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N-Methyl-D-aspartate Exposure Blocks Glutamate Toxicity in Cultured Cerebellar Granule Cells

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SUMMARY

Exposure of cultured cerebellar granule cells to glutamate results in a concentration-dependent ($EC_{50} = 22.7 \pm 0.4$ μM) and delayed (24–72 hr) neurotoxicity, which is blocked by the specific *N*-methyl-D-aspartate (NMDA) receptor antagonists 2-amino-5-phosphovalerate and MK-801 but is unaffected by the non-NMDA receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione and 6,7-dinitroquinoxaline-2,3-dione. Although glutamate toxicity in these cells is mediated by the NMDA subtype of glutamate receptor, pretreatment of cerebellar granule cells with subtoxic concentrations of NMDA markedly antagonizes the neurotoxic actions of glutamate, with an IC_{50} of 55 ± 4 μM. The neuroprotective effect of NMDA requires a preincubation time of approximately 120 min to be fully manifested and does not require the presence of NMDA during glutamate exposure. These data demonstrate that NMDA receptors mediate both neurotoxicity and neuroprotection in cerebellar granule cells. Among four glutamate receptor agonists tested (NMDA, quisqualate, ibotenate, and kainate), only NMDA was able to provide a robust neuroprotection against glutamate toxicity. Quisqualate was neither neurotoxic nor neuroprotective, whereas ibotenate, which was nontoxic by itself, induced a small degree of neuroprotection. In contrast, kainate, which was neurotoxic to cerebellar granule cells, also provided considerable neuroprotection against glutamate toxicity. Because preincubation of cerebellar granule cells with NMDA fails to alter NMDA receptor-mediated phosphoinositide hydrolysis or the specific binding of [^{3}H]MK-801 to NMDA receptors, it appears that the neuroprotective effects of NMDA are not due to NMDA receptor desensitization.

Glutamate, the major excitatory neurotransmitter in the brain, is also a potent excitotoxin, inasmuch as prolonged exposure of most cultured neurons to micromolar concentrations leads to either rapid or delayed-type neurotoxicity (for review, see Ref. 1). All three major subtypes of the glutamate receptor (quisqualate, kainate, and NMDA) can mediate excitotoxicity in susceptible populations of neurons; however, NMDA receptor-mediated neurotoxicity is the most well characterized. The latter involves excessive stimulation of receptor-gated cation channels, leading to a large and sustained rise in intracellular Ca^{2+}, as well as other, as yet unknown, events that produce cell death (1–5).

Recently, it has been reported that relatively high concentrations of NMDA or glutamate fail to induce acute neurotoxicity of cultured cerebellar granule cells when cells are exposed in a buffered salt solution containing Mg^{2+} and glucose, although omission of glucose and/or Mg^{2+} from the solution results in marked NMDA or glutamate toxicity in these cells (6, 7). NMDA has also recently been reported to have neurotrophic activity in differentiating cerebellar granule cells when the latter are cultured under partially depolarizing (5–15 mM KCl) conditions, presumably due to the ability of NMDA to induce Ca^{2+} influx (8, 9). However, the neurotrophic effects of NMDA are not observed when cerebellar granule cells are cultured in a fully depolarizing concentration of K^+, which is necessary for maintaining the long term survival and state of differentiation of these cells (8, 9). Because under certain incubation conditions NMDA has neurotrophic activity in cerebellar granule cells, we examined the effects of subtoxic concentrations of NMDA on glutamate-induced toxicity in these neurons. We now report that NMDA is a potent neuroprotectant in cerebellar granule cells, in that preincubation or coincubation of these neurons with NMDA markedly attenuates glutamate-induced toxicity. The neuroprotective effect of NMDA does not appear to be due to NMDA receptor desensitization and is independent of the degree of neuronal maturation.

Materials and Methods

Cerebellar granule cells were prepared from 8-day-old Sprague Dawley rats, as previously described (10). Briefly, cells were dissociated from the cerebellum by mechanical chopping and treatment with trypsin and DNase and were then plated in polylysine-precoated Costar

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; APV, 2-amino-5-phosphovalerate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DNQX, 6,7-dinitroquinoxaline-2,3-dione; PI, phosphoinositide; DPBS, Dulbecco's phosphate-buffered saline; DIV, days *in vitro*; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MPP^+, 1-methyl-4-phenylpyridinium ion.

(Cambridge, MA) 24-well clusters, at a density of 0.75×10^6 cells/well. Dissociated cells were cultured in basal Eagle medium (GIBCO, Grand Island, NY) containing 10% fetal bovine serum and 25 mM KCl. Cytosine arabinoside (10 μM) was added 24 hr later, to arrest the growth of non-neuronal cells. It has been well established that these cultures consist of ≥90% neurons after 8 DIV. For routine neurotoxicity assays, glutamate (50 μM) was added directly to the culture medium when cells were 5–6 DIV, and cell loss was assessed 72 hr later by using [^{3}H]ouabain binding, fluorescein production from fluorescein diacetate, and visual inspection with phase contrast microscopy (see below). For characterization of the neuroprotective effects of NMDA, the latter was routinely added 120 min before glutamate exposure and was coincubated with glutamate in the culture medium during the course of the experiment.

The specific binding of [^{3}H]ouabain to the Na^+,K^+-ATPase of intact cerebellar granule cells was measured using the method of Markwell *et al.* (11), except that the concentration of [^{3}H]ouabain (29 Ci/mmol; New England Nuclear, Boston, MA) was reduced to 25 nM. Nonspecific binding was measured in the presence of 12.5 μM ouabain, which represented no more than 5% of total binding. This method has been shown to be a simple, rapid, and sensitive technique to quantify neurons in mixed cultures and is particularly useful in cultures of cerebellar granule cells, which contain ≤5% glial cells (12). Cell survival was also assayed by measuring the production of fluorescein, formed from fluorescein diacetate by endogenous esterase present only in living cells, as described previously (13), with minor modification. Cells grown in 24-well clusters were washed with DPBS and then labeled with 0.3 ml of 10 μg/ml fluorescein diacetate (Sigma, St. Louis, MO), in the buffer solution described above. After incubation for 20 min at 37°, the reaction was terminated by addition of 1% sodium dodecyl sulfate in 5 mM Tris·HCl (pH 7.4). The fluorescein formed was measured with a Perkin Elmer LS-5 luminescence spectrometer. Visual assessment of neuronal morphology, using phase contrast microscopy, was carried out in all experiments.

NMDA receptor-mediated PI hydrolysis was measured in cells grown in 35-mm culture dishes, as described previously (14), with minor modifications. Cells were incubated for 20–24 hr with *myo*-[^{3}H]inositol (2.5 μCi/ml, 12 Ci/mmol; New England Nuclear) and then washed and preincubated in LiCl (5 mM)-containing physiological saline solution without added Mg^{2+} (118 mM NaCl, 4.7 mM KCl, 3.0 mM $CaCl_2$, 1.2 mM KH_2PO_4, 0.5 mM EDTA, 10 mM glucose, 20 mM HEPES, pH 7.4). The reaction was allowed to proceed for 45 min at 37° after stimulation with 100 μM NMDA or 10 μM glutamate. The accumulation of [^{3}H]inositol monophosphate was measured by anion-exchange chromatography (14). [^{3}H]MK-801 binding to intact cerebellar granule cells was measured in cultures grown in 24-well clusters. Cells were washed twice with 0.5 ml of DPBS and then incubated in DPBS containing 1 nM [^{3}H]MK-801 (24 Ci/mmol; New England Nuclear), at 2°, for 2 hr. The binding was terminated by washing the wells three times with DPBS. Nonspecific binding was measured in the presence of 10 μM unlabeled MK-801 and represented 40–50% of total binding.

Results

Exposure of cerebellar granule cells for 3 days to glutamate, in culture medium containing physiological concentrations of Mg^{2+} and glucose, resulted in a concentration-dependent delayed toxicity, as assessed by phase contrast microscopy and quantified by the loss of specific [^{3}H]ouabain binding (Fig. 1A). The maximal extent of glutamate-induced neuronal death was approximately 60%, and the EC_{50} value for glutamate (the concentration required to produce 50% of the maximal effect) was estimated to be 22.7 ± 0.4 μM (four experiments), which is similar to that reported previously by Lysko *et al.* (6), measured in the absence of glucose. The neurotoxic effects of glutamate were completely blocked by APV (200 μM) and MK-801 (10 μM) but not by the non-NMDA receptor antagonists CNQX (10 μM) and DNQX (10 μM) (Fig. 2). Exposure of cerebellar granule cells to APV, MK-801, CNQX, or DNQX alone did not produce significant neurotoxicity (data not shown). NMDA alone at concentrations of ≤100 μM had no effect on neuronal viability. Interestingly, pretreatment of cerebellar granule cells with NMDA (100 μM) almost completely blocked the neurotoxicity of glutamate observed at all concentrations examined (Fig. 1A). Similar neurotoxic effects of glutamate and neuroprotective effects of NMDA were observed when neuronal viability was measured by quantifying the fluorescein formed by deesterification of fluorescein diacetate in living cells (Fig. 1B). Morphological examination of these cultures using phase contrast microscopy revealed that glutamate toxicity resulted in the typical appearance of dead cells, which were round, smaller, and translucent, and a marked disintegration of neuronal proc-

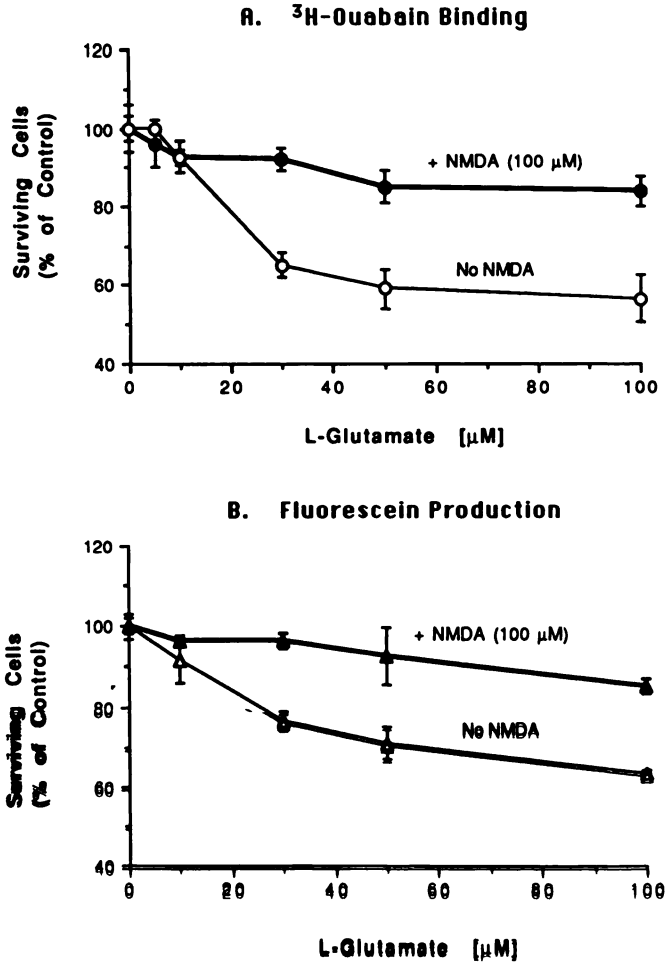

Fig. 1. Antagonism of glutamate-induced neurotoxicity in cerebellar granule cells by NMDA. Cultured cerebellar granule cells, after 5–6 DIV, were exposed to different concentrations of glutamate for 72 hr, and the neurotoxicity was determined by measuring the specific binding of [^{3}H]ouabain to intact cells (A) and by measuring the production of fluorescein from fluorescein diacetate by living cells (B). NMDA (100 μM) was added 2 hr before glutamate exposure. Details of experimental procedures are as described in Materials and Methods. The data shown represent the mean ± standard error of quadruplicate determinations and are from a representative experiment, which was repeated four times with similar results. Please note that the *ordinates* begin at 40%. The untreated control values (100%) for specific [^{3}H]ouabain binding and for fluorescein production were 23,077 ± 1,578 dpm (A) and 173 ± 3 fluorescence units (B), respectively.

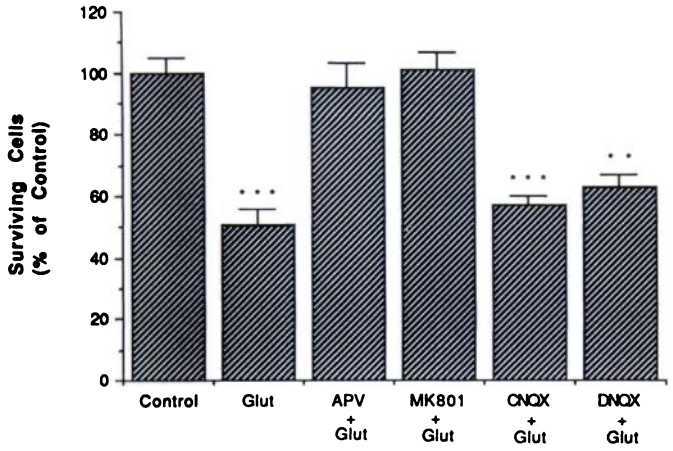

Fig. 2. Effects of NMDA and non-NMDA receptor antagonists on glutamate-induced neurotoxicity. Cultured cerebellar granule cells were exposed to 50 μM glutamate (*Glut*) in the absence or presence of APV (200 μM), MK-801 (10 μM), CNQX (10 μM), or DNQX (10 μM). The neurotoxicity was determined 3 days later by measuring specific binding of [^{3}H]ouabain to intact cells, as described in Materials and Methods. The data shown represent the mean ± standard error of quadriplicate determinations and are from a representative experiment, which was repeated three times with similar results. The untreated control (100%) value for [^{3}H]ouabain binding was 28,680 ± 1,435 dpm. ***, $p < 0.001$; **, $p < 0.01$, compared with the control, by Student's *t* test.

esses and cell bodies (Fig. 3B). Exposure of cerebellar granule cells to NMDA (100 μM) alone failed to affect neuronal survival adversely (Fig. 3C), confirming a previous report (8). However, pretreatment of cerebellar granule cells with NMDA markedly antagonized the morphological changes associated with glutamate toxicity (Fig. 3D).

To determine whether the neuroprotective effect of NMDA is selective for the NMDA subtype of glutamate receptors, we examined three other excitatory amino acid receptor agonists (quisqualate, ibotenate, and kainate) as possible neuroprotective agents (Fig. 4). Among the four agonists tested at 100 μM, only NMDA provided complete neuroprotection. Quisqualate was neither neurotoxic nor neuroprotective (Fig. 4). Ibotenate was nontoxic by itself but induced a small degree of neuroprotection. On the other hand, kainate (100 μM), which killed approximately 40% of granule cells, also significantly reduced the toxicity induced by glutamate alone (Fig. 4). The kainate-induced neurotoxicity of cerebellar granule cells was blocked by CNQX (10 μM) but was unaffected by APV (200 μM) and MK-801 (1 μM) (data not shown), indicating that the toxicity is not due to kainate-induced release of glutamate, which, in turn, acts on NMDA receptors. Moreover, we found that the toxic effect of kainate was not influenced by the presence of NMDA (100 μM) (results not shown). The neuroprotective effect of NMDA against glutamate toxicity was concentration dependent ($IC_{50} \simeq 55 \pm 4$ μM; three experiments) (Fig. 5A) and required approximately 120 min to be fully manifested (Fig. 5B). NMDA was only partially effective as a neuroprotectant when added simultaneously with glutamate and was virtually ineffective when added 2 hr after the excitotoxin (Fig. 5B). To examine the possibility that the neuroprotective effect of NMDA is related to agonist-induced receptor desensitization resulting from agonist preexposure, NMDA receptor-mediated PI hydrolysis was assayed in a Mg^{2+}-free solution. Preexposure of cerebellar granule cells to NMDA (100 μM) did not significantly alter basal or NMDA/glutamate-induced [^{3}H]inositol monophosphate accumulation (Fig. 6A). Moreover, [^{3}H]MK-801 binding to NMDA receptors of intact cerebellar granule cells was unchanged by NMDA pretreatment for 120 min (Fig. 6B). We have also observed that preexposure of granule cells to NMDA, followed by drug wash-out (as opposed to coincubation with glutamate), also resulted in significant neuroprotection (Fig. 7), indicating that the continuous presence of NMDA is not required for induction of the neuroprotective state. However, the effectiveness of NMDA-induced neuroprotection was reduced to some extent after removal of NMDA.

In order to examine the possibility that NMDA-induced neuroprotection is the result of enhanced uptake of glutamate, thereby reducing the availability of this excitotoxin, we measured the uptake of D-[^{3}H]aspartate (which labels glutamate uptake via the neuronal glutamate transporter) by cerebellar granule cells, with and without preexposure to NMDA (100 μM). The uptake of D-[^{3}H]aspartate into cerebellar granule cells was not significantly affected by NMDA pretreatment for time periods ranging from 2 to 72 hr (data not shown). To determine whether the neuroprotective effect of NMDA is dependent on the age or maturity of the granule cell neuron, we have also

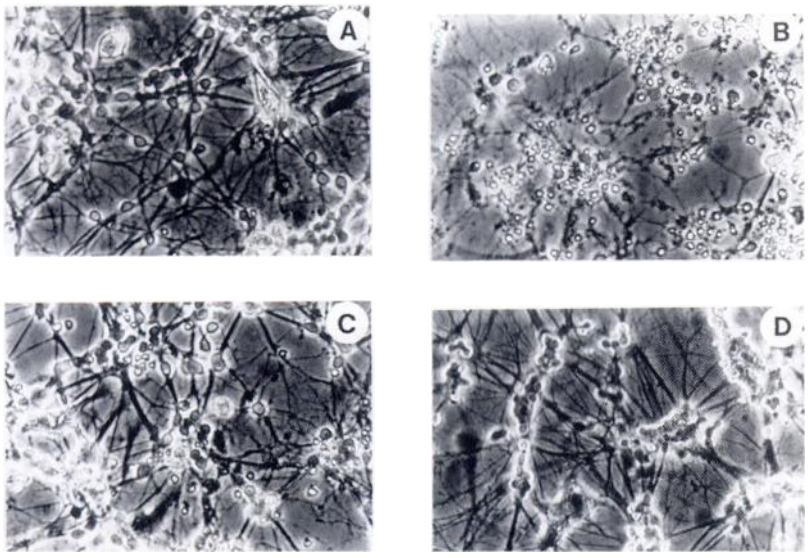

Fig. 3. NMDA-induced neuroprotection of cerebellar granule cells, as revealed by phase contrast microscopy. Cultured cerebellar granule cells were pretreated with NMDA (100 μM) for 2 hr. Glutamate (50 μM) or control vehicle was then added, and cell morphology was examined 3 days later, using a phase-contrast microscope, at 200× magnification. A, Untreated control; B, glutamate alone; C, NMDA alone; D, NMDA in combination with glutamate. Note the marked disintegration of neuronal processes and the change in neuronal perikarya morphology after glutamate exposure (B) and the essentially normal morphology in NMDA-pretreated cells (D, compared with A and C).

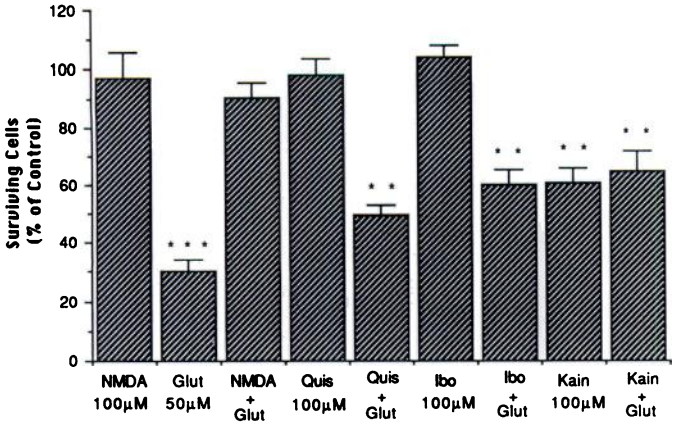

Fig. 4. Comparison of the effects of NMDA, quisqualate, ibotenate, and kainate on glutamate-induced neurotoxicity. Cultured cerebellar granule cells were pretreated with 100 μM NMDA, quisqualate (*Quis*), ibotenate (*Ibo*), or kainate (*Kain*) and then exposed to 50 μM glutamate (*Glut*) for 3 days. Surviving neurons were then quantified by measuring [^{3}H]ouabain binding to intact cells. The data shown represent the mean ± standard error of three independent quadruplicate experiments. ***, $p < 0.001$; **, $p < 0.01$, compared with the untreated controls, by Student's *t* test. A multiple comparison procedure after analysis of variance was also used to test the pairwise difference among the means. The Bonferroni *T* was used at an overall level of 0.05. The pairs that are statistically different with this procedure are glutamate versus NMDA; glutamate versus NMDA plus glutamate; glutamate versus quisqualate; glutamate versus ibotenate; glutamate versus ibotenate plus glutamate; glutamate versus kainate; glutamate versus kainate plus glutamate; NMDA versus quisqualate plus glutamate; NMDA versus kainate; NMDA versus kainate plus glutamate; quisqualate versus quisqualate plus glutamate; and ibotenate versus ibotenate plus glutamate. The pairs that are not significant are glutamate versus quisqualate plus glutamate; NMDA versus NMDA plus glutamate; and kainate versus kainate plus glutamate.

studied glutamate toxicity at various times after seeding. Initially, glutamate was added sequentially to cultures and all cells were harvested at 8 DIV, such that they varied in the time of exposure to the excitotoxin. The results showed that glutamate toxicity was evident within the first day after exposure but the toxicity was not fully manifested until 3–5 days later (Fig. 8). Treatment of cells for 6 or 7 days (i.e., addition of glutamate after 3 or 2 DIV, respectively) produced little or no cell death, consistent with a previous suggestion that glutamate is not neurotoxic and may even be neurotrophic when added to newly plated cerebellar granule cells, presumably because NMDA receptors are not yet expressed (15). Because mature cerebellar granule cells were more commonly used for pharmacological and biochemical studies, the neuroprotective effects of NMDA were compared in immature (6–9 DIV) versus mature (10–13 DIV) cultures (Fig. 9). NMDA-induced neuroprotection against glutamate (50 and 100 μM)-induced toxicity was seen in both preparations, suggesting that the neuroprotective effect could be induced in neurons at various stages of neuronal maturation or differentiation.

Discussion

In the present study, we have confirmed that glutamate added to primary cultures of cerebellar granule cells, maintained in serum-containing medium, results in delayed-type neurotoxicity that is mediated by NMDA receptors. Paradoxically, exposure of cerebellar granule cells to subtoxic concentrations of NMDA antagonizes glutamate toxicity, in a concentration- and time-dependent manner. Both glutamate-induced neurotoxicity

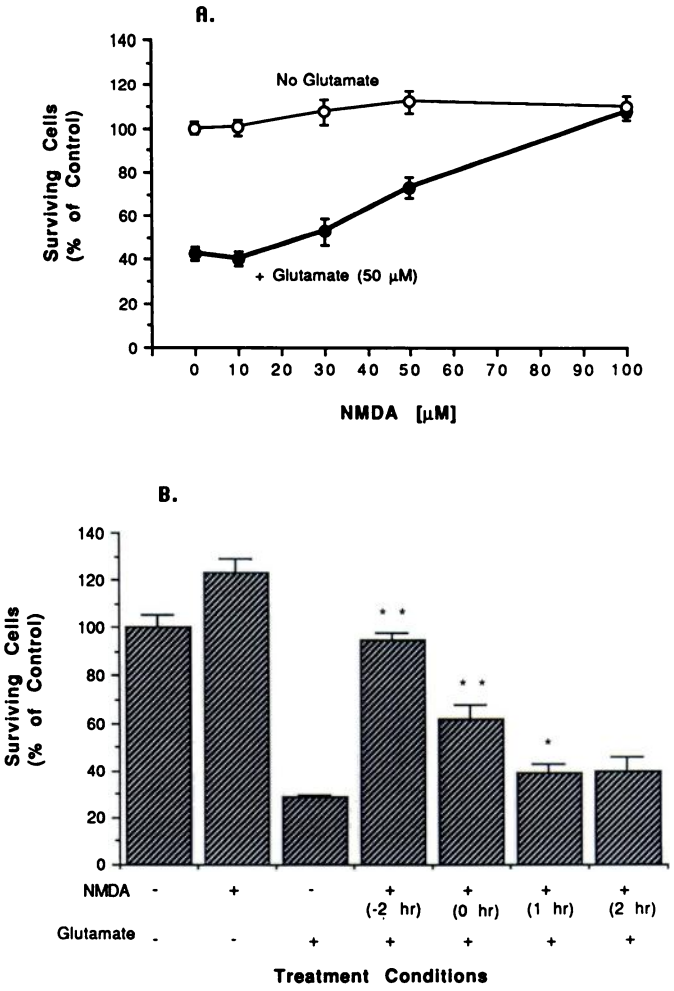

Fig. 5. Antagonism of glutamate-induced neurotoxicity by NMDA: effects of NMDA concentration and preincubation time. A, Cerebellar granule cells were pretreated with the indicated concentrations of NMDA for 2 hr and then exposed to control vehicle or glutamate (50 μM). Note that NMDA alone is devoid of neurotoxic effects at concentrations of ≤100 μM and dose-dependently blocks glutamate toxicity. B, Cerebellar granule cells were exposed to NMDA (100 μM), glutamate (50 μM), or NMDA (100 μM) plus glutamate (50 μM). The time of addition of NMDA with respect to that of glutamate is shown in parentheses. In both A and B, specific [^{3}H]ouabain binding to intact cells was determined 3 days after glutamate exposure, as described in the text. Data shown are mean ± standard error of quadruplicate determinations from a representative experiment, which was repeated three to five times with similar results. The control (100%) value for specific [^{3}H]ouabain binding was 30,611 ± 1,674 dpm. **, $p < 0.01$; *, $p < 0.05$, compared with results of cells treated with glutamate alone (Student's *t* test).

and NMDA-induced neuroprotection were demonstrated by using three independent methods for quantifying neuronal survival.

The glutamate-induced toxicity of cerebellar granule cells observed in our experiments was completely blocked by the NMDA receptor antagonists MK-801 and APV but not by the non-NMDA receptor antagonists CNQX and DNQX, confirming previous reports that glutamate kills cerebellar granule cells via a NMDA receptor-mediated mechanism (6, 15). However, in our experiments, it should be emphasized that glutamate was added to culture medium that contained 10% fetal bovine serum and physiological concentrations of Mg^{2+} and glucose and that neurotoxicity was not evident for several hours or fully manifested until approximately 72 hr. In other studies,

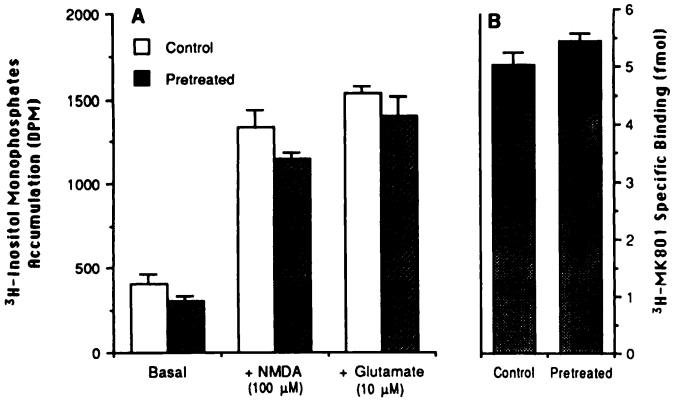

Fig. 6. Lack of significant effects of NMDA pretreatment on PI turnover and [^{3}H]MK-801 binding. Cultured cerebellar granule cells were pretreated with 100 μM NMDA for 2 hr, as described in Materials and Methods. A, PI turnover was assayed in the absence or presence of a stimulus; B, specific binding of [^{3}H]MK-801 to intact cells was measured. Details of the experimental conditions are as described in Materials and Methods. The data shown represent the mean ± standard error of quadruplicate determinations from a representative experiment, which was repeated at least three times with similar results. The specific activity of [^{3}H]MK-801 was 45 dpm/fmol.

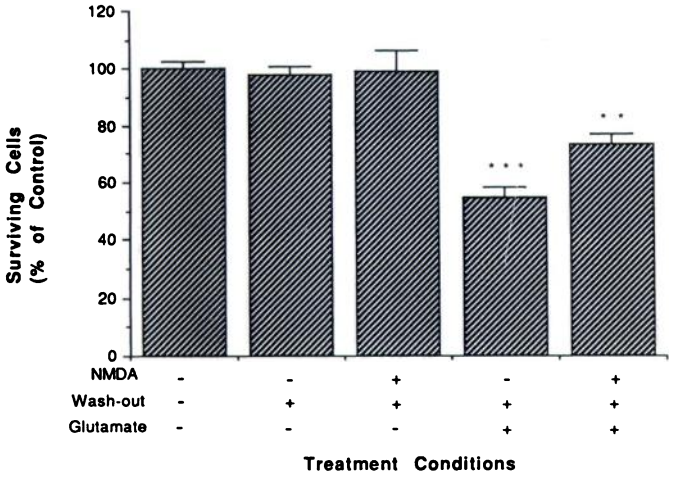

Fig. 7. Effects of NMDA wash-out on glutamate-induced neurotoxicity. Experimental conditions were as described in Materials and Methods, except that NMDA was removed after 2 hr of treatment by aspiration of the medium from the well. Cells were washed twice with conditioned medium derived from parallel cultures and were cultivated in the absence or presence of 50 μM glutamate for 48 hr before determination of neuronal survival by measurement of [^{3}H]ouabain binding. The data shown are the mean ± standard error of five independent quadruplicate experiments. Note that the drug wash-out with conditioned medium significantly reduced the glutamate-induced neurotoxicity, whereas wash-out *per se* did not cause neuronal death. ***, $p < 0.001$, compared with the untreated control; **, $p < 0.01$, compared with the glutamate-treated sample (Student's *t* test). A multiple comparison using analysis of variance was also used to test the pairwise difference among the means. The Bonferroni *T* was used at an overall level of 0.05. The pairs that are statistically significantly different with this procedure are untreated versus wash-out plus glutamate; untreated versus NMDA plus wash-out plus glutamate; wash-out plus glutamate versus NMDA plus wash-out plus glutamate; and NMDA plus wash-out versus NMDA plus wash-out plus glutamate.

brief exposure (≤30 min) of cerebellar granule cells to glutamate in a physiological salt solution without Mg^{2+} was sufficient to bring about a more rapid toxicity (3). At present, it is unclear why glutamate, but not NMDA, is so profoundly neurotoxic to cerebellar granule cells under our assay conditions. Although

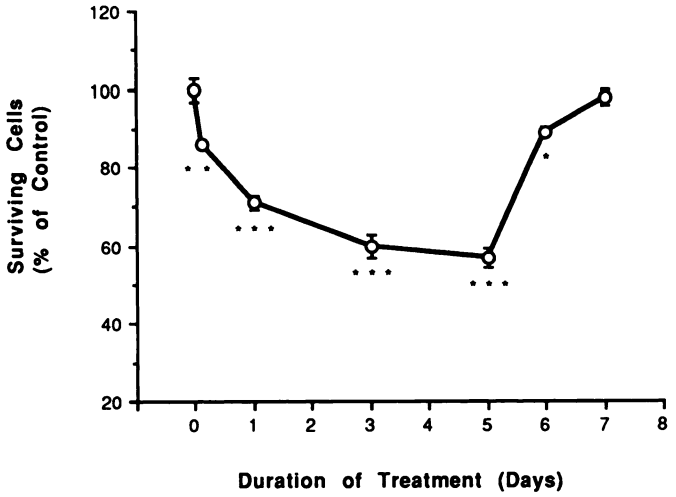

Fig. 8. Time-dependent neurotoxicity induced by glutamate. The length of glutamate exposure was varied by adding the excitotoxin sequentially to cultured cerebellar granule cells (i.e., cells treated for the longest duration were treated first in their culture period). All cultures were harvested at 8 DIV for determination of cell survival by measurement of the specific binding of [^{3}H]ouabain, as described in Materials and Methods. The data shown represent the mean ± standard error of percentage of the untreated control. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$, compared with the 0 time control (Student's *t* test).

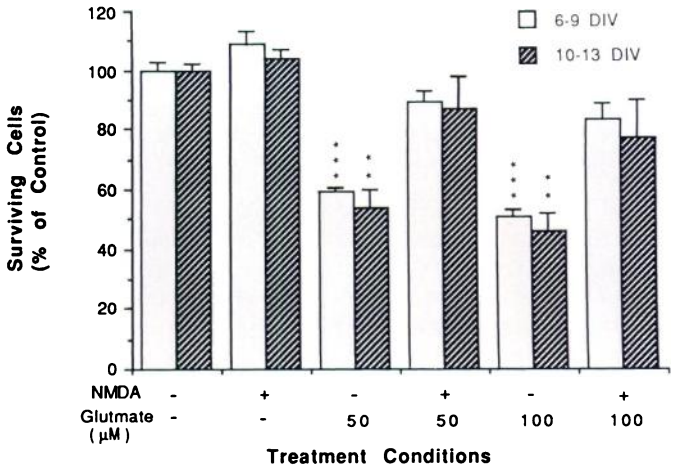

Fig. 9. Comparison of NMDA-dependent neuroprotection in immature and mature granule cells. Experimental conditions were as described in Materials and Methods, except that cells at 6 or 10 DIV were treated with glutamate (50 or 100 μM) and the neurotoxicity was assayed 3 days later, by measurement of [^{3}H]ouabain binding. The data shown represent the mean ± standard error of three independent quadruplicate experiments. The untreated control (100%) values for the specific binding were 28,950 ± 960 and 35,680 ± 1130 dpm for 6–9 and 10–13 DIV, respectively. Note that the NMDA neuroprotective effects were evident in both immature (6–9 DIV) and mature (10–13 DIV) cultures. ***, $p < 0.001$; **, $p < 0.01$, compared with the untreated control.

Mg^{2+} (0.8 mM) was included in our incubation culture medium, the presence of depolarizing concentrations of K^+ (25 mM) should have reduced the Mg^{2+}-induced blockade of NMDA receptor-gated cation channels (16). One might also argue that glutamate toxicity could be due to the concerted actions of both NMDA and non-NMDA receptors to trigger events such as a rise in intracellular Ca^{2+} or the release of endogenous glutamate. This possibility, however, seems unlikely, because the non-NMDA receptor antagonists CNQX and DNQX failed to affect glutamate-induced neurotoxicity. We have also found

that, even in the presence of 100 μM quisqualate or (*trans*)-1-aminocyclopentyl-1,3-dicarboxylic acid, a selective agonist of metabotropic glutamate (quisqualate) receptors, NMDA (10–100 μM) failed to kill granule cell neurons (data not shown), suggesting that activation of metabotropic receptors by glutamate fails to potentiate the toxic effects of NMDA receptor activation.

The neuroprotective effect of NMDA against glutamate toxicity is an interesting and unexpected finding. Because the neuroprotective effect of NMDA was most apparent when cells were preincubated with NMDA before glutamate exposure, it is possible that NMDA-induced neuroprotection is due to desensitization and/or down-regulation of NMDA receptors that mediate glutamate toxicity. However, we believe this explanation is unlikely, for several reasons. First, pretreatment of cerebellar granule cells with NMDA does not significantly reduce NMDA receptor-mediated PI hydrolysis or alter the number of NMDA receptors measured with [^{3}H]MK-801. We also found that similar pretreatment fails to alter NMDA-induced release of preloaded D-[^{3}H]aspartate from granule cells.[1] Second, the continuous presence of NMDA does not seem to be required for the neuroprotective effect, inasmuch as significant protection can be achieved by using cells pretreated with NMDA, with drug wash-out before glutamate exposure. Third, in separate studies, we found that NMDA is also able to prevent granule cell death induced by long term treatment with the psychotropic drug carbamazepine (30–100 μM) (12) or with MPP^+ (17). Because NMDA receptor antagonists do not block neurotoxicity induced by MPP^+ (17) or carbamazepine in granule cells,[1] it appears that desensitization cannot account for the neuroprotective actions of NMDA against these neurotoxins. Moreover, the neuroprotective effects of NMDA against glutamate and MPP^+ toxicity are blocked by either an RNA or protein synthesis inhibitor, suggesting that NMDA receptor activation can induce the expression of a neuroprotective protein (17). It is also noteworthy that rat cortical and hippocampal neurons are profoundly susceptible to the neurotoxic actions of both glutamate and NMDA (1, 4). In these neurons, NMDA is a potent neurotoxin and has no neuroprotective actions over a broad range of concentrations.[2] Thus, these neurons, which presumably would respond to desensitizing concentrations of glutamate/NMDA in a manner similar to cerebellar granule cells, are not protected by preincubation with NMDA and, therefore, receptor desensitization is, again, an unlikely explanation for NMDA-induced neuroprotection.

What are the cellular mechanisms responsible for the neuroprotective effects of NMDA reported here? We cannot exclude the possibility that at least two distinct subtypes of NMDA antagonist-sensitive receptors exist, one being responsible for neurotoxicity and the other for neuroprotection. It is noteworthy that the existence of at least two pharmacologically distinct NMDA receptor subtypes in the rat brain has been previously suggested (18–21) and that cerebellar NMDA receptors have lower affinity for MK-801 and diminished regulation of [^{3}H]MK-801 binding by glutamate and glycine. Alternatively, previous studies have demonstrated that the trophic action of NMDA observed in cerebellar granule cells results from an influx of Ca^{2+} through NMDA receptor-gated channels (8) and is accompanied by increased expression of the nuclear proto-oncogene c-*fos* (22). More recently, NMDA receptor activation has also been shown to stimulate tyrosine kinase activity in cultured hippocampal cells (23), an event commonly induced by activation of growth factor receptors. Conceivably, therefore, subtoxic concentrations of NMDA may induce the expression of second and third messengers, resulting in both neurotrophic and neuroprotective actions. It remains to be determined, however, whether these NMDA-induced cellular responses underlie the neuroprotective effects of NMDA in cerebellar granule cells.

Acknowledgments

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References

1. Choi, D. W. Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. *Trends Neurosci.* **11**:465–469 (1988).
2. Cheung, J. Y., J. V. Bonventre, C. D. Malis, and A. Leaf. Calcium and ischemic injury. *N. Engl. J. Med.* **314**:1670–1676 (1986).
3. Manev, H., M. Favaron, A. Guidotti, and E. Costa. Delayed increase of Ca^{2+} influx elicited by glutamate: role in neuronal death. *Mol. Pharmacol.* **36**:106–112 (1989).
4. Meldrum, B., and J. Garthwaite. Excitatory amino acid neurotoxicity and neurodegenerative disease. *Trends Pharmacol. Sci.* **11**:379–387 (1990).
5. Garthwaite, G., and J. Garthwaite. Differential dependence on Ca^{2+} of *N*-methyl-D-aspartate and quisqualate neurotoxicity in young rat hippocampal slices. *Neurosci. Lett.* **97**:316–322 (1989).
6. Lysko, P. G., J. A. Cox, M. A. Vigano, and R. C. Henneberry. Excitatory amino acid neurotoxicity at the *N*-methyl-D-aspartate receptor in cultured neurons: pharmacological characterization. *Brain Res.* **499**:258–266 (1989).
7. Kato, K., P. S. Puttfarcken, W. E. Lyons, and J. T. Coyle. Developmental time course and ionic dependence of kainate-mediated toxicity in rat cerebellar granule cell cultures. *J. Pharmacol. Exp. Ther.* **256**:402–411 (1991).
8. Balázs, R., O. S. Jorgensen, and N. Hack. *N*-Methyl-D-aspartate promotes the survival of cerebellar granule cells in culture. *Neuroscience* **27**:437–451 (1988).
9. Balázs, R., N. Hack, O. S. Jorgensen, and C. W. Cotman. *N*-Methyl-D-aspartate promotes the survival of cerebellar granule cells: pharmacological characterization. *Neurosci. Lett.* **101**:241–246 (1989).
10. Gallo, V., M. T. Ciotti, A. Coletti, F. Aloisi, and G. Levi. Selective release of glutamate from cerebellar granule cells differentiating in culture. *Proc. Natl. Acad. Sci. USA* **79**:7919–7923 (1982).
11. Markwell, M. A. K., H. Z. Sheng, D. E. Brenneman, and S. M. Paul. A rapid method to quantify neurons in mixed cultures based on the specific binding of [^{3}H]ouabain to neuronal Na^+,K^+-ATPase. *Brain Res.* **538**:1–8 (1991).
12. Gao, X.-M., and D.-M. Chuang. Carbamazepine-induced neurotoxicity and its prevention by NMDA in cultured cerebellar granule cells. *Neurosci. Lett.* **135**:159–162 (1992).
13. Didier, M., M. Heaulme, P. Soubrié, J. Bockaert, and J.-P. Pin. Rapid, sensitive and simple method for quantification of both neurotoxic and neurotrophic effects of NMDA on cultured cerebellar granule cells. *J. Neurosci. Res.* **27**:25–35 (1990).
14. Xu, J., and D.-M. Chuang. Muscarinic acetylcholine receptor-mediated phosphoinositide turnover in cultured cerebellar granule cells: desensitization by receptor agonists. *J. Pharmacol. Exp. Ther.* **242**:233–244 (1987).
15. Schramm, M., S. Eimerl, and E. Costa. Serum and depolarizing agents cause acute neurotoxicity in cultured cerebellar granule cells: role of the glutamate receptor responsive to *N*-methyl-D-aspartate. *Proc. Natl. Acad. Sci. USA* **87**:1193–1197 (1990).
16. Mayer, M. L., G. L. Westbrook, and P. B. Guthrie. Voltage dependent block by Mg^{2+} on NMDA responses in spinal cord neurones. *Nature* (*Lond.*) **309**:261–263 (1984).
17. Marini, A. M., and S. M. Paul. *N*-Methyl-D-aspartate receptor-mediated neuroprotection in cerebellar granule cells requires new RNA and protein synthesis. *Proc. Natl. Acad. Sci. USA* **89**:655–659 (1992).
18. Monaghan, D. T., H. J. Olverman, L. Nguyen, J. C. Watkins, and C. W. Cotman. Two classes of *N*-methyl-D-aspartate recognition sites: differential distribution and differential regulation by glycine. *Proc. Natl. Acad. Sci. USA* **85**:9836–9840 (1988).
19. Reynolds, I. J., and A. M. Palmer. Regional variation in [^{3}H]-MK801 binding to rat brain *N*-methyl-D-aspartate receptors. *J. Neurochem.* **56**:1731–1740 (1991).
20. Ebert, B., E. H. F. Wong, and P. Krogsgaard-Larsen. Identification of a novel NMDA receptor in rat cerebellum. *Eur. J. Pharmacol.* **208**:49–52 (1991).
21. Yoneda, Y., and K. Ogita. Heterogeneity of the *N*-methyl-D-aspartate receptor ionophore complex in rat brain, as revealed by ligand binding techniques. *J. Pharmacol. Exp. Ther.* **259**:86–96 (1991).
22. Didier, M., P. Roux, M. Piechaczyk, B. Verrier, J. Bockaert, and J.-P. Pin.

[1] X.-M. Gao and D.-M. Chuang, unpublished observations.
[2] F. Finiels-Marlier and S. M. Paul, unpublished observations.

Cerebellar granule cell survival and maturation induced by K^+ and NMDA correlate with c-*fos* proto-oncogene expression. *Neurosci. Lett.* **107**:55–62 (1989).
23. Bading, H., and M. Greenberg. Stimulation of protein tyrosine phosphorylation by NMDA receptor activation. *Science (Washington, D. C.)* **253**:912–914 (1991).

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