*Proc. Natl. Acad. Sci. USA*
Vol. 85, pp. 7351–7355, October 1988
Neurobiology

# Gangliosides prevent glutamate and kainate neurotoxicity in primary neuronal cultures of neonatal rat cerebellum and cortex

(glutamate receptors/neuronal death/phencyclidine/cationic channels/c-fos protein)

M. FAVARON, H. MANEV, H. ALHO, M. BERTOLINO, B. FERRET, A. GUIDOTTI, AND E. COSTA

FIDIA Georgetown Institute for the Neurosciences, Georgetown University, 3900 Reservoir Road, N.W., Washington, DC 20007

*Contributed by E. Costa, June 8, 1988*

ABSTRACT **Using a sensitive histofluorescence staining method that allows for a quantitation of neuronal death, we compared the protective effects of gangliosides (a group of naturally occurring glycosphingolipids), phencyclidine (PCP), and MK-801 (dibenzocycloheptenеimine) on glutamate- and kainate-induced neuronal death in primary cultures of cortical and cerebellar neurons prepared from neonatal rats. PCP and MK-801 block neurotoxicity induced by glutamate doses 50 times higher than the $LD_{50}$ ($LD_{50}$ in $Mg^{2+}$-free medium, 10 $\mu$M) but only partially block the kainate neurotoxicity ($LD_{50}$ in presence of $Mg^{2+}$, 100 $\mu$M). In contrast, pretreatment with gangliosides (GT1b > GD1b > GM1) results in complete and insurmountable protection against the neurotoxicity elicited by glutamate or kainate. In primary cultures of cerebellar granule cells gangliosides, unlike PCP and MK-801, fail to block glutamate-gated cationic currents and the glutamate-evoked increase of (*i*) inositol phospholipid hydrolysis, (*ii*) c-fos mRNA content, and (*iii*) nuclear accumulation of c-fos protein. Protection of glutamate neurotoxicity by gangliosides does not require their presence in the incubation medium; however, it is proportional to the amount of glycosphingolipid accumulated in the neuronal membranes. The ganglioside concentration (30–60 $\mu$M) that blocks glutamate-elicited neuronal death also prevents glutamate- and kainate-induced protein kinase C translocation from cytosol to neuronal membranes.**

High-affinity glutamate binding sites that recognize *N*-methyl-D-aspartate (NMDA) are located on a specific excitatory amino acid receptorial domain that operates ionotropic (1–4) and/or metabolotropic (4–10) signal transduction. These glutamate receptorial domains, in addition to the recognition site for the putative excitatory neurotransmitter, include two kinds of noncompetitive inhibitory sites regulated by $Mg^{2+}$ and by an unknown natural ligand acting on a site that recognizes phencyclidine (PCP) (1, 4, 11–13). Moreover, positive and/or negative allosteric centers modulate NMDA-sensitive glutamate recognition sites (13–15); these are believed to have glycine (13, 14) or kynurenate (15) as putative endogenous modulators, respectively. These NMDA-sensitive recognition sites operate cationic channels (1, 4), stimulate inositol phospholipid hydrolysis (4–6), or release arachidonic acid (7) and cause a $Ca^{2+}$-dependent accumulation of cGMP (4, 8). Transduction mechanisms operated by activation of NMDA-sensitive glutamate recognition sites elicit a sequelae of events, including an increase in c-fos protooncogene mRNA content (9) and the nuclear accumulation of c-fos protein, which in turn promotes the coordinated expression of selected mRNAs encoding proteins (16) presumably mediating long-term responses to NMDA-sensitive glutamate receptor stimulation, such as neuronal plasticity (9).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Persistent stimulation of these glutamate receptors and of other excitatory amino acid receptors sensitive to kainate and/or quisqualate activates protein kinase C (PKC) (10, 17), a cytosolic enzyme that in the presence of excitatory amino acid-mediated $Ca^{2+}$ influx (4, 18) translocates to the neuronal membranes (10, 17). In addition to PKC, other $Ca^{2+}$-dependent enzymes may be activated by the persistent stimulation of excitatory amino acid receptors. Since such stimulation also causes neurotoxicity and neuronal death (19–23), it can be surmised that the subversion of important cellular functions caused by excitatory amino acid receptor activation of $Ca^{2+}$-dependent enzymes (19, 22) may be operative in glutamate neurotoxicity. With mounting evidence that *in vitro* isosteric antagonists (aminophosphonovalerate and its cogeners) of NMDA-sensitive glutamate receptors, and noncompetitive inhibitors [PCP, dibenzocycloheptenеimine (MK-801)] acting on the PCP recognition sites attenuate or block glutamate and kainate neurotoxicity (19, 23–25), a possible therapeutic application of these drugs to limit secondary neurotoxicity occurring in brain regions surrounding ischemic areas has been proposed (19, 24, 25). Gangliosides and sphingosine applied to primary cultures of neonatal rat cerebellar granule cells prevent the glutamate receptor-mediated activation and translocation of PKC by a mechanism that does not involve a direct interaction with glutamate recognition sites (10). The present report demonstrates a protective effect of gangliosides against glutamate and kainate neurotoxicity in primary cultures of neonatal rat cerebellar and cortical neurons.

## MATERIALS AND METHODS

**Primary Neuronal Cultures.** Cerebral cortex or cerebellar neurons grown on 35-mm dishes for 8 days *in vitro* were prepared from 1- and 8-day-old Sprague–Dawley rats, respectively (26, 27). The cerebellar cultures contain >95% neurons and <5% glia cells (26); the cortical cultures contain ≈92% neurons and ≈8% glia cells (27). Glial proliferation was prevented with cytosine arabinofuranoside (26, 27).

**Exposure to Excitotoxins and Gangliosides.** Before application of the excitatory amino acids, the culture medium (culture-conditioned medium) was collected and the cells were washed once with Locke's solution (154 mM NaCl/5.6 mM KCl/3.6 mM $NaHCO_3$/2.3 mM $CaCl_2$/1 mM $MgCl_2$/5.6 mM glucose/5 mM Hepes, pH 7.4). $MgCl_2$ was omitted as indicated. The incubation with excitatory amino acids was terminated by washing the monolayer with Locke's solution three times. The cells were returned to the culture-conditioned medium and further incubated at 37°C in 95% air/5% $CO_2$ for 24 hr.

Gangliosides (1–200 $\mu$M) were serially diluted in methanol/$H_2O$ (95:5), dried under a stream of $N_2$, and an appropriate volume of Locke's solution was added to reach the final

Abbreviations: NMDA, *N*-methyl-D-aspartate; PCP, phencyclidine; PKC, protein kinase C; $PBt_2$, phorbol dibutyrate.

concentration. After preincubation with gangliosides (2 hr at 37°C), the cultures were washed three times with Locke's solution and challenged for the time indicated with glutamate or kainate.

**Intravital Staining of the Culture.** Monolayers were washed with Locke's solution and stained for 3 min at 22°C with a fluorescein diacetate (15 μg/ml) and propidium iodide (4.6 μg/ml) mixture (27, 28). The stained cells were examined immediately with a standard epi-illumination fluorescence microscope (Vanox, Olympus; 450 excit., 520 barrier). Fluorescein diacetate, a nonpolar ester, crosses the cell membranes and is hydrolyzed by intracellular esterases to produce a green–yellow fluorescence. Neuronal injury curtails fluorescein diacetate staining (29) and facilitates propidium iodide penetration and interaction with DNA to yield a bright red fluorescent complex (30).

After treatment with excitatory amino acid receptor agonists, some neurons may detach from the dish after degeneration. Therefore, we estimated the loss of cells by comparing the number of intact or degenerated neurons in one identified field photographed before and 24 hr after application of the receptor agonist under study. The percentage of surviving neurons in the monolayer was computed by assessing the fluorescein acetate/propidium iodide staining ratio in photomicrographs of four representative fields from each monolayer.

**Ganglioside Determination.** After preincubation for 2 hr with gangliosides, the monolayers were washed two times with 9% NaCl solution containing 10% fetal calf serum and three times with Locke's solution. The gangliosides were extracted according to Chigorno *et al.* (31). *N*-Acetyl neuraminic acid was determined by the resorcinol method (32) and after high-performance thin-layer chromatography, the ganglioside content was estimated by the densitometric method (31).

**c-fos Protein Immunocytochemistry.** Granule cell monolayers were washed with Locke's solution and fixed with 4% formaldehyde (20 min at 22°C). Fixative was removed by three washes with 0.1 M phosphate-buffered saline (PBS) containing 10 mM glycine (pH 7.4). The cells were permeabilized with 0.5% Nonidet P-40 (Sigma) in PBS and then incubated at 4°C for 24 hr with polyclonal rabbit c-fos peptide antiserum (1:150) (33) (a generous gift from T. Curran, Hoffmann–La Roche). The bound antibody was detected by the antibiotin-peroxidase system (Vectastain, ABC kit, Vector Laboratories, Burlingame, CA) and reacted with diaminobenzidine tetrahydrochloride (0.2 mg/μl) for 15 min. In the same experiments, the c-fos immunoreactivity was determined after inhibition of protein synthesis with 70 μM cycloheximide. The extent of protein synthesis inhibition was established by measuring [$^{14}$C]arginine incorporation into proteins (34). In 3 hr, cycloheximide reduced by 70% the incorporation of [$^{14}$C]arginine into trichloroacetic acid-precipitable proteins.

**[$^3$H]Phorbol Ester Binding to Neurons in Primary Culture.** Monolayers were washed once with Locke's solution and incubated in 1 ml of Locke's solution containing 4-β-[$^3$H]phorbol 12,13-dibutyrate ([$^3$H]PBt$_2$) in 0.1% fatty acid-free bovine serum albumin. The binding was carried out for 15 min at 22°C (10). Nonspecific binding was determined in the presence of 2 μM phorbol 12-tetradecanoate 13-acetate.

**Inositol Phospholipid Hydrolysis.** This was estimated by measuring the accumulation of [$^3$H]inositol 1-monophosphate in the presence of 7 mM lithium to inhibit the conversion of [$^3$H]inositol 1-monophosphate into [$^3$H]inositol (4).

**Glutamate-Activated Cationic Channels.** Glutamate-activated single-channel currents were recorded by the tight-seal patch-clamp technique from outside-out patches excised from granule neurons, as described by Bertolino *et al.* (13). Records of single-channel currents were sampled at the 10-kHz rate by the INDEC data acquisition system (Sunnyvale, CA) after filtering with a low-pass 8-pole Bessel filter set at 3 kHz and stored in the memory of a Digital Equipment (DEC; Maynard, MA) LSI 11/73 computer.

A semiautomated procedure generated distributions of amplitudes and open times. The threshold setting to validate channel opening and closing was determined by using half the amplitude of the channel current. Single exponential fitting of the distribution of kinetic parameters with probability density curves was performed by the method of maximum likelihood, with optimization of the fit being provided by a Simplex algorithm.

## RESULTS

**Neurotoxicity of Glutamate and Kainate: Effect of Gangliosides.** Figs. 1 and 2 show the neurotoxic effects of glutamate in primary cultures of cerebellar granule or cortical neurons stained with a fluorophor that gives bright green–yellow fluorescence in vital neurons and an intense red fluorescence (nuclear staining) in severely damaged neurons. The neurotoxic potency of glutamate and kainate depends on temperature, pH, and the age of the culture (see also refs. 21 and 22); the following experimental protocol was therefore adopted: cell monolayers (8 days *in vitro*) were exposed at 22°C for 15 min to glutamate or for 30 min to kainate in Locke's solution. The excitatory amino acids were removed by washing three times with Locke's solution, and the neurons were returned to the culture-conditioned medium and placed at 37°C in an atmosphere of 5% $CO_2$/95% air. The neurotoxic effects of glutamate and kainate developed gradually within several hours after their application. Hence, the extent of cell damage was assessed 24 hr after glutamate or kainate application. The number of cells damaged by glutamate (in the absence of $Mg^{2+}$) or kainate (in the presence of $Mg^{2+}$) was concentration dependent, with approximate $LD_{50}$ values of 10 and 100 μM, respectively (Fig. 3).

The $LD_{50}$ for glutamate increased by 50-fold when glutamate was either applied to the cells in Locke's solution containing 1 mM $Mg^{2+}$ or when the application was carried out at 37°C rather than 22°C (data not shown). In contrast, the $LD_{50}$ for kainate was $Mg^{2+}$ independent. The morphology and viability of the few glial cells in the culture were unaffected by treatment with either glutamate or kainate.

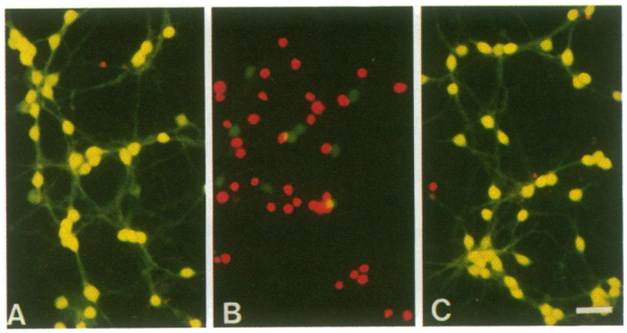


FIG. 1. Ganglioside GT1b prevents glutamate-evoked neurotoxicity in primary cultures of rat cerebellar granule cells. Photomicrographs of monolayers stained with fluorescein diacetate/propidium iodide mixture. This intravital staining yields green–yellow fluorescence for normal neurons and red fluorescence for dead neurons. (*A*) Control neurons: preincubated for 2 hr at 37°C with Locke's solution; washed three times and then incubated for 15 min with $Mg^{2+}$-free Locke's solution at 22°C; returned to culture-conditioned medium and examined 24 hr later. (*B*) Neurons treated for 15 min with 50 μM glutamate in $Mg^{2+}$-free Locke's solution and examined 24 hr later. (*C*) Neurons preincubated 2 hr at 37°C with 60 μM GT1b prior to glutamate exposure in $Mg^{2+}$-free Locke's solution. (Bar = 20 μm.)

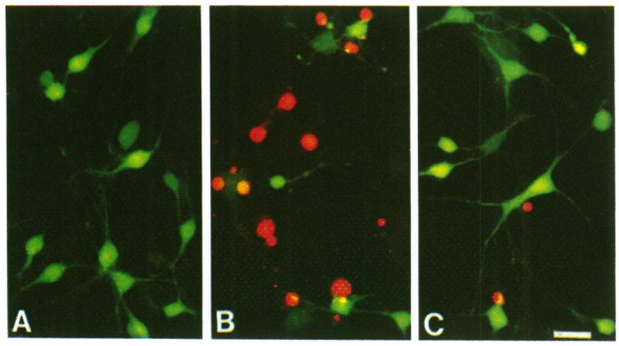


FIG. 2. Ganglioside GT1b prevents glutamate-evoked neurotoxicity in primary cultures of rat cortical neurons. (*A*) Control neurons. (*B*) Neurons treated for 15 min with 50 $\mu$M glutamate in $Mg^{2+}$-free Locke's solution. (*C*) Neurons preincubated with 60 $\mu$M GT1b prior to glutamate exposure. Experimental and staining conditions are the same as those described in Fig. 1. (Bar = 20 $\mu$m.)

When cerebellar neuronal cultures were preincubated with the ganglioside trisialosylgangliotetraglycosylceramide (GT1b; 60 $\mu$M) for 2 hr, and this treatment was followed by a thorough washout of the compound, glutamate neurotoxicity was inhibited (Figs. 1 and 2). This protective effect could not be surmounted by increasing the glutamate concentration from 10 to 500 $\mu$M (Fig. 3). The protective effect of GT1b against 50 $\mu$M glutamate was dose related (Fig. 4).

Preincubation of granule cell monolayers with GT1b induced a dose-dependent increase in the cell content of GT1b (Table 1). The extent of the GT1b uptake and the protection against glutamate-induced neurotoxicity are related (Table 1). In a separate group of experiments (not reported here), we observed that the protective effect of GT1b (60 $\mu$M) against glutamate-induced neurotoxicity lasted for 24 hr after the removal of ganglioside from the medium and that the time course of the protection was associated with that of the GT1b accumulation in neurons.

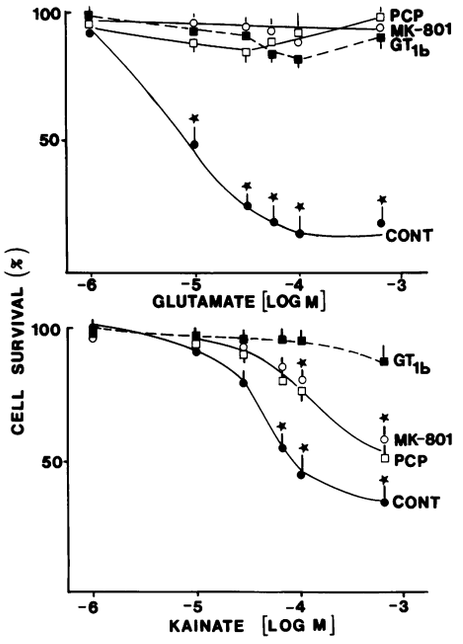


FIG. 3. GT1b (60 $\mu$M), PCP (1 $\mu$M), and MK-801 (0.1 $\mu$M) reduce glutamate- and kainate-induced cerebellar granule cell death. With the paradigm described in the text, 15-min exposure to glutamate (in $Mg^{2+}$-free Locke's solution) and 30-min exposure to kainate (in Locke's solution containing 1.2 mM $Mg^{2+}$) induces neuronal death 24 hr later. Pretreatment with GT1b (2 hr at 37°C in Locke's solution, followed by washing) or treatment with PCP and MK-801 prevented the glutamate- and kainate-induced granule cell death. Each point is the mean ± SEM of at least six different monolayers. *, $P < 0.01$ when compared with the respective controls.

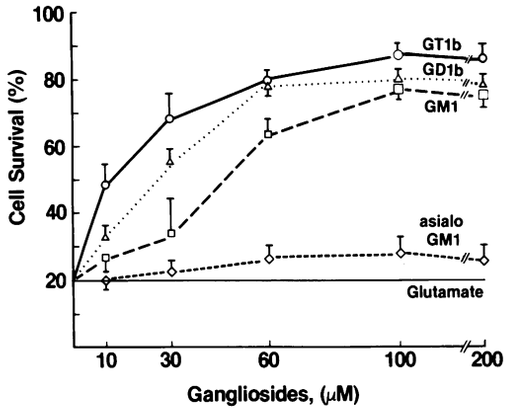


FIG. 4. Sialogangliosides prevent neuronal death induced by glutamate in primary culture of cerebellar granule cells. After 2 hr preincubation at 37°C with gangliosides, the monolayers were washed three times with Locke's solution, and 50 $\mu$M glutamate in $Mg^{2+}$-free medium was added for 15 min. Monolayers were then returned to culture-conditioned medium and neuronal death was measured 24 hr later. Each value is the mean ± SEM of five or six experiments.

GT1b was more potent than GD1b (disialosylgangliotetraglycosylceramide) or GM1 (monosialosylgangliotetraglycosylceramide); asialo-GM1 failed to protect neurons from glutamate neurotoxicity (Fig. 4). GT1b (60 $\mu$M) also prevented the neurotoxicity induced by kainate (Fig. 3). The potency of GT1b, GD1b, and GM1 against kainate and glutamate neurotoxicity is similar. In contrast, PCP completely prevents the neurotoxicity induced by glutamate but it fails to completely protect the neurons from kainate neurotoxicity (Fig. 3). Although MK-801, which appears to act at the same site as PCP (12), is at least 10 times more potent than PCP, the selectivity profile of its protective action against glutamate and kainate neurotoxicity is similar to that of PCP (Fig. 3). The gangliosides prevention of glutamate and kainate neurotoxicity has been replicated in primary culture of rat cortical neurons (see Fig. 2).

**Effect of Gangliosides on Signal Transduction Mechanisms at Glutamate Receptors.** *Glutamate-activated cationic channels.* In outside-out patches of cerebellar granule cell membranes, the glutamate-operated cationic channels were not affected by ganglioside GT1b (60 $\mu$M). The channel current

Table 1. Correlation between neuronal content of GT1b and protection of glutamate-elicited neuronal death in cerebellar granule cell cultures after 2 hr of preincubation with increasing concentration of GT1b

| Preincubation with GT1b, $\mu$M | GT1b, nmol per mg of protein | Neuronal death, % |
|---|---|---|
| 0 | 0.46 ± 0.042 | 80 |
| 2 | 0.61 ± 0.055 | 70 |
| 10 | 0.91 ± 0.082 | 53 |
| 30 | 1.6 ± 0.16 | 33 |
| 60 | 2.1 ± 0.22 | 20 |

Monolayers were preincubated for 2 hr at 37°C with GT1b in Locke's solution. At the end of 2 hr, the dishes were washed several times and gangliosides were extracted and their content was determined. Each value is the mean ± SEM of three experiments. The ganglioside composition (nmol per mg of protein) in control cells is as follows: GM3, 0.20; GM2, 0.040; GM1, 0.14; GD3, 0.19; GD1a, 0.49; GD1b, 0.22; GT1bO-AC, 0.14; GT1b, 0.46; GQ, 0.037. Neuronal death induced by glutamate (50 $\mu$M, 15 min) applied after ganglioside washout was quantified 24 hr later in separate cultures.

amplitude, channel opening frequency, and duration were unchanged by ganglioside pretreatment in doses that inhibit glutamate neurotoxicity in an insurmountable manner.

In 10 membrane patches, the channel amplitude at −50 mV holding potential was 2.5 ± 0.3 pA (mean ± SD) before and 2.5 ± 0.4 pA (mean ± SD) after GT1b pretreatment. From the same patches, the duration of channel opening was 5.6 ± 0.8 ms and 6.8 ± 1.5 ms (mean ± SD) before and after treatment, respectively. In addition, we failed to detect significant changes in the channel opening rate, measured as the number of channel openings per time unit, before and after ganglioside treatment (data not shown).

*Glutamate-induced activation of inositol phospholipid hydrolysis.* The basal inositol phospholipid hydrolysis by granule neurons was greatly enhanced by glutamate (50 μM in the absence of $Mg^{2+}$) (4, 5). This effect was blocked by PCP (1 μM) (4), but not by preincubation of the cells with 60 μM GT1b (i.e., [$^3$H]inositol 1-monophosphate accumulated is 2.5 × $10^3$ dpm per 100 μg of protein per 5 min preincubation with 50 μM glutamate and 2.6 × $10^3$ dpm per 100 μg of protein per 5 min when 50 μM glutamate was applied after preincubation with GT1b).

GT1b also failed to prevent the stimulation of inositol phospholipid hydrolysis induced by 50 μM carbachol.

*Glutamate- and kainate-mediated PKC translocation.* In primary cultures of cerebellar granule cells, we compared the potency of various gangliosides to that of PCP and MK-801 in preventing PKC translocation induced by neurotoxic doses of glutamate and kainate. PKC translocation was estimated by the increase of [$^3$H]$PBt_2$ binding in intact culture (10).

Fig. 5 demonstrates that PCP and MK-801 block the increase of [$^3$H]$PBt_2$ binding evoked by glutamate (50 μM in the absence of $Mg^{2+}$) but are less potent and less efficacious in blocking the increase evoked by kainate (100 μM in the presence of 1.2 mM $Mg^{2+}$). However, ganglioside preincubation virtually abolished PKC translocation elicited by either kainate or glutamate. The most potent ganglioside is GT1b, followed by GD1b and by GM1. Asialo-GM1 is ineffective up to doses of 200 μM.

*Glutamate-induced c-fos expression.* As shown in Fig. 6 the appearance of c-fos protein immunoreactivity in nuclei of cerebellar granule neurons elicited by exposure to 50 μM glutamate for 50 min (in the absence of $Mg^{2+}$) was reduced by PCP but not by pretreatment with 60 μM GT1b, a dose that prevents glutamate-induced neuronal death. If the neuronal cultures are pretreated with 70 μM cycloheximide for 3 hr, the incorporation of [$^{14}$C]arginine into proteins is inhibited by 70%; such a pretreatment prevents the increase of nuclear

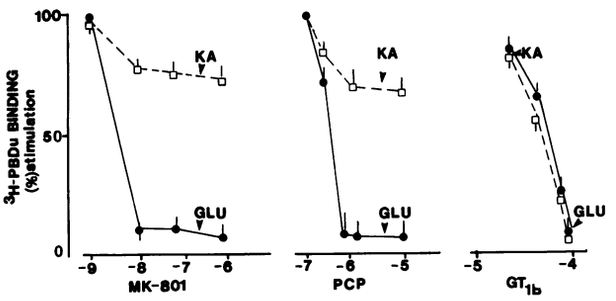


FIG. 5. Pretreatment with GT1b and treatment with PCP or MK-801 decrease glutamate- (50 μM) and kainate- (100 μM) induced enhancement of [$^3$H]$PBt_2$ ($^3$H-PBDu) binding in cerebellar granule cells. Values are expressed as percent of the stimulated binding, which is ≈3 times the control binding. Value of control binding is 350 ± 22 fmol per mg of protein, established by using 1 nM [$^3$H]$PBt_2$. Results are the mean ± SEM of at least three experiments. Glutamate (●) was added in $Mg^{2+}$-free medium; kainate (□) was added in medium containing 1.2 mM $Mg^{2+}$.

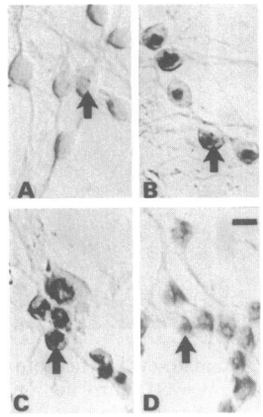


FIG. 6. c-fos immunoreactivity in cerebellar granule cell cultures. (*A*) Control culture shows sporadic weak nuclear immunoreactivity. (*B*) Culture treated with glutamate (50 μM for 50 min). (*C*) Culture pretreated with GT1b (60 μM for 2 hr) followed by glutamate. (*D*) Culture treated with glutamate and PCP (1 μM). Arrows point to nuclei. (Bar = 10 μm.)

c-fos immunoreactivity but not the neurotoxicity induced by glutamate.

## DISCUSSION

In brain, the stimulation of glutamate-operated cationic channels elicits a number of short- and long-lasting responses, including increase of (*i*) $Ca^{2+}$ influx (4, 35, 36), (*ii*) long-term posttetanic potentiation (37), and (*iii*) formation of dendritic spines (37). In addition, a prolonged stimulation of excitatory amino acid receptors leads to high levels of free cytosolic $Ca^{2+}$ (38), which, by a number of mechanisms, all presumably related to the activation of $Ca^{2+}$-dependent enzymes, cause neuronal toxicity and death (19).

It has been proposed that the edema that surrounds cerebral ischemic areas leads to a protracted uncontrolled increase in extraneuronal glutamate, resulting in a continuous stimulation of excitatory amino acid receptors and consequent perifocal neuronal degeneration (12, 24, 25). The idea that persistent stimulation of glutamate receptors may cause damage to neurons after brain ischemia has prompted the study of the beneficial effects of compounds that prevent the activation of NMDA-sensitive glutamate receptors. PCP and MK-801, which are potent noncompetitive antagonists of NMDA-sensitive glutamate receptors, prevent neuronal death induced by glutamate in primary neuronal cultures (19) and *in vivo* (12, 25).

However, the drawbacks of such pharmacological intervention are that MK-801 is both a dissociative anesthetic and a selective antagonist for the NMDA-sensitive glutamate receptor. Presumably, this is not the only excitatory amino acid receptor responsible for neuronal death during ischemia (19, 39).

Here, in primary neuronal culture of neonatal rat cerebellum and cortex, we show that gangliosides, which belong to a group of naturally occurring glycosphingolipids, prevent equally well glutamate and kainate neurotoxicity and neuronal death secondary to the stimulation of both types of excitatory amino acid receptors without blocking the ionotropic and metabolotropic signal transduction at these receptors.

Cerebellar granule cells and cortical neurons in culture respond to the standardized application of glutamate and kainate with early signs of neurotoxicity, followed by slowly occurring neuronal death that terminates after 24 hr. In the absence of $Mg^{2+}$, glutamate has an $LD_{50}$ of 10 μM and is ≈10 times more efficacious than kainate ($LD_{50}$, ≈100 μM), whereas, in the presence of 1.2 mM $Mg^{2+}$, kainate is more efficacious than glutamate in eliciting neuronal death. Since kainate acts at specific excitatory amino acid receptors that are stimulated by neither glutamate nor NMDA, and since $Mg^{2+}$ is physiologically present in brain, one cannot disregard the importance of controlling the neuronal death caused

by kainate receptor stimulation in testing drugs to be used in the treatment of neuronal degeneration in brain areas perifocal to cerebral ischemias.

PCP and MK-801 completely protect against the neurotoxicity induced by glutamate (doses 50 times the $LD_{50}$ value) but only partially protect neurons against kainate-induced neurotoxicity. In contrast to PCP and MK-801, GT1b, GD1b, and GM1 (pretreatment for 2 hr followed by washing) exert complete and insurmountable protection against glutamate- and kainate-induced neurotoxicity.

Interestingly, the protective actions of gangliosides relate to their accumulation in neuronal membranes, while they do not need to be present in the culture media at the time of glutamate application. In contrast, such a presence is important for the protective action of PCP and MK-801.

The abundance of sialic acid groups in the structure of gangliosides is an important determinant of their potency. GT1b (three sialic acids) is slightly more potent than GD1b (two sialic acids) and GM1 (one sialic acid), while the asialo-GM1 is ineffective.

The observation that gangliosides are equally effective against glutamate- and kainate-induced neurotoxicity, while they fail to block glutamate-gated cationic currents or the increase in inositol phospholipid hydrolysis, indicate that the site of action of these compounds is on neither the transmitter recognition sites, the cationic channels, nor the mechanism that couples the transmitter recognition site with phospholipase C, but rather is at the sites of action of the second messenger generated by the signal transduction. In this regard, two glutamate-evoked responses seem particularly relevant: the $Ca^{2+}$-dependent translocation of PKC (10), and the expression of c-fos mRNA (9) and the nuclear accumulation of the c-fos protein (16). Gangliosides fail to prevent the nuclear accumulation of c-fos protein but are potent antagonists of glutamate- and kainate-mediated PKC translocation.

Several laboratories have reported that glycosphingolipids block PKC activity *in vitro* (40, 41). We recently reported that gangliosides antagonize phosphorylation by the PKC that is activated and translocated by glutamate (10). In primary cultures of cerebellar granule neurons, there is a good correlation between ganglioside inhibition of glutamate- and kainate-induced PKC translocation and neuronal death, supporting the view that gangliosides act by counteracting the activation and/or translocation of $Ca^{2+}$-dependent enzymes (i.e., PKC, proteases) elicited by prolonged stimulation of excitatory amino acid receptors.

Since gangliosides can prevent glutamate- and kainate-induced neurotoxicity without affecting receptor-operated signal transduction (i.e., inositol phospholipid hydrolysis, opening of cationic channels), these molecules could be considered a class of negative modulators of specific enzyme activation caused by the persistent stimulation of excitatory amino acid receptors. They could prevent excitatory amino acid-induced neuronal death following brain ischemia by a mechanism that, differently from PCP, does not modify signal transduction at excitatory amino acid receptors, but inhibits specific responses (PKC translocation, for instance) generated by second messenger(s) produced during continuous stimulation of the excitatory amino acid receptors. Hence, gangliosides do not affect glutamate receptor operation in normal brain areas but inhibit the consequences of uncontrolled and persistent glutamate receptor activation in brain tissue surrounding ischemic areas. Because their effect is insurmountable and long-lasting (24 hr *in vitro*), they could specifically block the sequelae of events that induce neurotoxicity.


1. Nowak, L., Bregestovski, P., Ascher, P., Herbet, A. & Prochiantz, A. (1984) *Nature (London)* **307,** 462–465.
2. MacDermott, A. B., Mayer, M. L., Westbrook, G. L., Smith, S. J. & Barker, J. L. (1986) *Nature (London)* **321,** 519–522.
3. Jahr, C. E. & Stevens, C. F. (1987) *Nature (London)* **325,** 522–525.
4. Wroblewski, J. T., Nicoletti, F., Fadda, E. & Costa, E. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 5068–5072.
5. Nicoletti, F., Wroblewski, J. T., Novelli, A., Alho, H., Guidotti, A. & Costa, E. (1986) *J. Neurosci.* **6,** 1905–1911.
6. Sladeczek, F. J., Pin, J. P., Recasens, M., Bockaert, J. & Weiss, S. (1985) *Nature (London)* **317,** 717–719.
7. Lazarewic, J., Wroblewski, J. T., Palmer, M. & Costa, E. (1988) *Neuropharmacology* (1968) **27,** 765–769.
8. Novelli, A., Nicoletti, F., Wroblewski, J. T., Alho, H., Costa, E. & Guidotti, A. (1987) *J. Neurosci.* **7,** 40–47.
9. Szekely, A. M., Barbaccia, M. L. & Costa, E. (1987) *Neuropharmacology* **26,** 1779–1782.
10. Vaccarino, F., Guidotti, A. & Costa, E. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 8707–8711.
11. Anis, N. A., Berry, S. C., Burton, N. R. & Lodge, D. (1983) *Br. J. Pharmacol.* **79,** 565–575.
12. Kemp, J. A., Foster, A. C. & Wang, H. F. (1987) *Trends Neurosci.* **10,** 294–298.
13. Bertolino, M., Vicini, S., Mazzetta, J. & Costa, E. (1988) *Neurosci. Lett.* **4,** 351–355.
14. Johnson, J. W. & Ascher, P. (1987) *Nature (London)* **325,** 529–531.
15. Bertolino, M., Vicini, S. & Costa, E. (1988) *Neurosci. Abstr.*, in press.
16. Sagar, S. M., Sharp, F. R. & Curran, T. (1988) *Science* **240,** 1328–1331.
17. Akers, R. F., Lovinger, D. M., Colley, P. A., Linden, D. J. & Routtenberg, A. (1986) *Science* **231,** 587–589.
18. Connor, J. A., Wadman, W. J., Hockberger, P. E. & Wong, R. K. (1988) *Science* **240,** 649–653.
19. Rothman, S. M. & Olney, J. W. (1987) *Trends Neurosci.* **10,** 299–302.
20. Coyle, J. T., Bird, S. J., Evans, R. H., Gulley, R. L., Nadler, J. W., Niklas, W. J. & Olney, J. W. (1981) *Neurosci. Res. Program Bull.* **19,** 330–427.
21. Choi, D. W., Maulucci-Gedde, M. M. & Kriegstein, A. R. (1987) *J. Neurosci.* **7,** 357–368.
22. Choi, D. W. (1987) *J. Neurosci.* **7,** 369–379.
23. Choi, D. W., Koh, J. Y. & Peters, S. (1988) *J. Neurosci.* **8,** 185–196.
24. Simon, R. P., Swan, J. H., Griffiths, T. & Meldrum, B. S. (1984) *Science* **226,** 850–852.
25. Gill, R., Foster, A. C. & Woodruff, G. N. (1987) *J. Neurosci.* **7,** 3343–3349.
26. Vaccarino, F. M., Alho, H., Santi, M. R. & Guidotti, A. (1987) *J. Neurosci.* **7,** 65–76.
27. Alho, H., Ferrarese, C., Vicini, S. & Vaccarino, F. (1988) *Dev. Brain Res.* **39,** 193–204.
28. Jones, K. H. & Senft, J. A. (1985) *J. Histochem. Cytochem.* **33,** 77–79.
29. Rotman, B. & Papermaster, B. W. (1966) *Proc. Natl. Acad. Sci. USA* **55,** 134–141.
30. Krishan, J. (1975) *Cell Biol.* **66,** 188–193.
31. Chigorno, V., Sonnino, S., Ghidoni, R. & Tettamanti, G. (1982) *Neurochem. Int.* **4,** 397–404.
32. Svennerholm, L. (1957) *Biochim. Biophys. Acta* **25,** 604–611.
33. Curran, T., VanBauvveren, C., Ling, N. & Verma, I. M. (1986) *Mol. Cell. Biol.* **5,** 167–172.
34. Kumakura, K., Guidotti, A. & Costa, E. (1979) *Mol. Pharmacol.* **16,** 865–876.
35. MacDermott, A. B. & Dale, N. (1987) *Trends Neurosci.* **10,** 280–283.
36. Murphy, S. N., Thayer, S. A. & Miller, R. J. (1987) *J. Neurosci.* **7,** 4145–4158.
37. Collingridge, G. L. & Bliss, T. V. P. (1987) *Trends Neurosci.* **10,** 288–293.
38. Wong, E. H. F., Kemp, J. A., Priestly, T., Knight, A. R., Woodruff, G. N. & Iverseen, L. L. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 7104–7108.
39. Foster, A. C., Gill, R., Kemp, J. A. & Woodruff, G. N. (1987) *Neurosci. Lett.* **76,** 307–311.
40. Hannun, Y. A. & Bell, R. M. (1987) *Science* **235,** 670–674.
41. Kruetter, D., Kim, J. Y. H., Goldenring, J. R., Rasmussen, H., Ukomadu, C., DeLorenzo, R. J. & Yu, R. K. (1987) *J. Biol. Chem.* **262,** 1633–1634.