effects of glutamate in 6-, 12-, and 18-day-old cultures which were examined before and after 1–3 h of exposure to glutamate. Although younger cultures demonstrated little susceptibility to glutamate relative to controls, the number of neurons in older cultures was significantly depleted in the presence of glutamate. Glutamate toxicity was dose-dependent, with an ED_{50} of approximately 300 μm glutamate, and a maximal effect was observed within 3 h of initial exposure. Affected neurons demonstrated somal swelling within 1 h of glutamate exposure and disruption of neuritic processes and somal integrity within 3 h. Cell death was significantly increased by raising the extracellular calcium concentration and could be decreased by the addition of magnesium to the incubation medium. Moreover, the *N*-methyl-D-aspartate (NMDA) receptor agonist, quinolinic acid, showed a toxicity profile similar to that of glutamate. The NMDA receptor competitive antagonist, 2-amino-5-phosphonovalerate (APV) significantly reduced toxicity, albeit incompletely. An additional component of glutamate mediated toxicity in striatal cultures could be explained by activation of non-NMDA receptor subtypes. These in vitro studies indicate that glutamate is toxic to a subset of mature striatal neurons in the absence of a glutamatergic afferent input, and that this toxicity is mediated partially by the NMDA receptor, with an additional component due to non-NMDA receptors.

INTRODUCTION

In addition to its role in intermediary metabolism, the dicarboxylic amino acid, glutamate, acts as a putative excitatory neurotransmitter in many regions within the mammalian nervous system[39,70]. Glutamate is believed to act post-synaptically at 3 classes of receptors: the *N*-methyl-D-aspartate (NMDA) receptor and the two non-NMDA receptors, kainate and quisqualate[26,65]. Of these, the NMDA receptor is of particular interest since it is postulated to play a role in long-term potentiation and may mediate neuronal degeneration in a variety of neurological conditions[2,10,11,59,61,71].

A number of in vivo and in vitro studies have examined NMDA-receptor-mediated neurotoxicity in several brain regions, including the cortex, hippocampus, and striatum[2,8,17,33,59]. Following injection into the striatum of rodents, NMDA agonists such as quinolinic acid and homocysteic acid can produce some of the anatomical and neurochemical alterations seen in the genetic, neurodegenerative disorder, Huntington's disease (HD)[2,6,14,50,59].

Similarly, in primary striatal cultures, quinolinic acid results in a pattern of toxicity which spares some types of neurons, a feature also seen in HD[3,5,15,16,34]. Although toxic effects of glutamate have been observed by in vitro studies of other brain regions[7–9,56], they have not been demonstrated or characterized in the neostriatum where glutamate is the putative neurotransmitter of the corticostriatal pathway[18], and thus the most likely candidate for NMDA receptor-mediated toxicity.

We have undertaken a study aimed at characterizing the toxicity of glutamate in primary striatal cultures derived from neonatal rats. Despite the absence of extrinsic inputs, cultured neonatal striatal neurons develop a variety of cell types, synaptic connections and immunohistochemical and biochemical features of the in vivo neostriatum[31,42,51–55,61,62,66,72] that make them a suitable in vitro system for the study of glutamate-mediated toxicity. Understanding the mechanism and

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correlates of such toxicity may lend insight into the pathogenesis of Huntington's disease, as well as other neurologic disorders, such as stroke, epilepsy and hypoglycemia in which glutamate may play a role.

MATERIALS AND METHODS

Striatal cultures

Mixed striatal cultures, with both glial and neuronal elements, were prepared from postnatal day (PND) 0 (newborn) or PND 6 rat pups (Sprague–Dawley, Zivic Miller, Zelienople, PA). Using sterile technique, each pup was decapitated, and the brain was removed and placed into a Petri dish under a dissecting microscope (American Optical Stereo Star A0580). The anterior striatum was removed bilaterally and placed into a Petri dish containing Tyrode's buffer (NaCl 140 mM, glucose 11 mM, KCl 4 mM, NaH_2PO_4 360 μM, KH_2PO_4 180 μM, pH adjusted to 7.4 with bicarbonate) on ice. In each dissecting session, 2–3 litters (10–15 pups/litter) were used. After all the rat pups were sacrificed and the brains dissected, the Tyrode's buffer was aspirated in a laminar flow hood, and approximately 1 ml of media (50% DMEM, 50% HAMS F12, 10% heat inactivated horse serum, supplemented with 100 units/ml penicillin/streptomycin, 4 g/l glucose, Gibco, NY) was added per 10 striata. The tissue was grossly dissociated by trituration 5 times with a 10 ml Pasteur pipette. To produce about 20 dishes/litter, approximately 10^6 cells were plated into each of 35 mm dishes (Nunclon dishes, Nunc, Denmark) pretreated for 3 h with poly-L-lysine (M_r 75 000–150 000, 20 mg/l, Sigma Chemical, St. Louis, MO) and subsequently washed twice with distilled water and once with Tyrode's buffer. Culture dishes were then supplemented with 1.5 ml of media and placed into a 37 °C, 5% CO_2, humidified atmosphere in an incubator (Forma Scientific). After 24 h, the media in the dishes were replenished with fresh media and after 5 days in culture (DIC), approximately 75% of the media volume was aspirated and replaced with media containing cytosine arabinoside (40 μM, Sigma Chemical), an inhibitor of mitosis, to prevent overgrowth by glial cells. Thereafter, two-thirds of the media volume in each dish was replaced every 3–4 days by fresh media.

Determination of neuronal elements in culture

To confirm the presence of neurons in culture, Cresyl violet staining and immunohistochemical localization of neurofilament antigen was used. For these purposes, some of the cultures were grown on polylysine-coated Aclar coverslips. All cultures were fixed for 15 min in a 4% paraformaldehyde/phosphate-buffered saline (PBS) solution at 37 °C, after which some cultures were treated with 1% Cresyl violet for 15 min; other cultures were incubated with primary antibody (1:500 of mouse antineurofilament, courtesy of Dr. C. Marotta and 1:5000 of M33, Sternberger-Meyer) in PBS. After 48 h, the avidin-biotin method (Vectastain, Vector Lab, Burlingame, CA), followed by a diaminobenzidine reaction, was used for immunohistochemical localization.

Further confirmation of the neuronal nature of our cultured cells was obtained by ultrastructural examination of 12-day-old cultures which showed the presence of axons with vesicles and synaptic contacts (DiFiglia, M. and Folsom, unpublished observations).

Assessment of toxicity

Cultured cells, 6, 12, and 18 DIC were used in all experiments. Using a marker pen, a circular coordinate system was drawn underneath each dish which enabled relocation of selected fields with phase contrast microscopy (32× objective). A field with approximately 20–30 neurons was randomly chosen from each dish and photographed using a Zeiss inverted microscope with a modified Polaroid camera (Newton Plastics, Newton, MA) and Polaroid 665 positive/negative film before, during, and after exposure to different solutions. The solutions used in this study contained differing concentrations of glutamate, $CaCl_2$, $MgCl_2$, quinolinic acid, 2-amino-5-phosphonovalerate (APV), glycine, kynurenic acid, quisqualic acid, and/or kainic acid. All reagents were obtained from Sigma Chemical, and were prepared in Tyrode's buffer (pH adjusted to 7.4 at 37 °C in the presence of 5% CO_2). In a typical experiment, a field was selected and photographed, the media were aspirated from the culture dish, 1.5 ml of the experimental solution was added, and the dish was immediately replaced into the 37 °C, humidified, 5% CO_2 incubator. At the appropriate time points, the dish was removed from the incubator and the same field was relocated and photographed within 1 min. Control dishes were handled identically.

In preliminary studies, we found that the use of vital dyes to determine neuronal viability was unreliable. Therefore, a conservative set of morphological criteria was used to assess toxicity, before and after toxin exposure. A neuron was considered no longer viable only if the integrity of the cell body and at least two or all processes were destroyed (Fig. 2). Because somal swelling was found to be reversible (see results in Fig. 4), cavitation and shrinking of the cell body, as well as beading and/or disappearance of processes were required as criteria for cell death. These same criteria were applied at all culture ages throughout all experiments.

Measurement of neuronal swelling

Negatives were projected with a photographic enlarger and the outlines of cell bodies were traced with a pencil. The cross-sectional areas of the somata were measured from the drawings (Sigma Scan, Jandel Scientific, Corte Madera, CA) and values expressed as the mean percentage change as compared to control for each time point.

Data analysis

The mean (± S.E.M.) percentage survival of cells for experimental values was plotted against the concentrations of different compounds or time. Statistical comparisons were made using ANOVA with the StatView 512 program (BrainPower, Calabasas, CA), with posthoc statistical analysis using the Fisher PLSD.

RESULTS

General features of the cultures

Primary striatal cultures derived from newborn rats contained a variety of morphological types of neurons, similar to those described elsewhere in detail[31,42,51–54]. These cultures matured with age, and after 3–4 DIC, processes emanated from all neurons and continued to extend and elaborate with succeeding DIC (Figs. 1–3). A steady drop off in cell number occurred as the cultures aged, a feature characteristic of all primary neuronal cultures including striatal cultures[31]. In our study, no attempt was made to quantify this decrease.

Cells that exhibited cytoplasmic staining of Nissl substance with the Cresyl violet stain in bright field microscopy corresponded to the phase-positive bipolar and multipolar cells which possessed extensive processes (compare Fig. 1a and b). In contrast, the cytoplasm of the large flat cells which formed the support matrix of the cultures failed to stain with Cresyl violet. Immunohistochemical localization of neurofilament antigen further confirmed that the phase-positive cells with elaborated processes were neurons (Fig. 1c).

Characterization of toxicity

Neurons damaged by glutamate demonstrated initial somal swelling within 1 h after exposure and complete

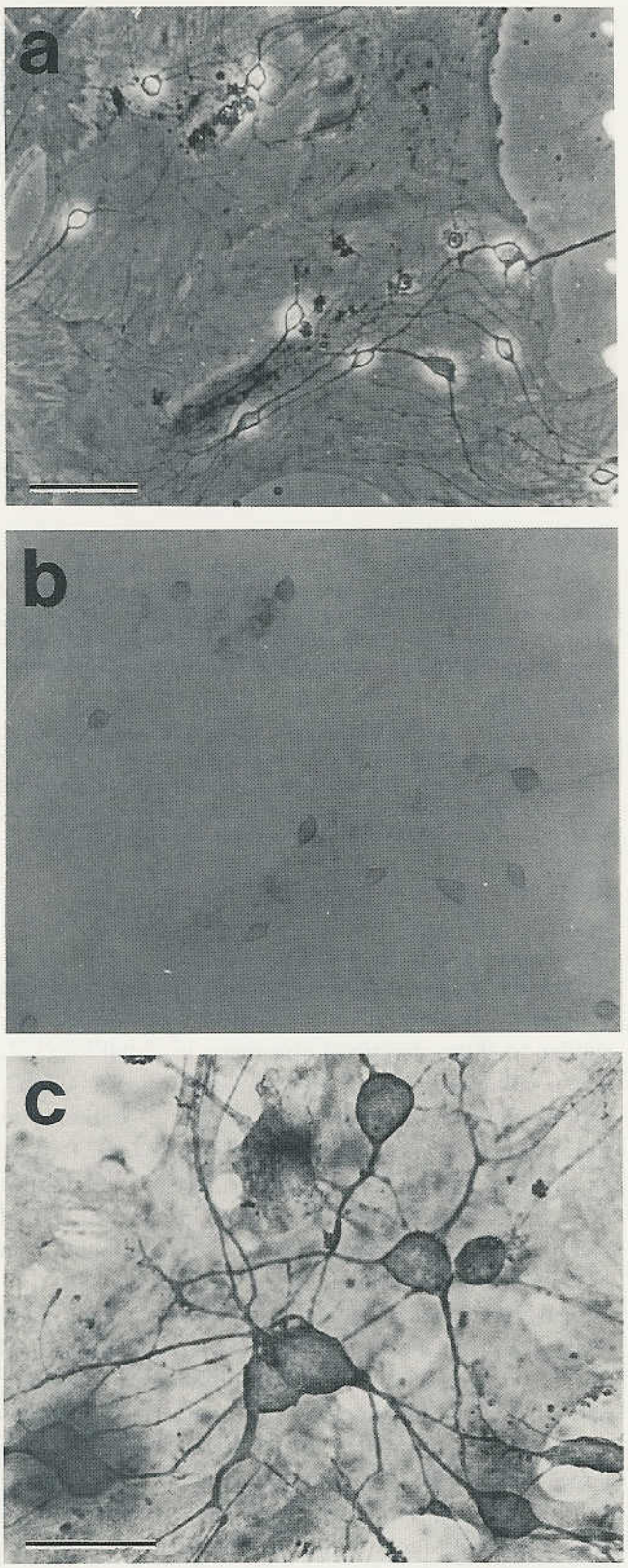

Fig. 1. a,b: Cresyl violet stained neurons shown in phase-contrast (a) and bright-field (b) microscopy. Only cells exhibiting long elaborated processes in phase optics show cytoplasmic Nissl stain in bright field. c: immunohistochemical localization of neurofilament antigen with DAB reaction product shown in bright field with 100× oil objective lens. Scale bar in a is 50 μm and also applies to b; scale bar in c is 20 μm.

disruption of neuritic processes and somal membrane integrity by 2–3 h (Figs. 2c,d and 3). Disappearance of neuritic processes was always preceded by their swelling and blebbing, which typically also occurred before the cell body demonstrated membrane invaginations and further degeneration. In most cases, after 3 h, all that remained of a destroyed neuron was a shrunken, irregularly shaped fraction of the cell body and occasional particulate blebs where the processes once existed (Figs. 2d and 7d). In contrast, neurons that survived glutamate exposure demonstrated intact cell bodies and processes, and patch-clamp analysis of these cells confirmed their viability (Koroshetz et al., companion paper)[35]. Fig. 3 shows the time course of neurotoxicity in 18-day-old cultures exposed to 1 mM glutamate. Using the morphological criteria for cell death outlined in the Materials and Methods section, significant cell loss ($\bar{x}$ = 72.9% cell survival ± 3.7; $P < 0.001$) was observed after 1 h glutamate exposure and was much greater after 3 h ($\bar{x}$ = 54.3% cell survical ± 3.9; $P < 0.001$). Neuronal loss increased slightly with additional hours of glutamate exposure (after 8 h, $\bar{x} = 45.9 \pm 4.9$), but because of cell migration relocating neurons after glutamate exposure was more accurate within shorter time intervals. Consequently, a maximum of 3 h of glutamate incubation was used for these toxicity experiments.

Somal swelling was examined in the same group of neurons before and after exposure to 1 mM glutamate (Fig. 4) in the presence of low concentrations of extracellular Ca^{2+} (see results below and Fig. 8 on the effects of Ca^{2+} and glutamate toxicity). Results showed that in 12-day-old cultures, neuronal cross sectional area increased significantly (n = 19 neurons; $\bar{x}$ = 160% of control ± 33; $P < 0.05$) after 1 h glutamate exposure and then returned to normal values after 2 h of glutamate treatment. These results suggest that neuronal swelling represented a relatively early response to glutamate exposure which could be reversed within 2 h.

Although exposure to glutamate (0.5–10 mM) produced neuronal toxicity in striatal cultures of all ages (6, 12 and 18 DIC), the degree of cell death was markedly influenced by the age of the cultures (Fig. 5). When exposed to 3 mM glutamate, 6-day-old cultures showed the least toxicity ($\bar{x}$ = 90.7% survival ± 3.1); striatal cells 12 DIC exhibited intermediate toxicity ($\bar{x}$ = 75.5% survival ± 3.6); and the oldest cultures examined (18 DIC) demonstrated maximal toxicity with a loss of about half of the neuronal population ($\bar{x}$ = 50.4%, survival ± 5.1). The ED_{50} for glutamate-induced neurotoxicity in the 18-day-old cultures was approximately 300 μM. Concentrations of glutamate greater than 3 mM did not significantly increase the loss of neurons in cultures of any age.

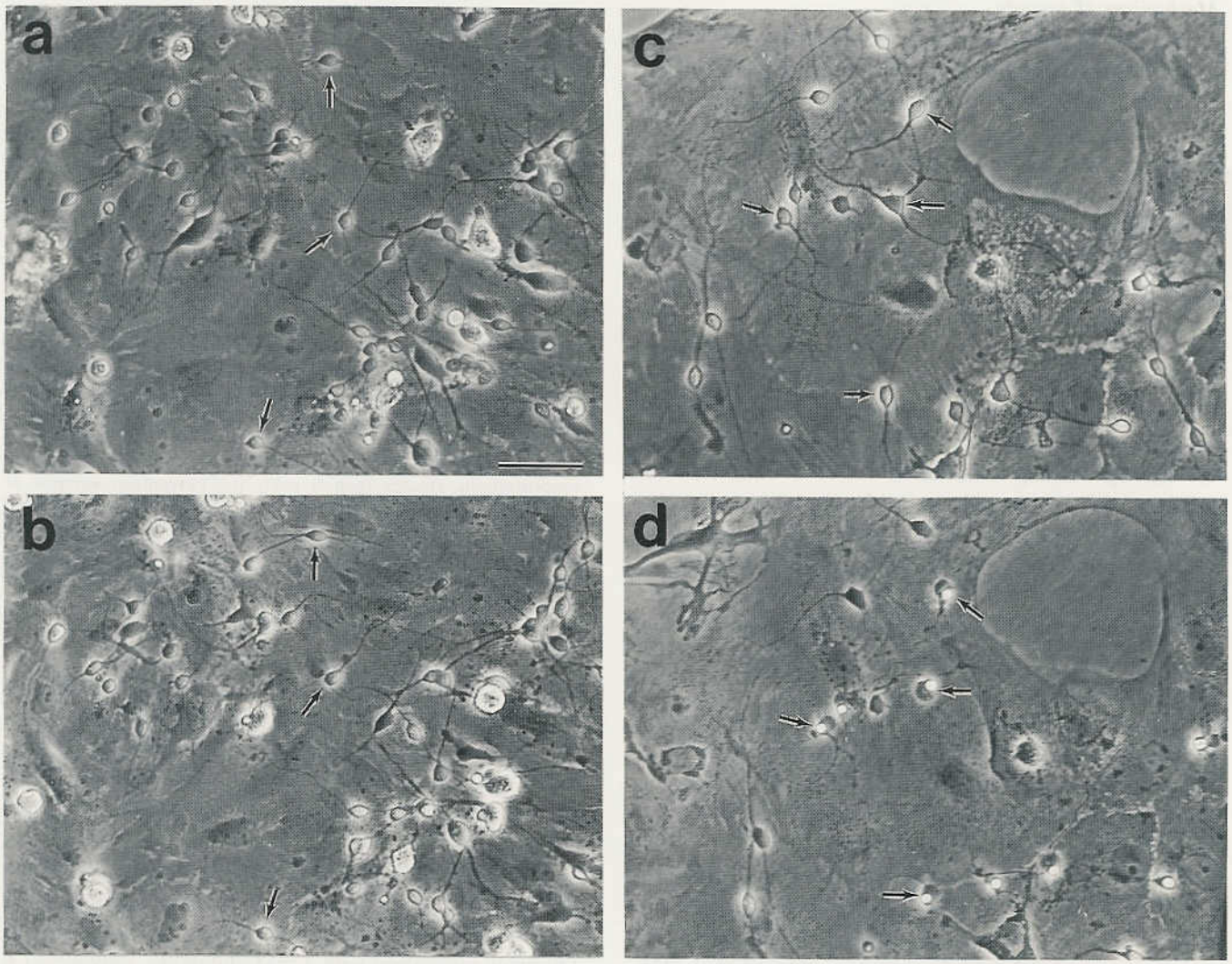

Fig. 2. Striatal cells at day 6 (a,b) and day 18 (c,d) in culture before (a,c) and after (b,d) exposure to 1 mM glutamate for 3 h. Arrows identify some of the cells examined before and after exposure to glutamate. Note the marked change in the cell bodies and disappearance of processes in the 18-day-old cells after exposure to glutamate (compare arrows in c before glutamate with arrows in d after glutamate); in contrast glutamate exposure results in little change in the 6-day-old cells (compare arrows in a and b). Scale bar in a is 50 μm and also applies to b–d.

To determine whether the apparent age-dependence of susceptibility of cultured cells to glutamate was due solely to time in culture, or could be meaningfully correlated to the ontogenetic age of the cells, another experiment was performed. Rat pups from the same litters were sacrificed either at PND 0 or 6, and cultures plated identically. Subsequently, the effects of glutamate were examined in cultures 12 DIC (from PND 6 pups) and in cultures 12 and 18 DIC (from PND 0 pups). Results showed that upon exposure to glutamate concentrations of 0.5–10 mM, the toxicity profile for cultures 12 DIC derived from PND 6 rats was virtually superimposeable upon the profile for the cultures 18 DIC from PND 0 rats (Fig. 6). These results suggest that the summation of the age of the striatal cells, in vivo plus in vitro, determined the age-dependence of glutamate toxicity, rather than the age in vitro alone.

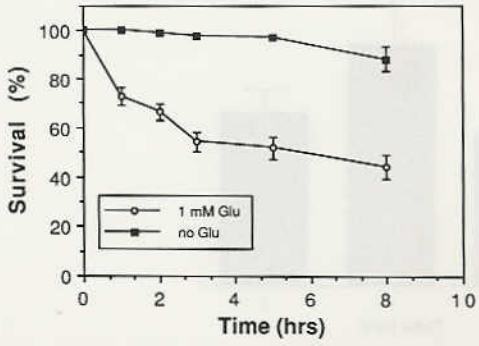

Fig. 3. Time course of glutamate toxicity: primary striatal cells 18 DIC in the presence (empty circles) and the absence (filled squares) of 1 mM glutamate. Each value represents the mean (± S.E.M.) of 12 dishes and 4 experiments. The same dishes were examined at each time point. Incubation media contained 1.8 mM $CaCl_2$. Beyond 0 time, each value in the presence of glutamate is significantly different (1-way ANOVA: $F_{5,66} = 16.2$, $P = 0.0001$, posthoc analysis at $P < 0.05$) from corresponding values in the absence of glutamate.

Mechanism of toxicity

Previous studies have shown that the *N*-methyl-D-aspartate (NMDA) glutamate receptor subtype activates a calcium-permeable cation channel which can be blocked by Mg^{2+} (ref. 48). In addition, reports have indicated that activation of the NMDA receptor is dependent on the presence of glycine[28,32] in the extracellular fluid. In the presence of 1 mM glutamate, striatal neurons 12 DIC demonstrated a significant toxicity to glutamate that was affected in a dose-dependent manner by increasing concentrations of Ca^{2+} in the incubation medium (Figs. 7 and 8); 82% (± 3) of the cells survived in the presence of 0.9 mM Ca^{2+}, and only 31% (± 2) of the neurons survived in the presence of 18 mM Ca^{2+}. In contrast, control cultures (without glutamate) were unaltered by varying extracellular Ca^{2+} concentrations between 0.45 mM and 18 mM. However, all cells disintegrated in the complete absence of extracellular Ca^{2+}, presumably due to the loss of the glial matrix. Of interest, addition of glycine (1 μM–1 mM) to the extracellular media had no effect on the neurotoxic action of 1 mM glutamate on striatal neurons 12 DIC (results not shown).

Glutamate-mediated toxicity could be largely blocked in a dose-dependent manner by the addition of Mg^{2+} to the incubation medium (Fig. 9). Only 17% (± 6) of neurons were destroyed in the presence of the highest concentration of Mg^{2+} tested (8 mM).

The specific NMDA agonist, quinolinic acid, was also shown to be toxic to striatal cultures (18 DIC), with an ED_{50} of approximately 700 μM (Fig. 10). Similar to the response seen to glutamate, neurons exposed to 1 mM quinolinate demonstrated a significant cell loss within 3 h

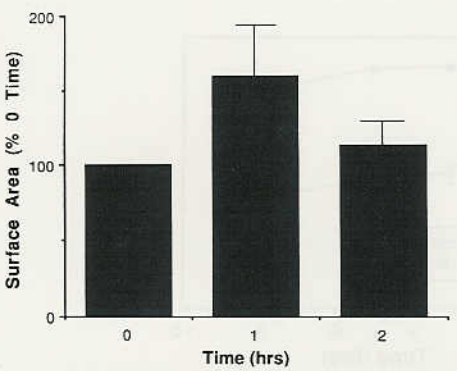

Fig. 4. Neuronal surface area changes during glutamate exposure: projections of neurons were traced and surface areas measured using Sigma Scan (Jandel Scientific, Corte Madera, CA). The surface area of 19 cells (18 DIC) was monitored before, and 1 h and 2 h after exposure to 1 mM glutamate in the presence of 0.9 mM extracellular Ca^{2+}. The abcissa indicates the mean percentage (± S.E.M.) of 0 time surface area for each cell. Following 1 h of glutamate exposure, cell surface area was significantly increased by 60% ($P < 0.05$, unpaired Student's *t*-test [two-tailed]).

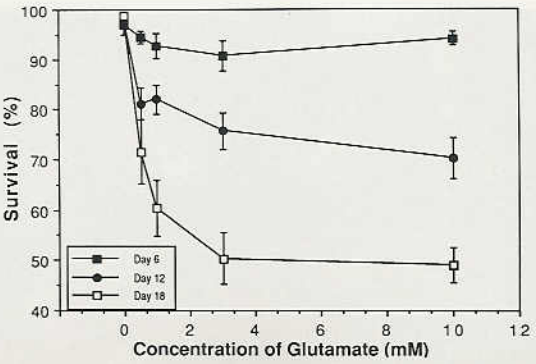

Fig. 5. Concentration and age dependence of glutamate toxicity: shown is the mean percentage survival (± S.E.M.) at 3 h of primary striatal neurons exposed to the indicated concentrations of glutamate in the incubation media, which contained 1.8 mM $CaCl_2$ for cultures at 6 DIC (filled squares) $n = 7$; 12 DIC (filled circles), $n = 9$; and 18 DIC (empty squares), $n = 9$. For glutamate concentrations greater than 0 mM, the percentage survival of neurons 12 DIC are significantly reduced from those for neurons 6 DIC (2-way ANOVA: $F_{4,30} = 5.8$, $P = 0.0001$; posthoc statistical analysis at $P < 0.05$), and for glutamate concentrations greater than 500 μM, the percentage survival of neurons 18 DIC are significantly different than those for neurons 12 DIC ($P < 0.05$).

($\bar{x} = 57.1\%$ survival ± 4.0; $P < 0.001$). Morphological sequelae were analogous to those seen in the glutamate-treated cultures; those cells which were destroyed demonstrated process degeneration and eventual somal disruption, although antecedent somal swelling was less conspicuous.

The NMDA receptor competitive antagonist, APV, blocked approximately one-half of glutamate-mediated toxicity in a dose-dependent manner (Fig. 11). Three h following incubation with 1 mM glutamate, only 49% (± 3) of neurons survived in the absence of APV, whereas

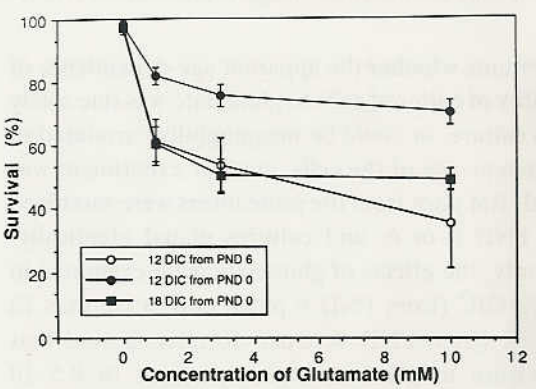

Fig. 6. Comparison between glutamate toxicity in cultures derived from PND 0 (Newborn) and PND 6 rats: cultures were incubated 12 DIC and 18 DIC in the presence of different concentrations of glutamate and 1.8 mM $CaCl_2$. Mean percentage survival (± S.E.M.) was monitored after 3 h for: cultures 12 DIC from PND 0 rats (filled circles, $n = 9$); cultures 18 DIC from PND 0 rats (filled squares, $n = 9$); and cultures 12 DIC from PND 6 rats (empty circles, $n = 4$). The percentage survival of cultures 12 DIC from PND 6 rats did not differ significantly from that of cultures 18 DIC from PND 0 rats.

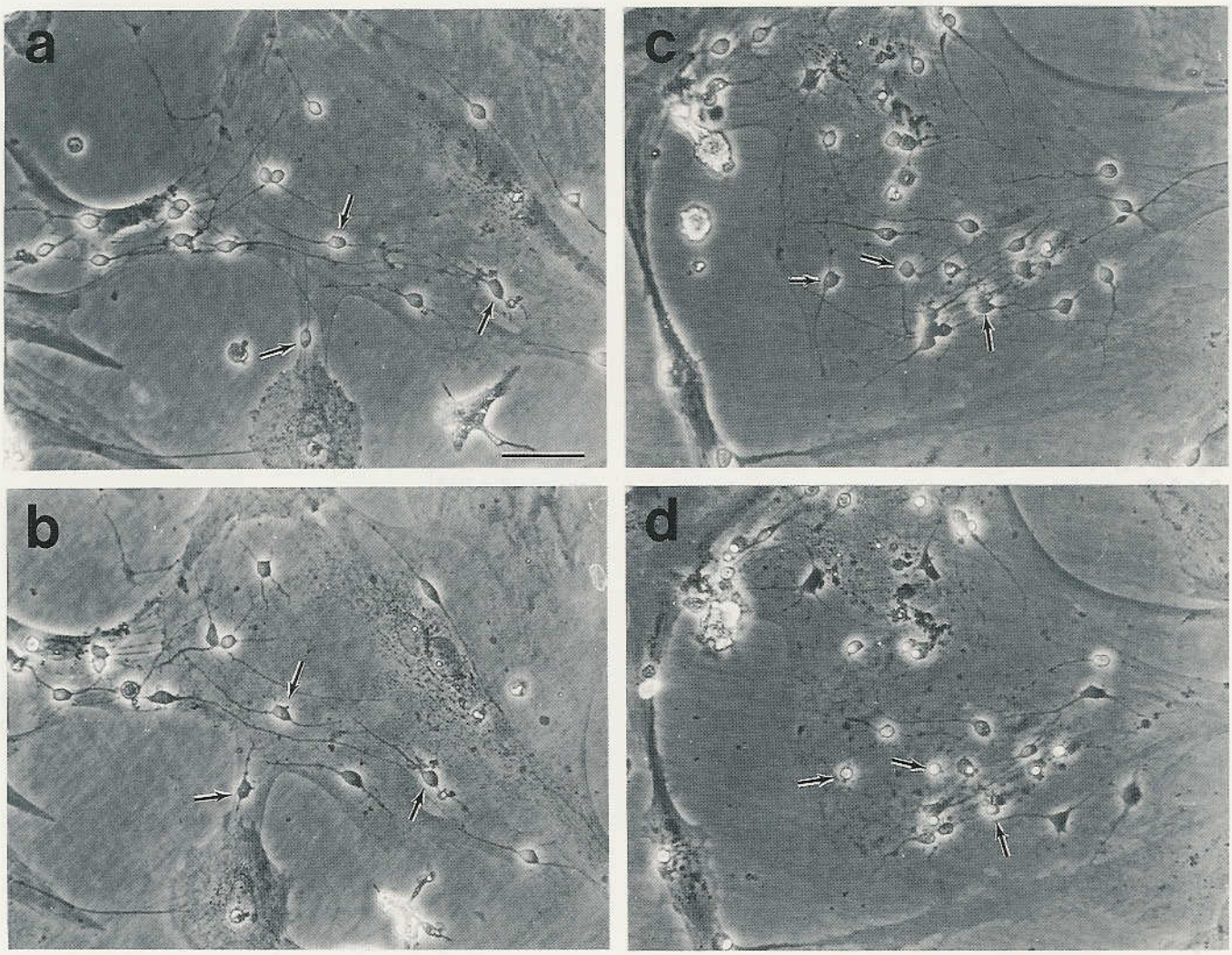

Fig. 7. Sister cultures of striatal neurons 12 DIC before (a,c) and after (b,d) exposure to 1 mM glutamate with low extracellular Ca^{2+} (0.9 mM) (a,b) and high extracellular Ca^{2+} (9 mM) (c,d) in the medium. Arrows at the same orientation in a and b and in c and d identify the same neurons. Scale in a is 50 μm and also applies to b–d. Note the marked changes in cell bodies and processes produced by exposing 12-day-old cells to high Ca^{2+} (compare arrows in c with those in d) whereas glutamate has relatively little influence when low Ca^{2+} is present in the media (compare a with b).

in the presence of 10 mM APV, 76% (± 7) of striatal neurons survived. Of note, the antagonist to all glutamate receptors, kynurenic acid (0.1–10 mM), effectively eliminated glutamate toxicity (results not shown).

The incomplete blocking effect of APV on glutamate-mediated toxicity, as well as the complete efficacy of kynurenic acid blockade, suggested that activation of non-NMDA receptors was also implicated in the toxicity of glutamate in striatal cultures. After 12 DIC, kainic acid was toxic in a dose-dependent yet time-independent manner (Fig. 12). Striatal cells either 12 or 18 DIC showed an equal susceptibility to kainate, with a maximal toxicity of 21 ± 6% after 3 h of exposure; cells 6 DIC were impervious to the presence of kainic acid. In turn, quisqualic acid (0.05–5 mM) had no toxic effect on striatal cultures 18 DIC (results not shown).

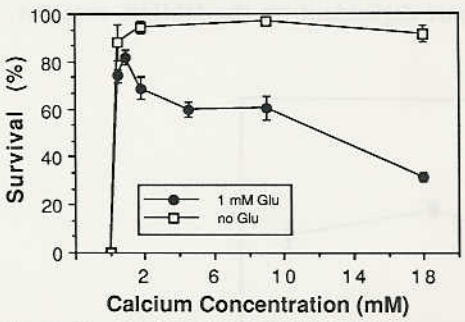

Fig. 8. The calcium dependence of glutamate toxicity: the survival of cultured primary striatal neurons 12 DIC was monitored in the presence of indicated concentrations of extracellular Ca^{2+}, and in the presence (filled circles) and absence (empty squares) of 3 mM glutamate. Values represent the mean (± S.E.M.) of 6 dishes and 3 experiments. In the absence of extracellular Ca^{2+}, all cellular elements (glial and neuronal) of the cultures disappeared. All values for Ca^{2+} concentrations greater than 0.9 mM are significantly different than that for 0.9 mM Ca^{2+} in the presence of glutamate and corresponding controls (1-way ANOVA: $F_{6,31} = 35.99$, $P < 0.001$; posthoc analysis $P < 0.05$).

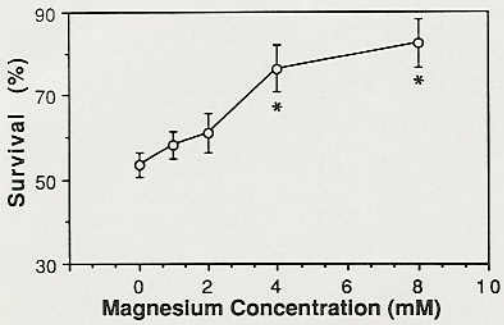

Fig. 9. The attenuation of glutamate toxicity by extracellular magnesium: toxicity of primary striatal cultures (18 DIC) at 3 h was determined in the presence of 1 mM glutamate and 1.8 mM $CaCl_2$ under differing extracellular Mg^{2+} concentrations. Values represent the mean (± S.E.M.) of 8 dishes and 4 experiments. Asterisks indicate those points that are significantly different from 0 mM Mg^{2+} (1-way ANOVA: $F_{4,39} = 8.39$, $P = 0.0001$; posthoc analysis $P < 0.05$).

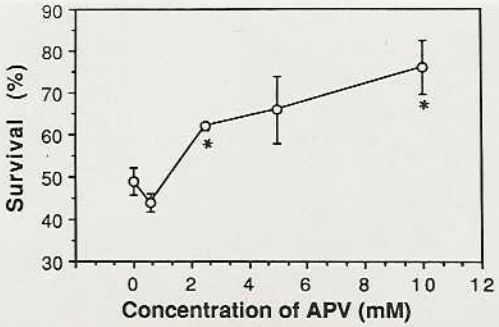

Fig. 11. Blockade of glutamate toxicity by the NMDA antagonist, APV: the survival of cultured cells (18 DIC) at 3 h was determined in the presence of 1 mM glutamate and 1.8 mM $CaCl_2$ with different concentrations of APV in the incubation media. Each value represents the mean (± S.E.M.) of 6 dishes and 3 experiments. Asterisks indicate those values which are significantly different from 0 mM APV (1-way ANOVA: $F_{4,23} = 12.77$, $P < 0.001$, posthoc analysis $P < 0.05$).

DISCUSSION

The application of tissue culture methods to studies on the nervous system has a number of advantages over in vivo methods. Included among these advantages is the ability to follow over time either a single cell or a given population of cells under defined extracellular conditions. We have attempted to take advantage of this asset of tissue culture in this series of experiments aimed at characterizing the toxicity of glutamate in primary striatal cultures derived from newborn rats. The results of this study show that glutamate is more toxic in a dose-dependent manner to older striatal cultures than to younger cultures. The toxicity is dependent on the extracellular Ca^{2+} concentration, can be blocked by increasing extracellular Mg^{2+} and APV, and can be induced by quinolinic acid – results consistent with a mechanism in part dependent on the NMDA receptor. An additional component of glutamate-mediated toxicity appeared to be mediated by non-NMDA receptors.

As with our findings, the concentration dependence of glutamate toxicity has been observed in primary cultures derived from cortex[7,8]. The latter studies showed that exposure of older cortical cultures, 15–24 days in vitro, to 500 μM glutamate for only 5 min was sufficient to produce widespread neural damage when cultures were examined 24 h later. These results contrast with our findings in striatal cells where significant cell loss was observed only after exposure to 500 μM glutamate for several hours. Although the findings from the two studies must be compared cautiously, due to differences in methods and in the assessment of toxicity, it appears that striatal cells in vitro are less susceptible to glutamate than cortical neurons. This is further supported by the work of Choi and coworkers who have used significantly higher concentrations or longer exposures of NMDA agonists to

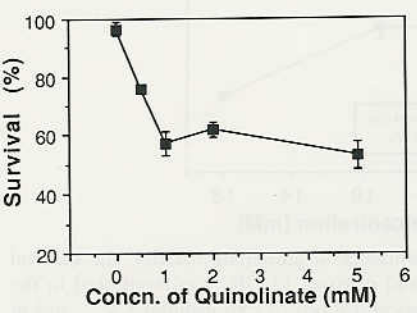

Fig. 10. Quinolinic acid toxicity dose dependence: the survival of cultures (18 DIC) at 3 h was monitored in the presence of 1.8 mM $CaCl_2$ and differing concentrations of quinolinic acid in the incubation media. Each value represents the mean (± S.E.M.) of 7 dishes and 3 experiments. The percentage survival of cultures exposed to concentrations greater than 0 mM quinolinic acid were significantly reduced from that at 0 mM quinolinic acid (1-way ANOVA: $F_{3,24} = 31.38$, $P = 0.0001$; posthoc analysis $P < 0.05$).

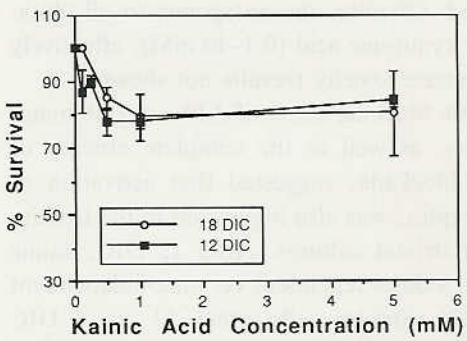

Fig. 12. The toxicity of kainic acid in striatal cultures, 12 and 18 DIC. The survival of cultured neurons [12 DIC (filled squares) and 18 DIC (empty circles)] at 3 h was determined in the presence of differing concentrations of kainic acid (0, 0.1, 0.25, 0.5, 1.0 and 5.0 mM) and 1.8 mM $CaCl_2$. Each value represents the mean (± S.E.M.) of at least 4 dishes and 2 experiments.

produce toxicity in striatal cultures[34] than in cortical cultures[7,8]. Unlike the striatum, where glutamate originates from extrinsic afferents, the cortex contains an intrinsic glutamate neuronal population[12] which may in culture favor the development of more glutamate receptors and thus a greater susceptibility to glutamate[31]. In addition, several studies have shown concentrations of NMDA receptors to be 2–3-fold higher in cerebral cortex than in striatum[27,38,43]. Recent studies[44] have suggested that the NMDA receptor in striatum is functionally distinct from that in neocortex, the former relatively independent of extracellular glycine concentration and the latter dependent on glycine. Our finding that NMDA-receptor-mediated toxicity in striatal cultures is independent of exogenously added glycine is consistent with this view, as is the preliminary finding in our laboratory (unpublished observations) that the in vivo toxicity of quinolinic acid in the striatum is also not glycine sensitive. However, since neuronal-glial cultures may release glycine into the extracellular media, this finding must be interpreted with caution.

It is of interest that neurotoxic concentrations of glutamate in vitro are significantly less than the millimolar concentrations of glutamate found normally in many areas of the mammalian brain[69]. Moreover, it has been shown that the direct injection of at least a 50 mM concentration of glutamate into the corpus striatum is necessary to achieve neurotoxicity[40]. It therefore seems likely that normal protective mechanisms in the brain which avert receptor mediated glutamate toxicity are either partly or completely inoperative in vitro. These include the absence in vitro of a normal glutamate uptake mechanism as observed in cortical cultures[8]. The observation of glutamate toxicity in striatal cultures in the absence of glutamatergic inputs suggests that the expression of glutamate receptors can occur independent of their afferent inputs, and raises the question whether receptor expression may influence normal glutamatergic synapse formation.

As shown in this study for primary striatal cultures, an age dependence for susceptibility to glutamate neurotoxicity has also been observed in cortical cultures[7,8]. The greater vulnerability of older striatal cultures may be due in part to an increase with age in the elaboration of neuritic processes where, based on in vivo anatomical studies[30], cortical inputs, which contain glutamate[18], are predominantly localized. In addition, electrophysiological studies suggest that glutamate receptors are concentrated on these processes[67]. Our morphological observations in vitro suggest that process degeneration precedes and may contribute to somal destruction and neuronal loss following glutamate exposure. Similarly, in vivo, within hours following injection of quinolinic acid into the striatum, marked changes in dendrites and spines occur prior to alterations in neuronal somata at the ultrastructural level (ref. 59; see also Roberts and DiFiglia, M., unpublished observations). Thus, the marked abnormalities in dendrites and spines of medium spiny neurons in Golgi impregnations of HD caudate nucleus[24] may be the result of an abnormality in glutamate receptor activation.

Similar to findings in cortical and hippocampal cultures[7,8,49,56–58], two distinct but overlapping phases in the neurotoxic response to glutamate are seen in striatal cells in vitro. The first phase of response to toxic levels of glutamate is reversible, occurs within 1 h and results in somal swelling even in the presence of low extracellular Ca^{2+} concentrations. Somal swelling in response to glutamate exposure is thought to result from the rapid influx of Na^+ and Cl^- ions[8,23,58], but the degree to which it contributes to cell death in culture is still unclear. The other phase of response to glutamate is marked by deterioration of neuritic processes and delayed disintegration of the cell body and, as discussed below, is at least in part dependent on activation of the NMDA receptor.

Our results suggest that glutamate-induced neurotoxicity in primary striatal cultures is in part mediated by the NMDA receptor which permits the influx of Ca^{2+} into the cell, ultimately causing its demise[7,19,20,23,36,37,39,45]. Our findings that toxicity was exacerbated by increasing extracellular Ca^{2+} concentrations and could be blocked by raising levels of Mg^{2+} in the incubation medium are consistent with the view that toxicity is partially mediated by the NMDA receptor. In addition, the NMDA agonist, quinolinic acid, was toxic to cultures in a dose-dependent manner, and the specific NMDA receptor antagonist, APV, could block much of the toxicity of glutamate in a concentration-dependent manner. Recently, it has been shown that NMDA receptor agonists do in fact induce intracellular accumulation of Ca^{2+} in primary murine striatal cultures[45]. The precise mechanism by which intracellular accumulation of Ca^{2+} can then contribute to cell death remains poorly understood; however, it is known that a variety of proteases and second messenger systems are modulated by intracellular Ca^{2+} levels[19–21,39,58,64,68]. For example, it has been shown that the intracellular uptake of Ca^{2+} which accompanies glutamate receptor stimulation can induce phosphatidylinositol turnover in neurons, liberating inositol phosphates and resulting in the translocation and activation of protein kinase C[46,47,63,73].

Physiological studies also support the view that glutamate induced toxicity may be partially mediated by the NMDA receptor. Patch-clamp analysis of striatal cultures in our laboratory (Koroshetz, W.J. et al., companion paper)[35] shows an age-dependent appearance in elec-

trophysiological responses to NMDA that in part parallels the apparent ontogeny of glutamate susceptibility; less than one-third of cells 6–7 DIC had detected electrophysiological responses to 10 μM NMDA, whereas over two-thirds of the cells greater than 8 DIC had detected responses to 10 μM NMDA. Analogous developmental changes in glutamate receptor-mediated electrophysiological responses have been observed in cerebellar- and hippocampal-derived tissue preparations[13, 22,25,29].

In our striatal cultures not all cells were susceptible to either glutamate or quinolinate-induced toxicity; in fact almost half of all cells 18 DIC survived short-term exposure to these neurotoxins. Recent studies of primary striatal cultures have shown that neurons exhibiting NADPH-diaphorase and acetylcholinesterase are among those neurons resistant to long-term exposure to quinolinic acid[34]. These in vitro results are strikingly similar to the neuropathological and biochemical data obtained in the striatum of Huntington's disease postmortem brain[1, 4,15,16,41]. Thus the striatal culture system may prove to be a useful model for understanding the neurodegeneration which occurs within the striatum of HD victims. In conclusion, we have shown that glutamate is a potent neurotoxin in mature primary striatal cultures, and that the toxicity of glutamate is to a large degree mediated by the NMDA receptor, with an additional component mediated by non-NMDA receptors. In addition to demonstrating the potential neurotoxicity of glutamate, this series of studies was directed towards developing a tissue culture model for the neuronal degeneration observed in Huntington's disease. Ongoing studies aimed at further characterizing the mechanism of toxicity in these cultures and developing manipulations to avert this toxicity may lead to advances in the understanding of the pathogenesis and treatment of Huntington's disease.

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