Vulnerability of Oligodendroglia to Glutamate: Pharmacology, Mechanisms, and Prevention.

グルタミン酸によるオリゴデンドログリアの障害に関する研究
―その薬理学的解析および未熟児における脳室周囲軟化症の予防の可能性

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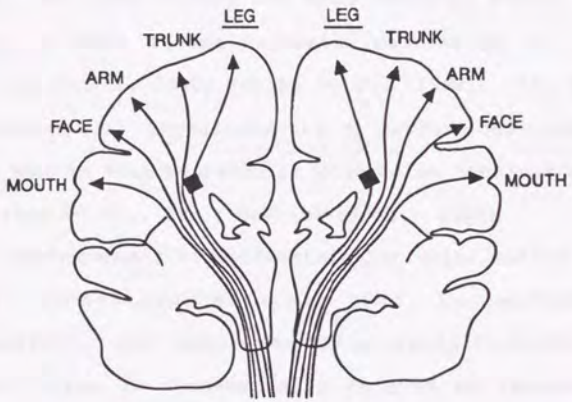
Introduction

Brain injury in premature infants and particularly the prevention of that injury is an enormous problem. With modern neonatal intensive care, most of these infants survive, and of the survivors approximately 5-15% exhibit major spastic motor deficit, i.e. cerebral palsy, and an additional 25-50% exhibit cognitive and attentional deficits that result in school failure (Volpe 1994). The principal spastic motor deficit, i.e., the type of cerebral palsy present in these infants, is so-called spastic diplegia, a type of spastic quadriplegia in which the lower extremities are affected much more than are the upper extremities. The neuropathology for the constellation of spastic diplegia and cognitive deficits in premature infants involves injury to the periventricular white matter, most commonly termed periventricular leukomalacia (PVL). Since the diagnostic efficiency of brain ultrasonography in detecting these white matter lesions has been established during the last decade, the high incidence of PVL among premature infants has come to be recognized. Because the incidence of intracranial hemorrhage, the other typical brain injury in premature infants, has declined remarkably due to the development of refined and less invasive technology to care for critically ill babies, PVL is currently cosidered to be the principal cause of motor deficit (Volpe 1994; Fujimoto 1994). In the more mature brain, however, white matter injury is less prominent and is overshadowed by injury to the gray matter, particularly in the cortex and hippocampus.

PVL is characterized by focal necrosis of periventricular white matter dorsal and lateral to the external angles of the lateral ventricles (DeVries et al., 1988; Gilles et al., 1983). The peculiar periventricular distribution of lesions tends to involve efferent motor axons to the lower extremities, which are located in the deep white matter adjacent to the lateral ventricles, rather than axons to the upper extremities, resulting in spastic diplegia as a typical clinical manifestation (see Figure below).



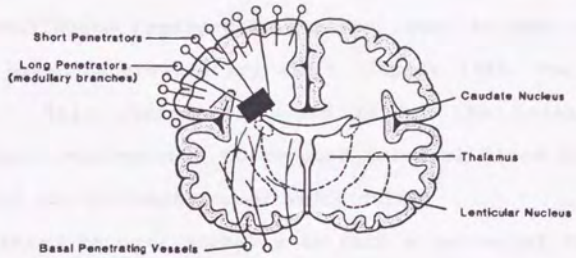
(Figure modified from Volpe 1994: Schematic diasgram of the corticospinal tracts from their origin in the motor cortex.)

The microscopic features emphasized in the original report of Banker and Larroche (Banker and Larroche, 1962) include, as the earliest features, coagulation necrosis, i.e., the appearance of an infarction, including disruption of axis cylinders. The early work, which focused on the

injury to glial cells characteristic of this lesion, showed that the injury was conspicuously absent in well-myelinated regions of the cerebrum and also in regions not expected to myelinate until later in the first year of life (Gilles and Murphy, 1969; Leviton and Gilles, 1971; Leviton and Gilles, 1973; Leviton and Gilles, 1974; Leviton et al., 1976; Leviton and Gilles, 1984). Myelinating white matter and presumably the glial cells involved in the process, i.e., oligodendroglia, appear to be particularly vulnerable. Moreover, the glial injury has been shown to extend beyond the foci of overt tissue necrosis (Dambska et al., 1989; Leviton and Paneth, 1990; Paneth et al., 1990). The chronic neuropathological appearance is a marked deficiency of cerebral myelin with ventricular dilatation (DeReuck et al., 1972; Gilles et al., 1983; DeVries et al., 1988).

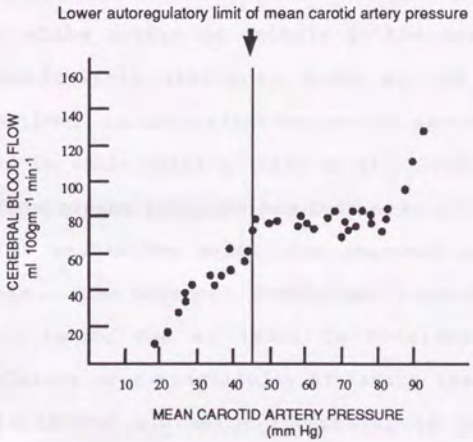
The pathogenesis of periventricular white matter injury is not understood entirely and is undoubtedly multifactorial. The most commonly accepted formulation is that the lesion is fundamentally related to ischemia and involes at least three principal factors (Volpe, 1989; Leviton and Paneth, 1990; Volpe, 1994).

The first of these is the presence of vascular border zones and end zones in the periventricular white matter (Rorke 1992, see Figure below; Volpe 1994). Until the development of anastomoses among penetrating vasculatures after 32 weeks of gestation, these periventricular zones would be most vulnerable to hypotension and reduced cerebral blood flow.



(Figure modified from Rorke. 1992: Diagrammatic representation of cerebral vascular supply; PVL indicated as square-shaped area.)

The second factor of importance is a pressure-passive cerebral circulation in premature infants, particularly in those who are severely ill (Volpe, 1989; Volpe 1994).



(Figure modified from Papile et al. 1985: Autoregulation of cccerebral blood flow in the preterm lamb.)

The autoregulation range of cerebral blood flow against the systemic pressure in the preterm lamb is narrower, and the normal blood pressure in preterm lambs is very near or at the lower autoregulatory limit (Papile 1985, see Figure above). This phenomenon would render the infant with hypotension susceptible to reduced cerebral blood flow and injury to the periventricular white matter .

A third factor, probably in fact a series of factors, must relate to the intrinsic vulnerability of glial cells of oligodendroglial lineage in the periventricular white matter. Presumably because of their developmental stage of active differentiation and myelination, they are particularly likely to be injured, perhaps by a variety of different insults, leading to the neuropathological features of PVL, such as the marked deficiency of myelination. Indeed, experimental data show that hypoxic-ischemic lesions in the white matter of animals in the developmental stage are particularly likely to occur at the sites of active myelination, in contradistinction to ischemic white matter lesions in adult animals (Rice et al., 1983).

Significant progress has been made in understanding the cellular mechanisms underlying neuronal death in hypoxia-ischemia. The neuronal death that occurs in this setting appears to be due at least in considerable part to an accumulation of extracellular glutamate leading to excessive stimulation of glutamate receptors, in particular the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors (Choi and Rothman, 1990; Meldrum and Garthwaite, 1990). Little is known, however, about the specific cellular

mechanisms underlying the death of oligodendroglia in the setting of hypoxia-ischemia in the developing brain. The particular importance of glutamate in periventricular white matter injury is postulated on the basis of the neuropathology of PVL, which includes axonal disruption among the earliest neuropathological features (Banker and Larroche, 1962), the fact that glutamate is present in millimolar concentration in cerebral tissue (Battistin et al., 1971; Fonnum, 1984; Beneveniste, 1991), and the presumption that local release of glutamate from injured axons into the periventricular white matter would result in a relatively high local glutamate concentration.

Thus it is clear that periventricular white matter injury in premature infants is highly prevalent, and leads to cerebral palsy in a large absolute number of those affected. This injury to developing white matter presumably involves oligodendroglial cells. The pathogenesis of periventricular white matter injury is unclear, although an important role for ischemia seems likely. An early event in overt PVL is axonal disruption, perhaps leading to an elevation of extracellular glutamate. A particular vulnerability of developing oligodendroglial cells to a variety of insults is also likely, as suggested by the lack of myelination.

The background hypothesis of this work is, therefore, that ischemia and perhaps another to-be-defined insults, such as extracellular glutamate generated locally from ischemic axonal injury, lead to oligodendroglial cell death, operating simultaneously or in sequence.

In this research, the vulnerability of oligodendroglia to glutamate is addressed. The developmental aspects of primary culture of oligodendroglia, isolated from mixed glial primary cultures derived from newborn rat brain, are characterized immunohistochemically and biochemically. Thereafter, oligodendroglia in culture are employed to assess the hypothesis, and it is shown that oligodendroglia from the developing rat brain are very sensitive to glutamate toxicity. The glutamate-induced oligodendroglial death is not caused by stimulating ionotropic glutamate receptors as occurs in neurotoxicity of glutamate. The prevention of oligodendroglial toxicity by the inhibition of glutamate uptake indicates the central importance of the uptake system of this amino acid. Further observations show that the toxicity is mediated by glutathione depletion, which is caused by the glutamate-cystine exchange system, leading to the accumulation of oxidative stress and cellular disintegration.

Materials and Methods

(1) Cultures of Oligodendroglia and Astrocytes. Mixed glial primary cultures were prepared by dissociation of brain obtained from 1-day-old Sprague-Dawley rats by a modification of an earlier method (Booher and Sensenbrenner, 1972; Hertz et al., 1982; Hertz et al., 1985) as previously described (Langan and Volpe, 1986; Ishii and Volpe, 1992). Cells were maintained in Dulbecco's modified Eagle's medium (DME)/Ham's F-12 (1:1), with 1.2 gm/liter (14mM) of $NaHCO_3$, 15mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid

(HEPES), and 10% fetal bovine serum, in 75 cm^2 flasks in an atomosphere of 95% air and 5% CO_2 at 37°C. After 7 d in culture, the flasks were shaken for 1 hr on an orbital shaker (37°C, 150 rpm) to remove macrophages. The medium was discarded and replaced with 9 ml of fresh medium. The flasks were then again shaken for 15-18 hours on an orbital shaker (37°C, 210 rpm). During the shaking process, phase-dark cells, which are principally oligodendroglia-type2-astrocytes (O2A) progenitors (McCarthy and de Vellis, 1980; Bologa-Sandru et al., 1981; de Vellis et al., 1983; Eccleston and Silberberg, 1984), were detached from the bed layer of the phase-light, type 1 astrocytes (Mounen et al., 1975; Manthorpe et al., 1979; Raff et al., 1979; McCarthy and de Vellis, 1980; Sensenbrenner et al., 1980; Giulian and Baker, 1986).

The cell suspensions of the phase-dark cells were combined, sedimented by centrifugation (1500rpm for 10 min), and suspended in a chemically defined medium (CDM) consisting of DME, $NaHCO_3$ (3.7gm/liter, 44mM), 15mM HEPES, selenium (5µg/liter), biotin (2.4µg/liter), triiodothyronine (10µg/l), hydrocortisone (3.6µg/liter), bovine serum albumin (fraction V) (1gm/liter), bovine insulin (5mg/liter), pyruvate (110mg/liter, 1mM), and human transferrin (iron-poor) (50mg/liter). The CDM was supplemented with 10 % fetal bovine serum and astrocyte extract (10 µg protein/ml) (Ishii and Volpe, 1992). The suspension was passed through a nylon mesh of 10 µm pore diameter to remove any contamination by astrocytes, and placed in an uncoated 75-cm^2 flask for 10 min to remove any contamination by

microglia (Giulian and Baker, 1986). Microglia adhere to the flask surface during this interval. The unattached cells were then seeded onto poly-D-lysine coated 35 mm culture dishes and 24-well plates (well diameter, 16 mm) at a seeding density of 2.7×10^4 cells/cm^2. For immunocytochemical staining, cells were seeded onto poly-D-lysine coated coverslips placed into 35 mm culture dishes. For autoradiography, cells were seeded onto poly-D-lysine coated culture slides. After the cells had attached the surface, the medium was replaced with fresh medium in which the fetal bovine serum had been replaced with boiled fetal bovine serum (Ishii and Volpe, 1992).

For astrocyte cell culture, cells of the bed layer of the mixed glial cultures remaining in the flasks after overnight shaking (95% glial fibrillary acid protein (GFAP) positive) were incubated with a salt solution consisting of 140mM NaCl, 3.5mM Tris HCl and 0.5mM Tris base (pH 7.5) supplemented with 0.6mM EDTA for 30 minutes on an orbital shaker (150 rpm) at 37°C. Cell suspensions were centrifuged; resusupended in DME/F-12 (1:1), with 1.2gm/liter $NaHCO_3$, 15mM HEPES, 10% fetal bovine serum; passed through a nylon mesh of 37 µm pore diameter and grown in poly-D-lysine coated 35 mm culture dishes at a seeding density of 2.7×10^4 cells/cm^2.

(2) Immunocytochemical Staining. For staining of galactocerebroside(GC) or A2B5, the procedures were essentially as previously described (Bansal and Pfeiffer, 1989; Gard and Pfeiffer, 1990). Briefly, nonspecific binding was blocked with 3% goat serum in PBS for 20 min.

Cells were then labeled with monoclonal antibody (mAb) O1 (anti-GC) (1:20) or A2B5 antibody, as crude hybridoma culture medium (1:5) for 20 min, followed by FITC-conjugated goat anti-mouse IgM (1:50) for 20 min. All washes between steps were carried out on ice three times for 5 min each in PBS with 1% goat serum. The entire procedure was done on ice to prevent internalization of antigen-antibody complex. For staining of GFAP, cells were fixed in cold 4% paraformaldehyde in PBS for 20 min, permeabilized with 0.5% Triton X-100, and labeled with mouse monoclonal anti-GFAP (1:100) for 30 min at room temperature, followed by FITC-conjugated goat anti-mouse IgG (1:25) for 30 min at room temperature. Cells were visualized with interference filters used for fluorescein detection.

(3) Determination of 2',3'-Cyclic nucleotide 3'-phosphohydrolase (CNPase) activity. CNPase activity was measured by a modification of the method of Prohaska et al. (1973) as previously described (Prohaska et al., 1973; Maltese and Volpe, 1979).

(4) Quantitation of viable cells. For the studies of oligodendroglial toxicity, the medium was aspirated and replaced with Earle's balanced salt solution (EBSS) (containing 5.5mM glucose). After additions to the dishes and incubations for the indicated times, oligodendroglial viability was determined using a modification of the technique of Persidsky and Baillie (1977) (Persidsky and Baillie, 1977). Cells were incubated with fluorescein diacetate (FDA) (60 mg/ml) for 5 min. The medium was then removed by aspiration and replaced with fresh EBSS. Cells

were observed immediately, and fluorescein-labeled cells were identified by fluorescence microscopy. Cell counts were made in five adjacent fields at 200x magnification. It should be noted that there was no evidence of loss of viability in control cells maintained for as long as 48 hours in EBSS when compared with those in complete growth medium. Astrocytes, as determined by GFAP staining and by their distinct morphology, were excluded readily from cell counts of oligodendroglia.

(5) L-^{3}H-glutamate Uptake. Glutamate uptake was determined in cells grown on 24-well plates. After the medium was aspirated from each dish, the adherent cells were washed once with 1 ml of EBSS and given 0.3 ml fresh EBSS containing 1.5-15.0 μCi of L-^{3}H-glutamate (49.0 Ci/mmol) and the indicated amounts of unlabeled L-glutamate. The cells were incubated for the indicated times at 37°C. After removal of medium, the cells were washed three times with ice-cold PBS, and then lysed with 0.1*N* NaOH. Protein concentration was determined by the method of Bradford (1976) (Bradford, 1976). The lysate was neutralized with HCl and radioactivity was determined by liquid scintillation spectroscopy. Counting efficiency was approximately 30%. In experiments investigating the ion dependency of transport, EBSS was replaced with Tris-buffered saline (TBS) consisting of 120mM Na^{+}, 5.4mM K^{+}, 130.6mM Cl^{-}, 1.8mM Ca^{2+}, 0.8mM Mg^{2+}, 16mM glucose, and 25mM Tris base. Sodium and chloride were replaced in isomolar amounts by *N*-methylglucamine and sulfate, respectively.

(6) L-^{3}H-glutamate Autoradiography. Autoradiography was performed using a modification of the method of Murphy et al. (1990) (Murphy et al., 1990). After the medium was aspirated from each culture slide, the adherent cells were washed once with 2 ml EBSS and given 1.5 ml fresh EBSS containing L-^{3}H-glutamate (1.7 mCi/ml; final glutamate concentration, 33nM). The cells were incubated for 5 min, washed three times in ice-cold PBS, fixed in 2.5% gluteraldehyde (0.2 M phosphate buffer, pH 7.0) for 30 min at room temperature, washed once with distilled water, air dried, dipped in Kodak NTB2 photographic emulsion, exposed for 12-14 days, and then developed with Kodak Dektol developer.

(7) Glutathione measurement. Glutathione concentration was determined by a modification of the method described by Murphy et al (1989) (Murphy et al., 1990). Cells were grown on 35mm culture dishes. The medium was aspirated and the cells were washed once with EBSS. Two milliliters of 3% perchloric acid were added to each dish and allowed to incubate for 15 min on ice. The supernatant solution was transferred to a test tube containing 0.6 ml of 3M potassium bicarbonate. The cells, still attached to the culture dishes, were harvested for protein determination as described below. For determination of glutathione, 0.3 ml of the supernatant solution was placed into a 1 ml cuvette with the following: 0.1 ml of 5,5'-dithio-bis [2-nitrobenzoic acid] (DTNB), 0.1 ml of NADPH (1.7 mg/ml), 0.1 ml of glutathione reductase (GR) (10.5 U/ml), and 0.4 ml of 0.1M phosphate buffer with 5mM EGTA (pH 7.5). The reduction

of DTNB was determined spectrophotometrically at the wavelength of 412 µm. Measurements were compared to glutathione standards treated identically. The protein was extracted by sonication in 2 ml of 20mM Tris buffer, 1% SDS (pH 7.4). Protein was determined by the method of Lowry (1951) (Lowry et al., 1951).

(8) Visualization of intracellular oxygen radicals. The formation of hydrogen peroxide was observed with 2'7'-dichloroflurescin diacetate (DCFH-DA) by a modification of the method of Murphy et al. (1989). Cells exposed with 0 or 2 mM glutamate were incubated with 10 µM DCFH-DA for 5 min at 37°C, washed three times with PBS, and viewed by a fluorescence microscope with fluorescein filters. DCFH-DA readily diffuses into cells, and is hydrolyzed to 2'7'-dichlorofluorescin, and thereby trapped within the cells. The intracellular 2'7'-dichlorofluorescin, a nonfluorescent fluorescein analogue, is oxidized to highly fluorescent 2'7'-dichlorofluorescein by hydrogen peroxide (Bass et al., 1983). The numbers of stained and total cells were counted in 8 visual fields in each well. Cells which generated superoxide were visualized with nitroblue tetrazolium (NBT) by the modified method of Sáez et al. (1987) (Saez et al., 1987). Cells exposed to 0 or 2 mM glutamate were incubated with 0.5 mM NBT in EBSS for 30 minutes at 37°C. In the presence of superoxide, insoluble blue formazan is produced.

(9) Efflux of [^{35}S] radioactivity. Cells grown in 35mm dishes were washed once with EBSS and incubated in EBSS containing 1.5 µCi of L-^{35}S-cystine (405.2 Ci/mmol) for 60 min. After washing three times in EBSS with 0.6% BSA, cells

were incubated in 1.2 ml of EBSS with 0 or 2 mM L-glutamate for the indicated times at 37°C on a rotary shaker (25 rpm). After incubation, media were collected, and cells were washed once and lysed in 0.1*N* NaOH. Radioactivity in media and cells was determined by liquid scintillation spectroscopy, and aliquots were immediately frozen at -80°C for chromatography. Counting efficiency was approximately 90%. The percentage of efflux of [^{35}S] radioactivity was defined as follows:

% efflux of [^{35}S] radioactivity=[^{35}S in medium]x100/([^{35}S in cells]+[^{35}S in medium])

(10) Chromatographic analysis of efflux of [^{35}S] radioactivity. Thin-layer chromatographic analysis was performed by a modification of States and Sagal (1969) (States and Segal, 1969). A 30 ml aliquot of medium derived from the efflux experiments described above was combined with 1.5 µg of cystine, 1.5 µg of cysteine, 1 µg of oxidized glutathione, 4 µg of reduced glutathiione, and 3 µg of *N*-methylmaleimide and analyzed by thin-layer chromatography on cellulose, using an isopropanol: methyl ethyl ketone: 1*N* HCl (3:2:3) solvent system. After detection by ninhydrin, spots were cut out and radioactivity determined by liquid scintillation spectroscopy.

(11) Materials. Tissue culture media (DME, DME/F-12) and HEPES were purchased from GIBCO; Earle's balanced salt solution, D,L-*threo*-β-hydroxyaspartic acid (BHA), L-glutamate, D-glutamate, L-aspartate, D-aspartate, L-cystine, L-cysteine, NMDA, kainic acid, CDM constituents, gluteraldehyde, GFAP antibody, FITC-conjugated goat anti-

mouse IgG, vitamin E, fluorescein diacetate (FDA), and NBT from Sigma; FITC-conjugated goat anti-mouse IgG and IgM, and Texas-Red conjugated horse anti-mouse IgG from Vector Laboratories; A2B5 hybridoma clone from American Type Culture Collection, fetal bovine serum from Hyclone; quisqualate and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) from Cambridge Research; MK-801 from Research Biochemicals Inc.; DCFH-DA from Molecular Probes; L-^{3}H glutamate and L-^{35}S-cystine from New England Nuclear; cellulose chromatography sheets and photographic materials from Kodak; nylon mesh from Small Parts; 35 mm culture dishes, 24 well plate, and 75-cm^2 culture flasks from Falcon; culture slides from Nunc; and Sprague-Dawley rats from Charles River. Idebenone was kindly donated by Takeda Chemical Co., and anti-GC antibody by Dr. Stephan Pfeiffer, University of Connecticut. All other chemicals were purchased from standard sources.

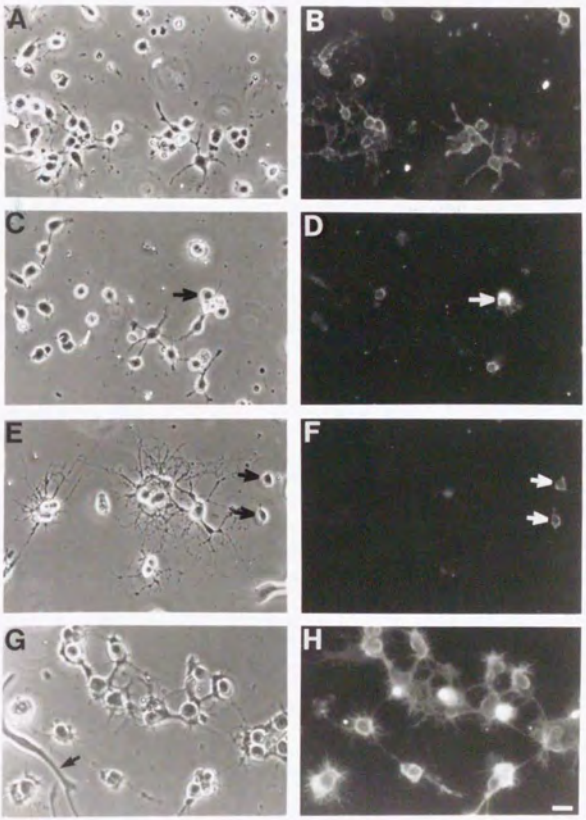
Results

(1) Immunohistochemical and biochemical characterization of oligodendroglial cultures. The immunohistochemical and biochemical characteristics of the cells were obtained by the newly modified procedure for culturing oligodendroglia (see Materials and Method). One day after plating, the culture consisted of 86% (±4% SD, *n*=8) A2B5-positive cells, presumed O2A progenitors, 7% (±3% SD, *n*=7) GC-positive oligodendroglia, and 4% GFAP-positive astrocytes (Fig. 1). On the third day, the cultures contained 81% (±4% SD, *n*=11) GC-positive oligodendroglia

(Fig. 1), with only 10% A2B5-positive cells. Concurrently, the activity of CNPase, which was negligible one day after plating, reached the mature level on the third day (Fig. 2).

Cells expressing GC on the surface displayed relatively small round soma and complex branched processes, whereas A2B5-positive presumed progenitors were small, phase-dark cells with one or two short processes but lacking the complex branched processes. These morphologies were entirely consistent with previously reported observations (McMorris and Dubois Dalcq, 1988; Gard and Pfeiffer, 1990; Ishii and Volpe, 1992). Astrocytes, as determined by GFAP staining, accounted for 4% of the cells on the third day after plating. These cells were flat and polygonal shaped, sometimes with long single processes. Astrocytes were easily distinguishable morphologically from oligodendroglia and progenitors. Moreover, oligodendroglia were easily distinguishable from presumed progenitors. All of the experiments described below employed oligodendroglia after 4-5 d of subculture.

Figure 1.(next page) Oligodendroglial cultures. Cells separated from mixed glial primary cultures derived from rat cerebral hemispheres (see Methods) were maintained in chemically defined medium supplemented with boiled fetal bovine serum and astrocyte extract for 1 day (A-D) or 4 days (E-H). Cells were labeled with A2B5 antibody (B, F) or mAb O1 (anti-GC antibody; D, H) followed by FITC-conjugated second antibody (see Materials and Methods). *A to D,* The phase contrast views of 1-day old cultures (A, C) showed cells that were small and phase-dark, lacking the complex network of processes. In corresponding views, most cells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were A2B5-positive (B) and GC-negative (D), indicative of presumed O2A progenitors. Less than 10% of cells were GC-positive oligodendroglia (arrow in C and D). *E to H,* On the 4th day after separation, the phase contrast views showed cells with finely branched processes (E, G). At this stage, most cells expressed GC on the surface (H), indicative of differentiated oligodendroglia. A cell with astrocytic morphology (arrow in G) was not stained with GC. Only a small number of A2B5-positive cells were seen at this stage (arrows in E and F). Scale bar: H, 20 μm for A-H.

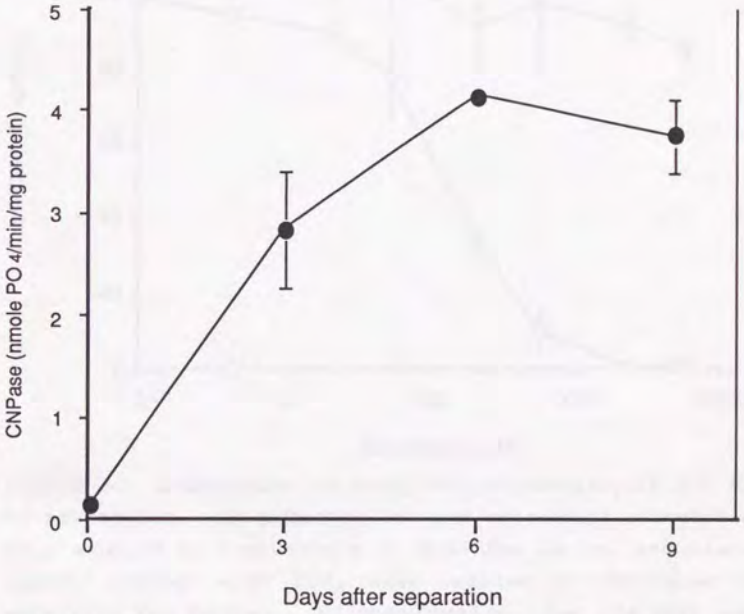

Figure 2. The development of CNPase activity in oligodendroglia *in vitro.* CNPase activity in oligodendroglia of different ages was determined as described in Materials and Methods. Values represent the mean ± SEM in three experiments performed in triplicate or quadruplicate.

(2) Toxicity of glutamate to oligodendroglia and to astrocytes. To evaluate the possibility that glutamate might be involved in the mediation of oligodendroglial cell death, whether glutamate exhibited toxicity in a purified preparation of oligodendroglia was determined.

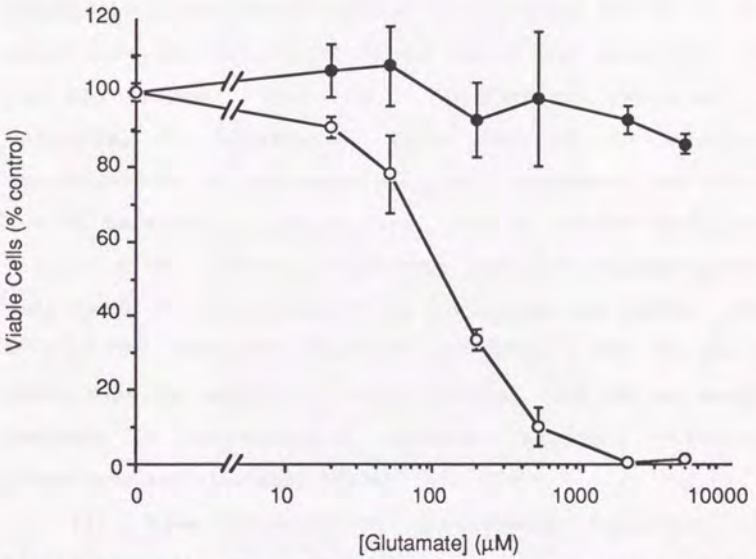

Figure 3. L-Glutamate is toxic to oligodendroglia but not to astrocytes. Oligodendroglial and astroglial subcultures were exposed to L-glutamate in EBSS for 24 hr, and viable cells, stained with FDA, were counted as described in Materials and Methods. Oligodendroglia (*open circles*) were highly vulnerable to L-glutamate (EC_{50}; 132 μM). No significant toxicity was observed in astrocytes (*closed circles*) in concentrations up to 5 mM of L-glutamate. Data are presented as the percentage of viable cells compared to control cultures (incubated in EBSS without L-glutamate). The number of viable oligodendroglia and astrocytes in control cultures was 332 and 153, respectively. Values represent the mean ± SD in an experiment performed in

triplicate. Similar results were obtained in three other experiments.

It was found that cultures of purified oligodendroglia were destroyed almost entirely by a 24 hr exposure to 2 mM glutamate. To determine the concentration dependence of the toxic effect of glutamate on oligodendroglia, cells were exposed to graded concentrations of glutamate for 24 hr, at which time the numbers of viable cells were determined by the FDA method. (Fig. 3). In parallel experiments, cultures of astrocytes were exposed to similar concentrations of glutamate (Fig. 3). Glutamate was found not to be toxic to astrocyters, even at concentration as high as 5 mM. However, glutamate led to oligodendroglial cell death at concentrations in the micromolar range. The EC_{50} in the experiment depicted in Figure 3 was 132 μM, a value that is similar to that observed with rat or mouse neurons in conventional cerebral cortical cultures (Rosenberg and Aizenman, 1989).

(3) Time course of glutamate toxicity to oligodendroglia. Next, the temporal characteristics of the toxic effects of glutamate on oligodendroglia (Fig. 4,5) were studied.

Control cultures incubated in EBSS with no L-glutamate for 24 hr were undamaged (Fig. 4A). After 12 hr, approximately 50% of cells exhibited membrane disintegration (Fig. 4B) and loss of viability by the FDA method (Fig. 5). By 24 hr, essentially all cells had disintegrated (Fig. 4C) and lost viability.

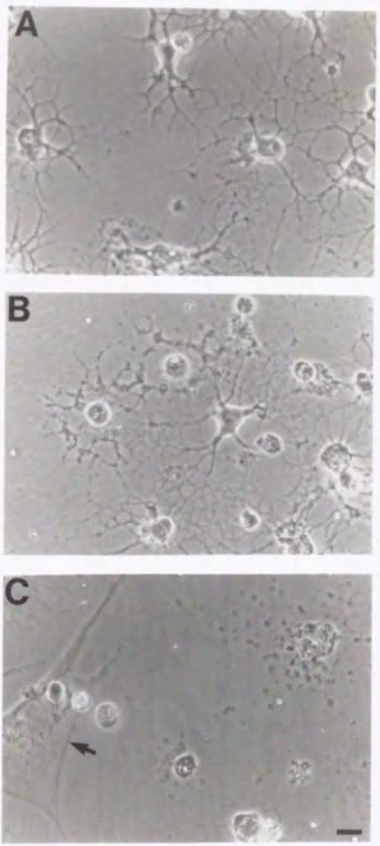
A
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Figure 4.(previous page) Phase contrast micrographs of oligodendroglia exposed to L-glutamate. Oligodendroglia were grown for 4 days after separation. The medium was aspirated and replaced with EBSS. Control cultures maintained for 24 hr (A) showed no morphological changes. In cultures exposed to 2 mM L-glutamate in EBSS for 12 hr (B), some cells had already undergone lysis, while others showed granular or swollen somata and processes marked with surface blebs. After 24 hr of exposure (C), almost all oligodendroglia had been killed. An astrocyte (arrow) still retained intact morphology. Scale bar: C, 10 μm for A-C.

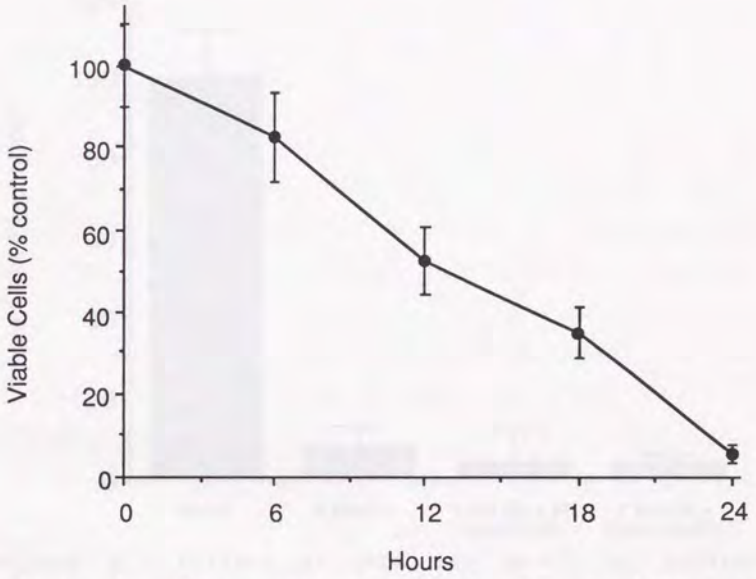


Figure 5 Time course of L-glutamate toxicity in oligodendroglia. Oligodendroglia from sister cultures were incubated with 2 mM L-glutamate in EBSS for the indicated time. Viable cells were counted (see Materials and Methods). Data are presented as the percentage of viable cells compared to control cultures (451 cells). Values represent the mean ± SD in an experiment performed in

quadruplicate. Similar results were obtained in two other experiments.

(4) Pharmacology of glutamate toxicity to oligodendroglia. Since the toxicity of glutamate to cultured cerebral neurons appears primarily because of activation of excitatory amino acid receptors (Choi, 1990), whether similar receptor-mediated mechanisms might underlie glutamate toxicity to oligodendroglia was investigated.

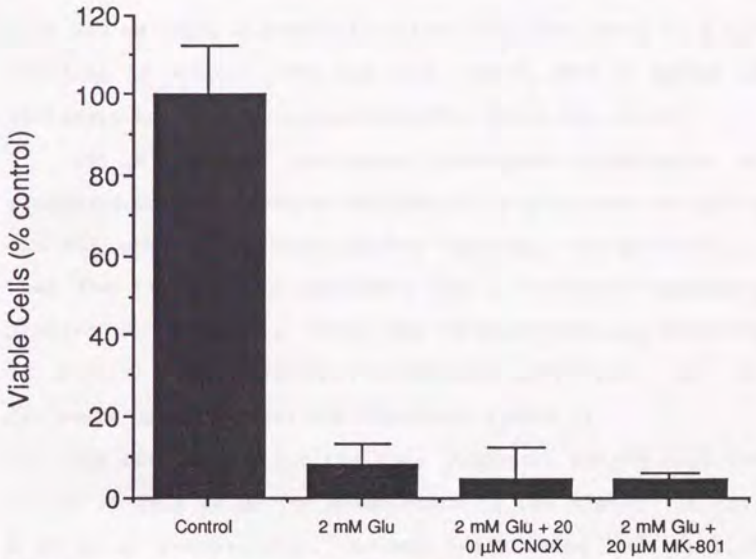


Figure 6. Failure of CNQX and MK-801 to protect oligodendroglia from L-glutamate toxicity. Oligodendroglia were incubated with 2 mM L-glutamate in EBSS in the presence of 200 µM CNQX or 20 µM MK-801 for 24 hr, and viable cells counted. Data are presented as the percentage of viable cells compared to control cultures (691 cells). CNQX and MK-801 were not able to block L-glutamate toxicity in oligodendroglia (no significant difference compared to cultures with 2 mM glutamate by ANOVA). Values represent

the mean ± SD in an experiment performed in triplicate. Similar results were observed in two other experiments.

It was found that the toxicity of 2 mM glutamate could not be blocked by 20 μM MK-801, an uncompetitive antagonist of the NMDA receptor (Wong et al., 1986; Huettner and Bean, 1988) (Fig. 6). CNQX, a competitive antagonist of non-NMDA receptors (Honore et al., 1988; Watkins et al., 1990), had no effect on glutamate toxicity to oligodendroglia up to concentrations of 200 μM (Fig. 6). Similarly, 20 mM MK-801 plus 200 mM CNQX, a combination that has been shown to block swelling in neurons (Koh and Choi, 1991), had no effect on glutamate toxicity to oligodendroglia (data not shown).

(5) Effect of glutamate transport substrates on oligodendroglia. Because activation of glutamate receptors did not seem to be required for toxicity, the possibility that the toxicity of glutamate was a transport-dependent process was examined. Thus, the oligodendroglial toxicity of several compounds with different affinities for the glutamate uptake system was evaluated (Table 1).

The EC_{50} values for the four compounds ranged from the lowest of 24 ± 12 μM for D-aspartate to the highest of 1107 ± 34 μM of L-aspartate. L- and D-glutamate had similar values for the EC_{50}, that is, 197 ± 29 μM and 174 ± 86 μM, respectively. L-Glutamate, L-aspartate, and D-aspartate in astrocytes and neurons are all transported by a sodium-dependent high-affinity transport system. However, the toxicity of D-glutamate suggests the involvement of a low

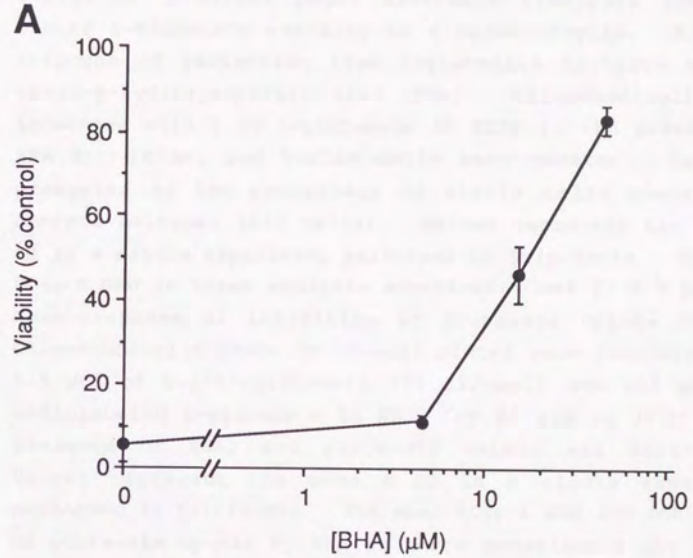
affinity transport system, recognized in a variety of neuronal and non-neuronal cells.

Table 1. The comparison of potency of glutamate transport substrates in oligodendroglia. Oligodendroglia were incubated with glutamate transport substrates in EBSS for 24 hr, and viability was quantified as described in Materials and Methods. Values represent the mean EC50 ± SEM of 4 individual experiments performed in triplicate.

Transport Substrate	EC50 (μM)
L-glutamate	197 ± 29
D-glutamate	174 ± 86
L-aspartate	1107 ± 34
D-aspartate	24 ± 12

The possibility that the cytotoxic effect of glutamate on oligodendroglia is caused by a nonspecific mechanism related to uptake of amino acids in general was excluded by the demonstration that lysine and glycine had no cytotoxic effect in concentrations of 5 mM after exposure for 24 hr (data not shown).

(6) Effect of inhibition of glutamate uptake on oligodendroglial toxicity. Because the data described above suggested that the oligodendroglial toxicity of glutamate required transport of glutamate into the cell, the effect of blockade of this transport with D,L-*threo*-β-hydroxyaspartic acid (BHA) was evaluated.


A
Viability (% control)
100
80
60
40
20
0
0
1
10
100
[BHA] (μM)

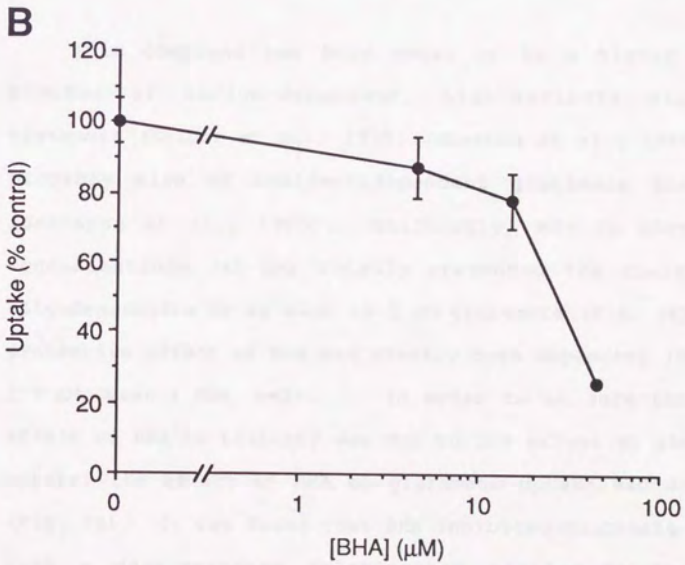

B
Uptake (% control)
120
100
80
60
40
20
0
0
1
10
100
[BHA] (μM)

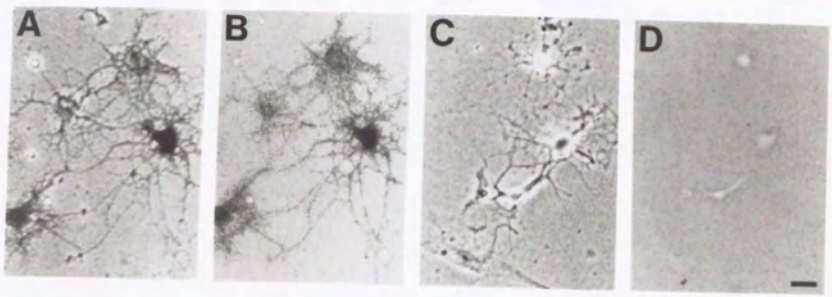
Figure 7. (previous page) Glutamate transport inhibitor blocks L-glutamate toxicity in oligodendroglia. *A*, Dose-response of protection from L-glutamate toxicity by D,L-threo-β-hydroxyaspartic acid (BHA). Oligodendroglia were incubated with 2 mM L-glutamate in EBSS in the presence of BHA for 24 hr, and viable cells were counted. Data are presented as the percentage of viable cells compared to control cultures (416 cells). Values represent the mean ± SD in a single experiment performed in triplicate. The mean EC_{50} ± SEM in three separate experiments was 20 ± 3 μM. *B*, Dose-response of inhibition of glutamate uptake by BHA. Oligodendroglia grown on 24-well plates were incubated with 1.5 μCi of L-[^{3}H]-glutamate (49 Ci/mmol) and 200 μM non-radiolabeled L-glutamate in EBSS for 20 min at 37°C in the presence of BHA, and glutamate uptake was determined. Values represent the mean ± SD in a single experiment performed in triplicate. The mean EC_{50} ± SEM for inhibition of glutamate uptake by BHA in three experiments was 18 ± 3 μM.

This compound has been shown to be a highly potent blocker of sodium-dependent, high-affinity glutamate transport (Balcar et al., 1977; Johnston et al., 1980), and probably also of sodium-independent glutamate transport (Johnston et al., 1980). Strikingly, BHA in micromolar concentrations (45 μM) totally prevented the toxicity to oligodendroglia by as much as 2 mM glutamate (Fig. 7A). The protective effect of BHA was clearly dose dependent (EC_{50}= 22 ± 3 μM, mean ± SEM, *n*=3). In order to be sure that this effect of BHA on toxicity was due to its effect on glutamate uptake, the effect of BHA on glutamate uptake was examined (Fig. 7B). It was found that BHA inhibited glutamate uptake with a dose-response relationship similar to that for inhibition of glutamate toxicity.

To ensure that BHA was inhibiting uptake into oligodendroglia and not just into the few contaminating astrocytes in cultures, uptake of ^{3}H-glutamate by autoradiography was studied (Fig. 8). Cell bodies and processes of control oligodendroglia were labeled clearly, but when incubated with BHA (45 µM), uptake was inhibited almost completely.

Figure 8.(next page) Glutamate uptake in oligodendroglia and the inhibition by BHA. L-[^{3}H]-glutamate autoradiography (33 nM L-[^{3}H]-glutamate for 5 min) was performed in oligodendroglia grown on culture slides as described in Materials and Methods. In control cells (A; phase contrast, B; bright field), processes as well as cell bodies of oligodendroglia were clearly labeled. In the presence of 45 µM BHA (C; phase contrast, D; bright field), uptake was inhibited. Scale bar: D, 20 µm for A-D.

(7) Relationship of cystine to the toxicity of glutamate to oligodendroglia. Coyle and colleagues have demonstrated a mechanism of glutamate-induced death in neurons that appears to be caused by glutamate inhibition of cystine uptake and that can be prevented by exogenous cystine (Murphy et al., 1989; Murphy et al., 1990). Because the phenomenon described above takes place in a cystine-free medium, itcannot be the result of blockade of cystine transport into the cells. However, since a transport system shared by glutamate and cystine that can act as an exchanger has been demonstrated in a number of extraneural cell types as well as in neurons (Bannai, 1986; Kesseler et al., 1987; Zaczek et al., 1987), it seemed possible that exogenous glutamate might be exchanging with endogenous cystine.


A
B
C
D

Therefore, to evaluate the possibility that the oligodendroglial toxicity of glutamate might be related to a disturbance of intracellular cystine levels, an attempt was made to prevent glutamate toxicity to oligodendroglia by providing the cells with exogenous cystine (Fig. 9).

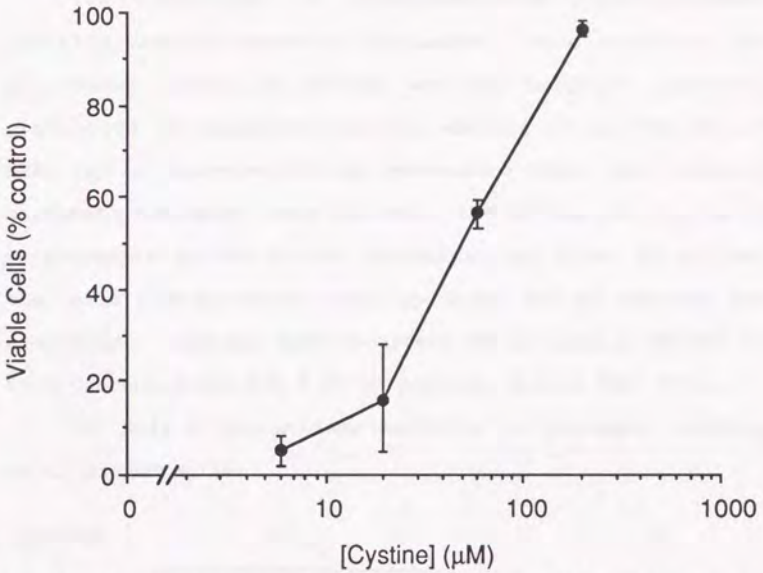


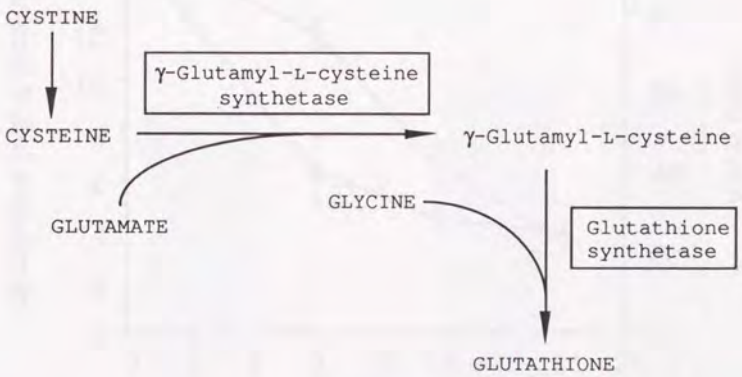
Figure 9. Dose-response of protection from L-glutamate toxicity by cystine in oligodendroglia. Oligodendroglia were incubated with 2 mM L-glutamate in EBSS for 24 hr in the presence of cystine, and viable cells were counted. Data are presented as the percentage of viable cells compared to control cultures (604 cells). Values represent the mean ± SD in an experiment performed in triplicate. The mean EC_{50} ± SEM in three experiments was 52 ± 9 μM.

As described in Materials and Methods, no cystine is present in the medium used for the toxicity studies; in the absence of glutamate, this absence of cystine has no toxic

effect, as judged by the FDA method. Cystine (200 μM) was shown to protect oligodendroglia fully from the toxicity of glutamate present in as high as a 2 mM concentration, and 50% protection required only approximately 50 μM cystine (EC_{50}= 52 ± 9 μM, mean ± SEM, n=3) (Fig. 9).

(8) Protection of oligodendroglia from glutamate toxicity despite uptake of glutamate. To ensure that the protective effect of cystine was not mediated simply by prevention of glutamate uptake, similar to the action of BHA, and to determine whether protection could occur despite glutamate transport into the cell, the effect of cystine on ^{3}H-glutamate uptake in the concentrations shown to protect the cells from glutamate toxicity (i.e., 200 μM cystine) was evaluated. Cystine had no effect on glutamate uptake at this concentration (95 ± 5% of control, mean ± SEM, *n*=3).

(9) Role of glutathione depletion in glutamate toxicity to oligodendroglia.



Scheme 1

The above data indicate that (1) glutamate transport into the cell is critical for the oligodendroglial toxicity of this amino acid, (2) glutamate toxicity can be blocked by addition of cystine, and (3) the cystine protection occurs despite cellular uptake of glutamate. Cystine is the essential substrate for the synthesis of γ-glutamyl-L-cysteine, the first part of the intracellular synthesis of glutathione as shown in Scheme 1. The intracellular pool of cystine or cysteine seems to be actually small, and it has been shown that deficiency of glutathione due to cystine depletion can lead to cell death in other systems (Murphy et al., 1989; Murphy et al., 1990; Cho and Bannai, 1990). The effect of exogenous glutamate on intracellular glutathione levels was therefore investigated.

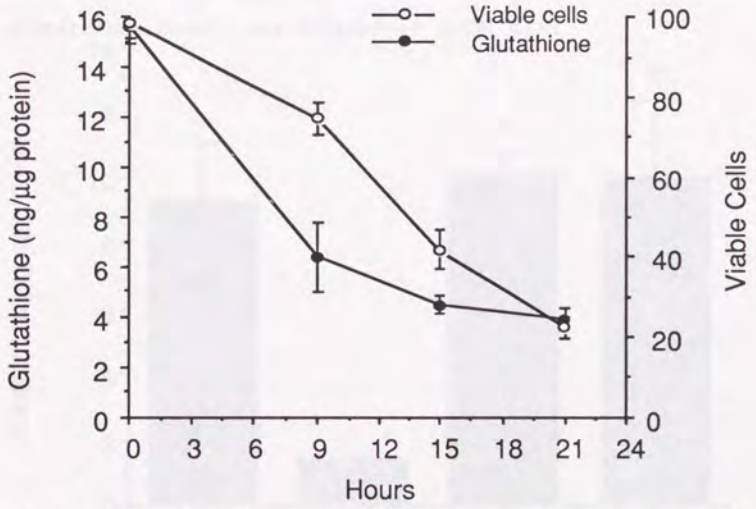


Figure 10. Glutathione levels in oligodendroglia decline prior to loss of viability in the presence of L-glutamate.

Oligodendroglia from the same sister cultures were incubated with 2 mM L-glutamate in EBSS for the indicated times, and glutathione levels and the number of viable cells were determined (see Materials and Methods). Data are presented as the percentage of viable cells compared to control cultures (361 cells). Values represent the mean ± SD in an experiment performed in triplicate. Similar results were obtained in two other experiments.

The data show a striking decrease of glutathione levels after glutamate exposure, and the greatest decrease occurred before oligodendroglial death (Fig. 10). In parallel experiments, D-glutamate led to a decrease in glutathione levels similar to that shown in Figure 10 for L-glutamate (data not shown).

To determine whether the cystine protection of oligodendroglia from glutamate toxicity is due to prevention of depletion of glutathione, the effect of cystine on glutathione levels was determined (Fig. 11).

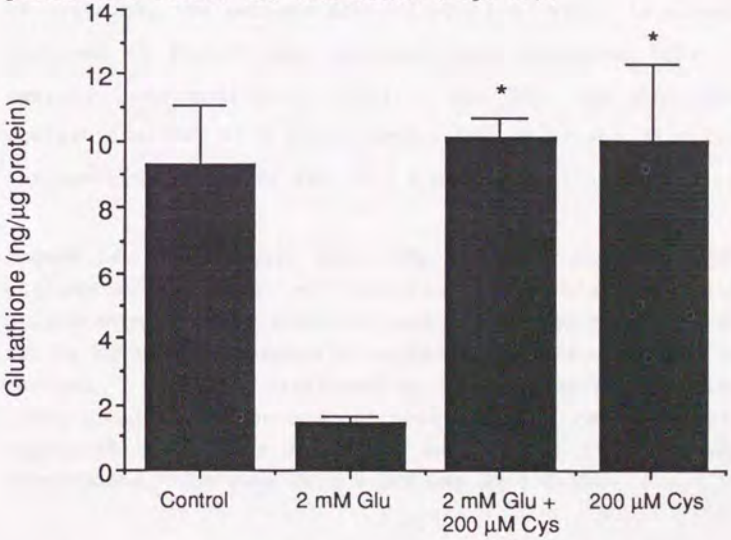
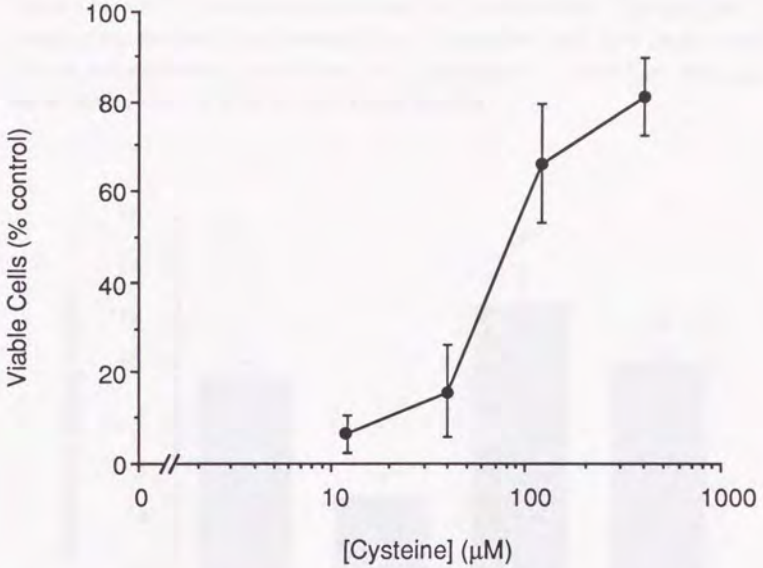


Figure 11. (previous page) Prevention of glutathione depletion by cystine. Oligodendroglia were incubated with 2 mM L-glutamate for 21 hr in the presence or absence of 200 µM cystine in EBSS, and intracellular glutathione was determined (see Materials and Methods). Cystine (200µM) sufficient to block glutamate toxicity (Figure 9) prevented glutathione depletion due to glutamate (* $p<0.001$, compared to cells with 2 mM glutamate and no cystine by two-tailed *t* test with Bonferroni correction). Values are the mean ± SD in an experiment performed in triplicate. Similar results were obtained in two other experiments.

Indeed, there was nearly complete prevention of glutathione depletion at a concentration of cystine (200 µM) that totally prevented the oligodendroglial toxicity of glutamate.

In further support of the conclusion that the protective effect of cystine was related causally to its prevention of glutathione depletion, the protective effect of cysteine, the reduced form of cystine, which is directly involved in glutathione synthesis was evaluated (Fig. 12) (Meister and Anderson, 1983). The EC_{50} for protection against toxicity of 2 mM glutamate for 24 hr was 99 ± 31 µM for cysteine (Fig. 12) and 52 ± 9 µM for cystine (Fig. 9).

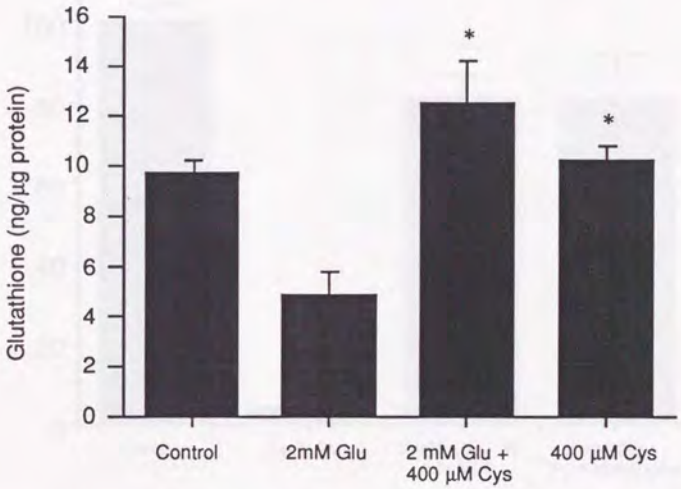
Figure 12. (next page) Dose-response of protection from L-glutamate toxicity by cysteine in oligodendroglia. Oligodendroglia were incubated with 2 mM L-glutamate in EBSS for 24 hr in the presence of cysteine, and viable cells were counted. Data are presented as the percentage of viable cells compared to control cultures (450-574 cells). Values represent the mean ± SD of six cultures in three separate experiments. The mean EC_{50} ± SEM was 99 ± 31µM.



The approximately twofold difference in EC_{50} is expected if one molecule of cystine, as expected, is converted to two molecules of cysteine (either intracellularly or extracellularly) for synthesis of glutathione. Moreover, it was also shown, as with cystine, that a concentration of cysteine (400 μM) sufficient to prevent the toxicity of 24 hr exposure to 2 mM glutamate also preserved intracellular glutathione levels (Fig. 13).

Figure 13. (next page) Prevention of glutathione depletion by cysteine. Oligodendroglia were incubated with 2 mM L-glutamate for 21 hr in the presence or absence of 200 μM cysteine in EBSS, and intracellular glutathione was determined (see Materials and Methods). Cysteine (400μM) sufficient to block glutamate toxicity (Figure 12) prevented glutathione depletion due to glutamate (* $p<0.001$, compared

to cells with 2 mM glutamate and no cysteine by two-tailed *t* test with Bonferroni correction). Values are the mean ± SD in an experiment performed in triplicate. Similar results were obtained in two other experiments.



(10) Role of oxidative stress and free radicals in glutamate toxicity to oligodendroglia. Because glutathione is an important cellular protective agent against oxidative stress (Floyd, 1990; Bast et al., 1991; Sies, 1991; Traystman et al., 1991), the possibility that the toxicity of glutamate to oligodendroglia involved a free radical mechanism was evaluated. The effect of the free radical scavengers, vitamin E (30 µM) (Burton and Ingold, 1989; Niki et al., 1989) or idebenone (1 µM) (Suno and Nagaoka, 1984), on oligodendroglial toxicity to glutamate was studied (Fig. 14). Both compounds totally protected oligodendroglia from the toxicity of as much as 2 mM glutamate. These data

suggest that cellular depletion of glutathione leads to vulnerability to oxidative stress and free radical-mediated toxicity.

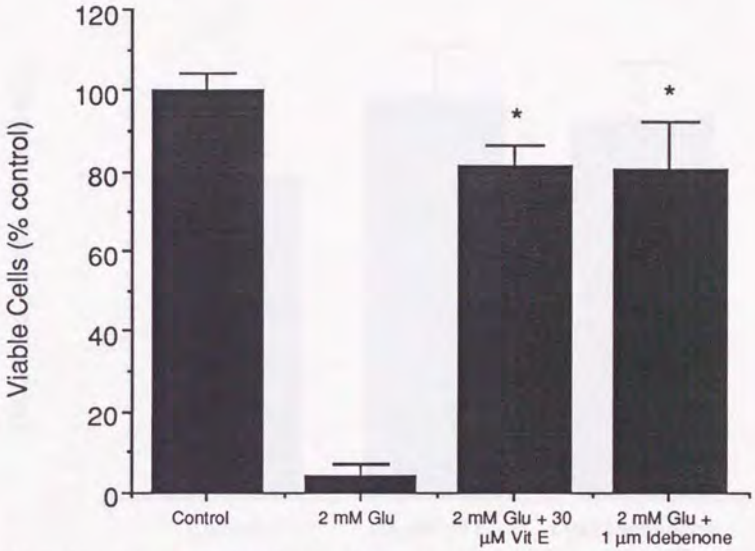


Figure 14. Protection from L-glutamate toxicity by antioxidants in oligodendroglia. Oligodendroglia were incubated with 2 mM L-glutamate in EBSS for 24 hr in the presence of 30 µM vitamin E or 1 µM idebenone, and viable cells were counted. Data are presented as the percentage of viable cells compared to control cultures (498 cells). Vitamin E and idebenone exhibited protective effects against L-glutamate toxicity (* $p<0.001$, compared to cells exposed to L-glutamate without antioxidants by two-tailed *t* test with Bonferroni correction). Values represent the mean ± SD in an experiment performed in triplicate. Similar results were obtained in two other experiments.

It was next determined whether glutamate toxicity could be prevented by the free radical scavengers despite uptake of glutamate into the cells and despite glutathione

depletion (Fig. 15 and 16). Glutamate uptake was unaffected by concentrations of vitamin E and idebenone that totally protect oligodendroglia from glutamate toxicity (Fig. 15).

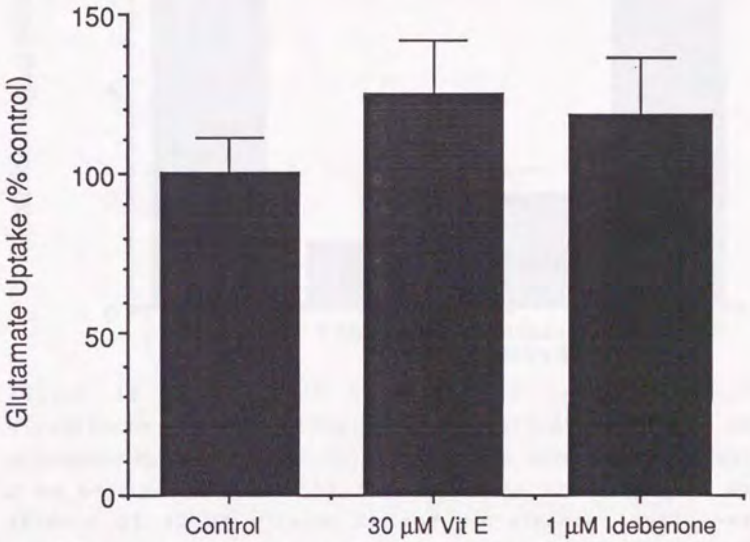


Figure 15. The effect of vitamin E on L-glutamate uptake. For determination of L-glutamate uptake, oligodendroglia grown on 24-well plates were incubated with 1.5 µCi L-[^{3}H]-glutamate (49 Ci/mmol) and 200 µM non-radiolabeled L-glutamate in EBSS for 20 min at 37^{o}C in the presence of vitamin E and 1 µM idebenone (see Materials and Methods). Data are presented as the percentage of uptake in cultures incubated in EBSS without antioxidants. There was no inhibition of L-glutamate uptake by vitamin E and idebenone (no significant difference by ANOVA).

Moreover, vitamin E was shown to protect the cells against glutamate toxicity despite the occurrence of glutathione depletion (Fig. 16).

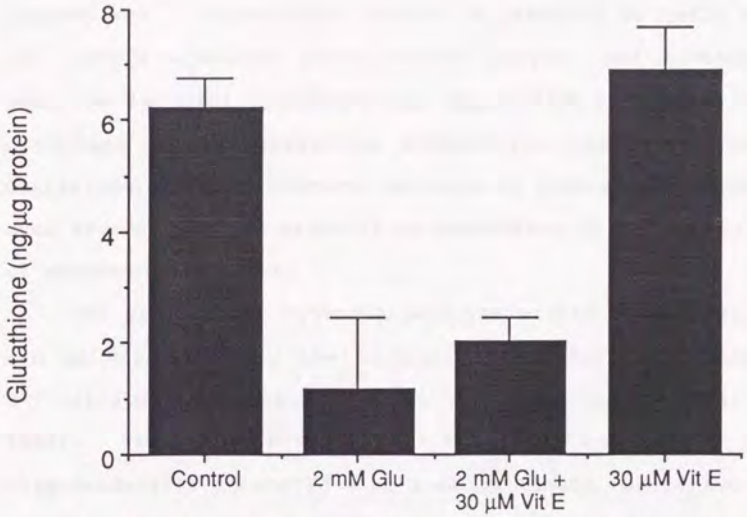


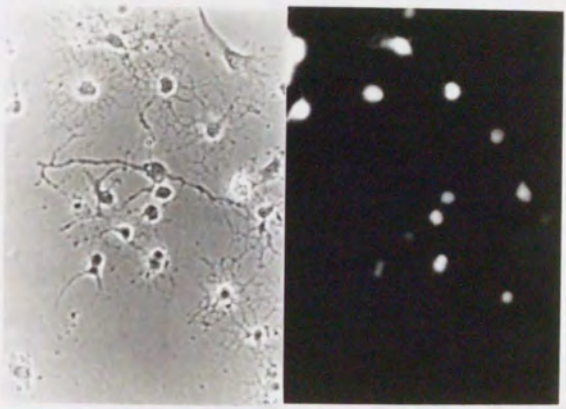
Figure 16. The effect of vitamin E on intracellular glutathione levels. Depletion of glutathione was not prevented by vitamin E. Oligodendroglia were incubated with 2 mM L-glutamate in EBSS for 21 hr in the presence and absence of 30 µM vitamin E, and glutathione levels were determined (see Materials and Methods). Vitamin E (30 µM), which protected cells from L-glutamate toxicity, did not inhibit glutathione depletion (* $p=0.342>0.05$, compared to cells with 2mM L-glutamate by two-tailed *t* test). Values represent the mean ± SD in an experiment performed in triplicate. Similar results were obtained in two other experiments.

(11) Accumulation of intracellular hydrogen peroxide induced by glutamate. It is likely that the intracellular production of oxygen radicals takes place spontaneously and constantly, and that the depletion of glutathione as an antioxidant leads to the accumulation of oxidative stress. Thereby, the presence of oxygen radicals such as superoxide radical or hydrogen peroxide in the cells treated with glutamate was studied by staining with NBT or DCFH-DA,

respectively. Superoxide radical is produced in cells by the single electron reduction of oxygen, and hydrogen peroxide is mainly produced from superoxide by superoxide dismutase or non-enzymatic dismutation (Halliwell and Gutteridge, 1990). Hydrogen peroxide is removed by enzymes such as catalase and glutathione peroxidase in the presence of reduced glutathione.

The presence of hydrogen peroxide within living cells can be visualized by the formation of highly fluorescent 2'7'-dichlorofluorescein (Bass et al., 1983; Murphy et al., 1989). Sixty-nine percent (± 5.1 %; mean ± sem, n=3) of oligodendroglia incubated with 2 mM glutamate for 18 hours were fluorescent, indicating the presence of hydrogen peroxide (Fig. 17), while only 11 % (± 1.2 %; mean ± sem, n=3) of control cells incubated with 0 mM glutamate were positive. On the other hand, almost no cells showed the production of blue formazan from NBT throughout the period of exposure to glutamate, and it was unlikely that superoxide accumulated in oligodendroglia incubated with glutamate (data not shown).

Figure 17. (next page) Hydrogen peroxide in oligodendroglia treated with glutamate. Oligodendroglia were incubated with 2 mM L-glutamate for 18 hours, and intracellular hydrogen peroxide was visualized by DCFH-DA as described in Materials and Methods. The presence of highly fluorescent 2'7'-dichlorofluorescein (left, phase contrast; right fluorescence filter) within cells indicated the accumulation of this oxygen derivative induced by L-glutamate.



(12) Glutamate transport in oligodendroglia. Taken together, these data suggest an important role for glutamate uptake in glutamate toxicity to oligodendroglia. Because of this central role of uptake, glutamate transport was investigated further. The ionic dependency of glutamate uptake in oligodendroglia in the absence of Na^+ or Cl^- or both was evaluated (Fig. 18), because of the reported presence in other cells of sodium-dependent (high-affinity, ouabain-sensitive) and sodium-independent, chloride-dependent transport systems.

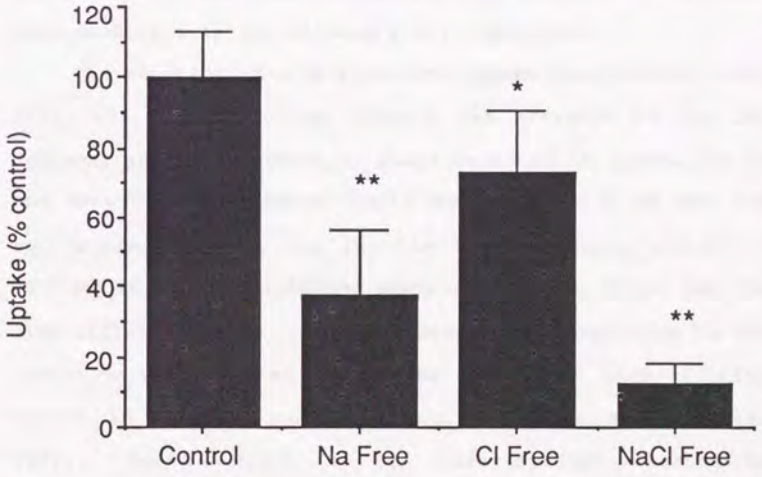


Figure 18. Glutamate uptake in Na^+ and/or Cl^- free solutions. Oligodendroglia grown on 24-well plates were incubated with 1.5 µCi L-[^{3}H]-glutamate (49 Ci/mmol) and 200 µM non-radiolabeled L-glutamate for 20 minutes at 37^{o}C. In control cultures, Tris buffered saline (TBS) consisting of 120 mM Na^+, 5.4 mM K^+, 130.6 mM Cl^-, 1.8 mM Ca^{2+}, 0.8 mM Mg^{2+}, 16 mM glucose, and 25 mM Tris base was used. For ion deficient media, sodium and chloride in TBS were replaced in isomolar amounts by *N*-methyl-glucamine and sulfate,

respectively. L-Glutamate uptake was determined as described in Materials and Methods. Data are presented as the percentage of uptake in cultures incubated in standard TBS. L-Glutamate uptake was inhibited in sodium- and/or chloride-free conditions (** $p<0.001$, * $p<0.01$, compared to control by two-tailed *t* test with Bonferroni correction). Values represent the mean ± SD of nine cultures in three separate experiments.

Both systems appear to be present in oligodendroglia, since the Na+-free condition results in approximately 65% inhibition of uptake, the chloride-free condition in approximately 25% inhibition, and the sodium and chloride free condition in approximately 90% inhibition.

The kinetics of L-^{3}H-glutamate uptake were studied next (Fig. 19). The findings support the presence of the two systems, and Lineweaver-Burk plots resulted in values for *Km* and Vmax for the high-affinity system of 3.9 µM and 3.4 nmol/min/mg protein, and for the lower-affinity system of 30.0 µM and 7.1 nmol/min/mg protein. The *Km* value for the high-affinity system in oligodendroglia is comparable to the lowest values reported for the Na^+-dependent high-affinity system in neurons and astrocytes (Nicholls and Attwell, 1990). The *Km* value for the lower-affinity system in oligodendroglia is similar to the *Km* of the glutamate-cystine transport system in C-6 glioma cells (Cho and Bannai, 1990), which in turn may be comparable to the low-affinity transport system studied in brain slices (Benjamin and Quastel, 1976), synaptosomes (Takagaki, 1976), and glioma cells (Henn et al., 1974).

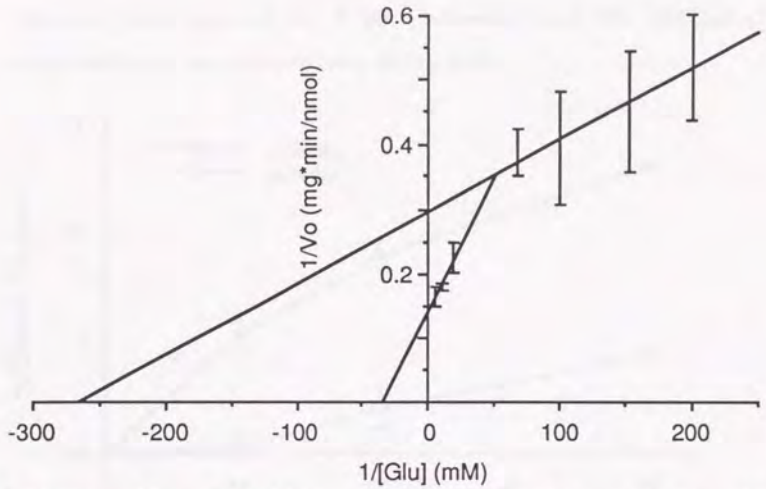


Figure 19. Kinetics of L-glutamate uptake in oligodendroglia (Lineweaver-Burk plots). Oligodendroglia grown on 24-well plates were incubated with the indicated concentrations of L-glutamate containing 1.5 to 15 µCi L-[^{3}H]-glutamate (49.0 Ci/mmol) in 0.3 ml EBSS for 2 min at 37^{o}C. L-Glutamate uptake was determined as described in Materials and Methods. Two uptake systems were apparent. Values of Km and Vmax were respectively 3.9 µM and 3.4 nmol/min/mg protein for the higher affinity system and 30.0 µM and 7.1 nmol/min/mg protein for the lower affinity system. Values represent the mean ± SEM in three experiments performed in duplicate.

(13) Promotion of cystine efflux by glutamate. Because the lower-affinity, glutamate-cystine transport system can act as an exchanger in neurons (Kesseler et al., 1987; Zaczek et al., 1987), the possibility that such a system was operative in oligodendroglia and that glutamate entrance into the cells promotes cystine efflux was evaluated. Cells prelabelled by preincubation with ^{35}S-

cystine were exposed to 2 mM glutamate and the efflux of radioactivity was determined (Fig. 20).

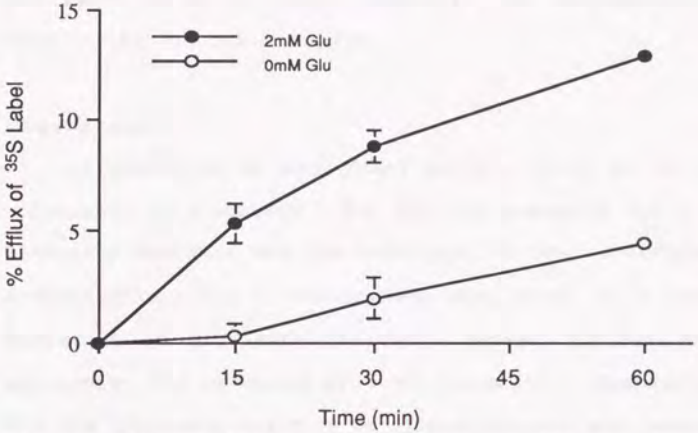


Figure 20. L-Glutamate promotes efflux of [^{35}S] radioactovity from oligodendroglia prelabeled with [^{35}S]. Cells grown in 35 mm dishes were incubated in EBSS containing 1.5 µCi of L-^{35}S-cystine (405.2 Ci/mmol) for 60 min. After washing three times, cells were incubated in 1.2 ml of EBSS with 0 or 2 mM L-glutamate for the indicated times at 37°C. After incubation, media were collected, and cells were washed once and lysed in 0.1*N* NaOH. Radioactivity in media and cells was determined by liquid scintillation, and percentage efflux of ^{35}S label was calculated as described in Materials and Methods. Values represent the mean ± SD in an experiment performed in duplicate. Similar results were obtained in three other experiments.

Glutamate clearly caused efflux of radioactivity into the medium; the rate of efflux in the glutamate-treated cultures was more than threefold higher than the rate of efflux in the control cultures. In a parallel experiment, D-glutamate led to an increase in cystine efflux similar to that shown

in Figure 20 for L-glutamate. ^{35}S-Cystine was isolated by thin-layer chromatography, and radioactivity was determined and found to be 4.9-fold higher in the glutamate-treated than in the control cultures.

Discussion

Oligodendroglia were found in this study to be highly vulnerable to glutamate. The EC_{50} for glutamate for a 24 hr glutamate exposure was approximately 200 μM. Astrocytes of a comparable stage of development were found to be markedly resistant to glutamate toxicity; indeed, no toxicity to astrocytes was observed at 5 mM glutamate. Moreover, the EC_{50} for glutamate toxicity to oligodendroglia was comparable to the EC_{50} for neuronal toxicity caused by this amino acid in conventional cerebral cortical cultures derived from the mouse (Koh and Choi, 1988) or rat (Rosenberg and Aizenman, 1989; Rosenberg et al., 1992). However, the temporal characteristics of glutamate-induced oligodendroglial death are different from those of neuronal death; the former requires hours of exposure whereas the latter occurs in less than an hour. The cultures of oligodendroglia in these experiments contain fewer astrocytes, and in this respect resemble more closely the astrocyte-poor cultures described by Rosenberg and Aizenman (1989) (Rosenberg and Aizenman, 1989), in which it was possible to measure the intrinsic vulnerability of neurons to glutamate. In the oligodendroglia-enriched, astrocyte-poor cultures, I believe it was possible to measure the intrinsic vulnerability of oligodendroglia to glutamate, and while these cells are less

sensitive to glutamate than are neurons, they are markedly more vulnerable to glutamate toxicity than are astrocytes. Whether astrocytes confer on oligodendroglia a similar degree of protection from glutamate toxicity to that which they confer upon neurons remains an important topic for future study. In any case, the unexpected finding is that oligodendroglia, as well as neurons, are potential targets for glutamate toxicity in the developing mammalian CNS.

The inability of MK-801 or CNQX to block the toxicity of glutamate to oligodendroglia suggests that the mechanism of oligodendroglial death caused by glutamate is not mediated by excitatory amino acid receptors, in contrast to the mechanisms of glutamate neurotoxicity (Choi and Rothman, 1990; Meldrum and Garthwaite, 1990). Instead, the oligodendroglial toxicity of glutamate appears to be mediated primarily by glutamate transport mechanisms. A similar conclusion was drawn concerning the neuronal toxicity of glutamate and quisqualate in studies of toxicity to immature neurons (Murphy et al., 1990).

The necessity of transport of glutamate into oligodendroglia to produce cell death was shown by the total prevention of cell death by blockade by BHA, an antagonist of glutamate transport. The protective effect of BHA exhibited the same dose dependency as did its inhibitory effect on glutamate uptake. BHA has been shown to be a highly potent blocker of the sodium-dependent, high-affinity glutamate transport system described in neurons and astrocytes (Balcar et al., 1977; Johnston et al., 1980). However, this system cannnot be exclusively involved in the

glutamate toxicity to oligodendroglia, as is demonstrated by the fact that D-glutamate, which is not transported by the sodium-dependent, high-affinity system (Benjamin and Quastel, 1976), is highly toxic. (The similar effects of D-glutamate and L-glutamate on cystine efflux and glutathione levels, however, do suggest that the mechanism of toxicity of the two stereoisomers is the same.) Moreover, BHA has been shown to block sodium-independent glutamate transport as well as the sodium-dependent system (Johnston et al., 1980). The only previous suggestion concerning a glutamate uptake system in oligodendroglia was the demonstration by autoradiography of the uptake of radioactive D-aspartate by oligodendroglia in mixed glial primary cultures by Reynolds and Herschkowitz (1986) (Reynolds and Herschkowitz, 1986). However, uptake of L-glutamate could not be demonstrated in those experiments. In the present report, uptake of L-glutamate into oligodendroglia was unequivocally demonstrated by autoradiography, as well as by tracer experiments. A kinetic analysis of the ^{3}H-glutamate uptake data that we obtained suggests the presence of two uptake systems in oligodendroglial cultures, with the *Km* of one nearly an order of magnitude greater than that of the other.

The present studies demonstrate in five ways that the toxicity of glutamate is mediated by depletion of intracellular cystine leading to depletion of glutathione. First, the addition of cystine totally prevented glutamate toxicity to oligodendroglia. This striking protective effect was comparable to that shown by Coyle and coworkers with immature neurons (Murphy et al., 1989; Murphy et al.,

1990). Second, the addition of cysteine, the reduced form of cystine that is involved in glutathione biosynthesis, also totally prevented glutamate toxicity to oligodendroglia, and the EC_{50} for this prevention was twofold that for cystine, the disulfide derivative from which cysteine is generated. Third, glutathione levels were shown to decrease markedly in cells exposed to glutamate, and this marked decrease preceded the loss of cell viability. Fourth, glutamate toxicity could be prevented totally by simultaneous exposure to two different free radical scavengers, vitamin E and idebenone. This observation is consistent with the fact that glutathione, a crucial cellular product of cysteine (Murphy et al., 1989; Murphy et al., 1990; Cho and Bannai, 1990), is an important protective agent against oxidative stress (for reviews; Floyd, 1990; Bast et al., 1991; Sies, 1991; Traystman et al., 1991). Fifth, intracellular accumulation of hydrogen peroxide was induced by glutamate, as visualized by the formation of highly fluorescent 2'7'-dichlorofluorescein in oligodendroglia.

The oligodendroglial cell death caused by glutamate thus appears to involve a mechanism that ultimately renders the cell vulnerable to oxidative stress, probably because of depletion of glutathione. How could glutamate lead to this depletion? The major possibility is that oligodendroglia contain the transport system described in extraneural cells that is shared by glutamate and cystine, and that can operate as an exchange system in neurons (Kesseler et al., 1987; Zaczek et al., 1987). The promotion of cystine efflux

and, thereby, glutathione depletion by glutamate uptake through such an exchange system would be consistent with the prevention of both glutamate toxicity and glutathione depletion by addition of cystine and cysteine. We showed that glutamate exposure does cause efflux of ^{35}S-cystine after oligodendroglia were prelabeled with this amino acid.

Since the development of neonatal intensive care has resulted in the survival of most premature infants, brain injury, which causes cerebral palsy or cognitive deficits as sequelae, has become a critical problem. The principal neuropathology is the necrosis in the white matter adjacent to the lateral ventricles, designated as periventricular leukomalacia. Although the pathology indicates an ischemic process, presumably due to the insufficiency of vascular supply to the white matter in prematures, the pathogenesis is not thoroughly elucidated, and no useful preventative measures against white matter injury have been established. Death of oligodendroglia, extending beyond the areas of focal necrosis, has been shown neuropathologically in the acute stage and has been deduced from the finding of subsequently impaired myelination by brain imaging as well as neuropathology (DeReuck et al., 1972; Gilles et al., 1983; DeVries et al., 1988; Dambska et al., 1989; Leviton and Paneth, 1990; Paneth et al., 1990).

In the present study, we have shown that differentiating oligodendroglia in culture are susceptible to glutamate. These observations in vitro raise the possibility that the oligodendroglial cell death associated with the periventricular leukomalacia of premature infants

is caused, at least in part, by glutamate. Although the accumulation of extracellular glutamate after ischemic insults in brain has been established, the concentration of this amino acid has been measured by microdialysis exclusively in gray matter, not in white matter (Beneveniste, 1991). The earliest neuropathological features of this lesion, however, are coagulation necrosis, which is infarction with disruption of axis cylinders (Banker and Larroche, 1962). It is hypothesized that this oligodendroglial death is caused, at least in part, by increased extracellular glutamate, released locally from injured axis cylinders, which contain millimolar concentrations of this amino acid (Battistin et al., 1971; Fonnum, 1984; Beneveniste, 1991). A similar formulation would be relevant to oligodendroglial injury in areas of cerebral infarction in older patients suffering from stroke. Concerning the temporal alterations of extracellular glutamate in white matter under ischemic conditions, further investigations in vivo are required.

The possibility is raised of novel therapies for white matter injury in premature infants. Since glutamate transport, the efflux of cystine, the depletion of glutathione, and finally the accumulation of oxidative stress lead to oligodendroglial death in cultures, therapeutic measures directed against each of these will be considered. For example, the use of antioxidants such as vitamin E, or the administration of cystine may be useful for the prevention of periventricular leukomalacia and subsequent neurological deficits.

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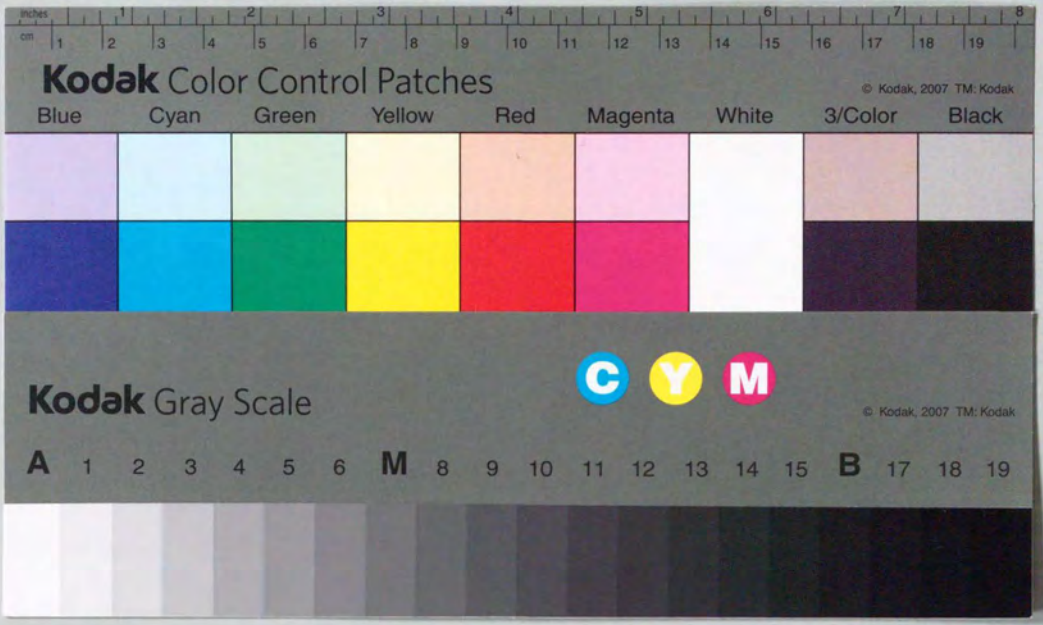
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