Glutamate carboxypeptidase II inhibition protects motor neurons from death in familial amyotrophic lateral sclerosis models

Ghanashyam D. Ghadge*, Barbara S. Slusher†, Amos Bodner‡§, Mauro Dal Canto¶, Krystyna Wozniak†, Ajit G. Thomas†, Camilo Rojas†, Takashi Tsukamoto†, Pavel Majer†, Richard J. Miller‡, Anna Liza Monti*, and Raymond P. Roos*‖

*Department of Neurology, University of Chicago School of Medicine, Chicago, IL 60637; †Department of Research, Guilford Pharmaceuticals, Baltimore, MD 21224; and Departments of ‡Molecular Pharmacology and Biological Chemistry and ¶Pathology, Northwestern University Medical School, Chicago, IL 60611

Edited by Solomon H. Snyder, Johns Hopkins University School of Medicine, Baltimore, MD, and approved May 29, 2003 (received for review January 10, 2003)

Approximately 10% of cases of amyotrophic lateral sclerosis (ALS), a progressive and fatal degeneration that targets motor neurons (MNs), are inherited, and ≈20% of these cases of familial ALS (FALS) are caused by mutations of copper/zinc superoxide dismutase type 1. Glutamate excitotoxicity has been implicated as a mechanism of MN death in both ALS and FALS. In this study, we tested whether a neuroprotective strategy involving potent and selective inhibitors of glutamate carboxypeptidase II (GCPII), which converts the abundant neuropeptide *N*-acetylaspartylglutamate to glutamate, could protect MNs in an *in vitro* and animal model of FALS. Data suggest that the GCPII inhibitors prevented MN cell death in both of these systems because of the resultant decrease in glutamate levels. GCPII inhibition may represent a new therapeutic target for the treatment of ALS.

Amyotrophic lateral sclerosis (ALS) is a progressive and fatal degeneration of motor neurons (MNs) in the spinal cord and cerebral cortex. About 10% of ALS cases are inherited in an autosomal dominant fashion, and ≈20% of these cases of familial ALS (FALS) are caused by a mutation in copper/zinc superoxide dismutase type 1 (SOD1) (1). Mice and rats that carry mutant (MT) SOD1 as a transgene manifest a progressive MN degeneration similar to that in patients with ALS (2–4). Several lines of evidence suggest that glutamate excitotoxicity is a pathogenic mechanism in both sporadic ALS and FALS (5–13).

N-acetylaspartylglutamate (NAAG) is one of the most abundant peptides in the mammalian central and peripheral nervous system (14), is present in neuronal vesicles, is released from neurons in a calcium-dependent manner, and functions as a high-affinity agonist at the group II metabotropic glutamate receptor subtype 3 (mGluR3). Activation of mGluR3 by NAAG has been shown to inhibit glutamate release (15), increase release of transforming growth factor β (TGFβ) from glial cells, and provide neuroprotection (16). NAAG is hydrolyzed to *N*-acetylaspartate and glutamate by glutamate carboxypeptidase II (GCPII) (EC 3.4.17.21; also termed *N*-acetylated-α-linked acidic dipeptidase or NAALADase; ref. 17), an enzyme localized on the plasma membrane of glial cells with its catalytic region facing the synapse (18). Therefore, inhibition of GCPII would be expected to provide neuroprotection by means of both decreasing glutamate and increasing NAAG (19).

We hypothesized that GCPII inhibition would protect MNs expressing MT SOD1 from cell death, as well as ameliorate the MN degeneration seen in FALS transgenic mouse. For *in vitro* studies, we used 2-(phosphonomethyl)pentanedioic acid (2-PMPA), a potent and selective GCPII inhibitor (K_i = 0.2 nM; ref. 20) that has been shown to selectively reduce ischemic glutamate and provide neuroprotection in cell culture and animal models of ischemia (19), diabetic neuropathy (21), and drug abuse (22, 23). For the animal studies, we used a recently discovered thiol-based GCP II inhibitor, 2-(3-mercaptopropyl)pentanedioic acid (2-MPPA) (24). Unlike 2-PMPA, 2-MPPA is orally bioavailable (fraction of the administered dose systemically available = 65–85%), offering an advantage over 2-PMPA in animal studies that require long-term daily dosing. Our studies showed that the GCPII inhibition prevented MN cell death both *in vitro* and *in vivo*, probably because of the effect on the level of glutamate.

Methods

Materials. 2-PMPA (20) and 2-MPPA (24) were synthesized as described previously. (+)-MK801 hydrogen maleate (MK801), (2*S*)-α-ethylglutamic acid (EGLU), and 6-cyano-2,3-dihydroxy-7-nitroquinoxaline (CNQX) were obtained from Tocris Cookson; TGFβ and anti-TGFβ from Sigma; radiolabeled NAAG from Perkin–Elmer; and Hoechst 33342 from Molecular Probes.

MN/Glia Cultures and Adenovirus (AdV) Transduction. MNs were isolated and cultured from rat embryos according to previously described methods (25) with some modifications. In brief, a ventral portion of spinal cord was dissected from 14-day-old Holtzmann rat embryos, and MNs were purified by centrifugation on a 6.5% metrizamide (Sigma) cushion. MNs were planted on poly-L-lysine/laminin-coated glass coverslips for 2 h; coverslips were then incubated facing down in a 60-mm Petri dish that had a previously planted glial feeder layer. MN growth medium consisted of DMEM (Invitrogen) supplemented with D-glucose (4.5 mg/ml), Hepes buffer (12.5 mM), N2 supplement (1%, Invitrogen), chicken egg albumin (1 mg/ml, Sigma), chicken embryo extract (5%, Invitrogen), and gentamicin (0.01%, Invitrogen). Immunohistochemical analysis of the MN cultures showed that they were generally >95% pure. For the infection, six or seven coverslips containing MNs were removed from the feeder layer 7–8 days after culture and placed so that the MNs faced up in a 60-mm culture dish containing 2.5 ml of medium. An aliquot of CsCl gradient-purified AdV (26) was then added for 2 h at a multiplicity of infection of 5,000 plaque-forming units

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: ALS, amyotrophic lateral sclerosis; CXQA, 6-cyano-7-nitroquinoxaline-2,3-dione; EGLU, (2*S*)-α-ethylglutamic acid; FALS, familial ALS; MNs, motor neurons; MT, mutant; SOD1, copper/zinc superoxide dismutase type 1; NMDA, *N*-methyl-D-aspartate; GCPII, glutamate carboxypeptidase II; NAAG, *N*-acetylaspartylglutamate; mGluR; metabotropic glutamate receptor; mGluR3, mGluR subtype 3; TGFβ, transforming growth factor β; 2-PMPA, 2-(phosphonomethyl)pentanedioic acid; AdV, adenovirus; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; MK801, (+)-MK801 hydrogen maleate; 2-MPPA, 2-(3-mercaptopropyl)pentanedioic acid.

§Deceased February 28, 2003.

‖To whom correspondence should be addressed at: Department of Neurology/MC2030, University of Chicago School of Medicine, 5841 South Maryland Avenue, Chicago, IL 60637. E-mail: rroos@neurology.bsd.uchicago.edu.

per cell (assuming 1.5×10^4 neurons per seven coverslips). Mock-infected cultures were treated identically except that they received no virus. Coverslips were then returned to the culture dish. This usually resulted in >95% of cells being transduced.

Drug Treatment. Half of the growth medium was replaced with phosphate-free growth medium 1 day before AdV infection and again after AdV infection, when one or more of the following were added: 2-PMPA (10 μM); MK801 (0.2 μM); CNQX (100 μM); EGLU (100 μM), anti-TGFβ antibody (10 μg/ml).

Cell Viability Determination. Cell viability was determined by Hoechst 33342 staining (26) 3–5 days after drug treatment. Both live and dead cells were counted from 6–10 randomly selected fields per coverslip by using fragmentation of the nuclear morphology and the presence of chromatin clumps as a measure of cell death. Cell death was presented as percent survival of MNs after normalization of values to mock-transduced cells.

NAAG Analysis. MN/glia were cultured as described above and [^{3}H]NAAG [2 μCi (50 Ci/mmol; 1 Ci = 37 Gbq) per 4 ml of medium, 10 nM final concentration] was added in the presence or absence of 2-PMPA. Three days later aliquots of culture medium were lyophilized and then reconstituted in methanol/water (80:20). Undissolved particles were removed by ultracentrifugation at 100,000 $\times$ *g* for 30 min at 4°C. Samples were analyzed for NAAG and glutamate on a Packard HPLC Radiomatic detector by using an anion-exchange column (ES Industries, West Berlin, NJ) with a 0.1 M KH_2PO_4/0.1 M KCl (pH 4.5) mobile phase at 1.5 ml/min flow rate (27).

Measurement of TGFβ. Medium was collected from mock- and AdV-transduced MNs after a 3-day exposure to 2-PMPA and AdV transduction. An ELISA based on the manufacturer's protocol (Promega) was used to measure the amount of TGFβ.

Animal Studies. Starting from 37–39 days of age, low expressor G93A FALS transgenic mice received a daily oral dose of either 2-MPPA (30 mg/kg in a volume of 10 ml/kg) or a similar volume of vehicle. G93A FALS transgenic mice were kept in individual cages and allowed a regular diet and water ad libitum. Clinical signs were monitored over time by a rater who was blinded to the treatment as previously described (2).

In a separate experiment, G93A FALS transgenic mice were given daily treatment with 2-MPPA or vehicle as detailed above and then killed by total body perfusion with freshly prepared, chilled 4% paraformaldehyde on days 164, 183, 206, 241, and 260. The spinal cord was dissected out and cross sections along its entire length were postfixed in the same fixative for an additional hour and then osmicated and processed for Epon embedding. One-micrometer-thick Epon-embedded sections were cut, stained with toluidine blue, and processed for histopathological changes under light microscopy.

Results

GCPII Inhibition Protects MN/Glia Cultures from MT SOD1-Induced Cell Death. To determine whether GCPII inhibition decreases cell death induced by MT SOD1, we measured the viability of MNs transduced with AdV expressing β-galactosidase (AdLacZ), WT SOD1 (AdSODWT), or MT SOD1 (AdSODA4V) in the presence or absence of 2-PMPA. AdSODA4V caused significant cell death compared with mock-, AdLacZ-, and AdSODWT-transduced cells ($P < 0.001$; $n = 3$; Fig. 1*A*). After treatment with 2-PMPA (1 nM to 10 μM), there was a statistically significant increase in survival of MNs expressing AdSODA4V ($P < 0.001$; Fig. 1*B*) with no effect on viability of the other transduced MNs (Fig. 1*A*). Similar results were found after terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining (data not shown).

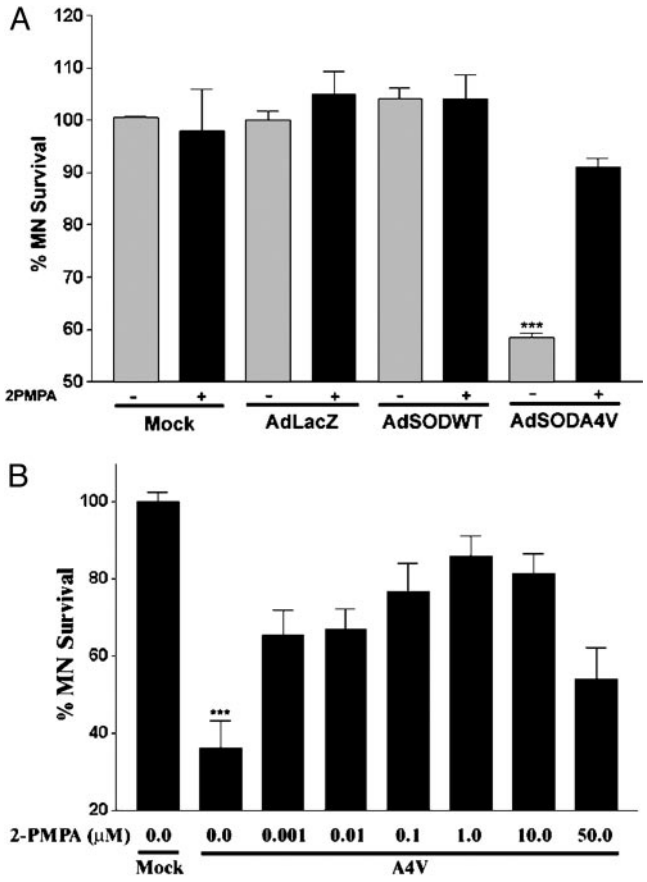


Fig. 1. GCPII inhibitors protect MNs from death induced by MT SOD1. (*A*) Effect of 2-PMPA on MT SOD1-induced MN death. MT SOD1 was expressed by using recombinant AdVs, and cell death was assayed by staining with Hoechst 33342. Data show percent MN survival 3 days after AdV transduction from a total of three experiments. AdSODA4V caused significant cell death compared with mock, AdLacZ, and AdSODWT ($P < 0.001$). Treatment with 2-PMPA significantly increased survival of MNs expressing AdSODA4V (***, $P < 0.001$). (*B*) Effect of various concentrations of 2-PMPA on MT SOD1-induced MN death. Data show percent MN survival 5 days after transduction of MT SOD1. 2-PMPA showed significant neuroprotection against AdSODA4V at a concentration as low as 1.0 nM (***, $P < 0.001$ for 0.001–10.0 μM, and $P < 0.05$ for 50.0 μM).

GCPII Inhibition Decreases Levels of Glutamate and Increases Levels of NAAG in MN/Glia Cultures. 2-PMPA is expected to inhibit the hydrolysis of NAAG into glutamate and *N*-acetylaspartate (20).

Table 1. Analysis of glutamate and NAAG production after AdV transduction with and without 2-PMPA

Conditions	% glutamate + metabolite	% NAAG
Mock	37	63
AdLacZ	40	59
AdSODWT	37	63
AdSODA4V	37	63
Mock + 2-PMPA	17	83
AdLacZ + 2-PMPA	15	83
AdSODWT + 2-PMPA	14	85
AdSODA4V + 2-PMPA	16	83

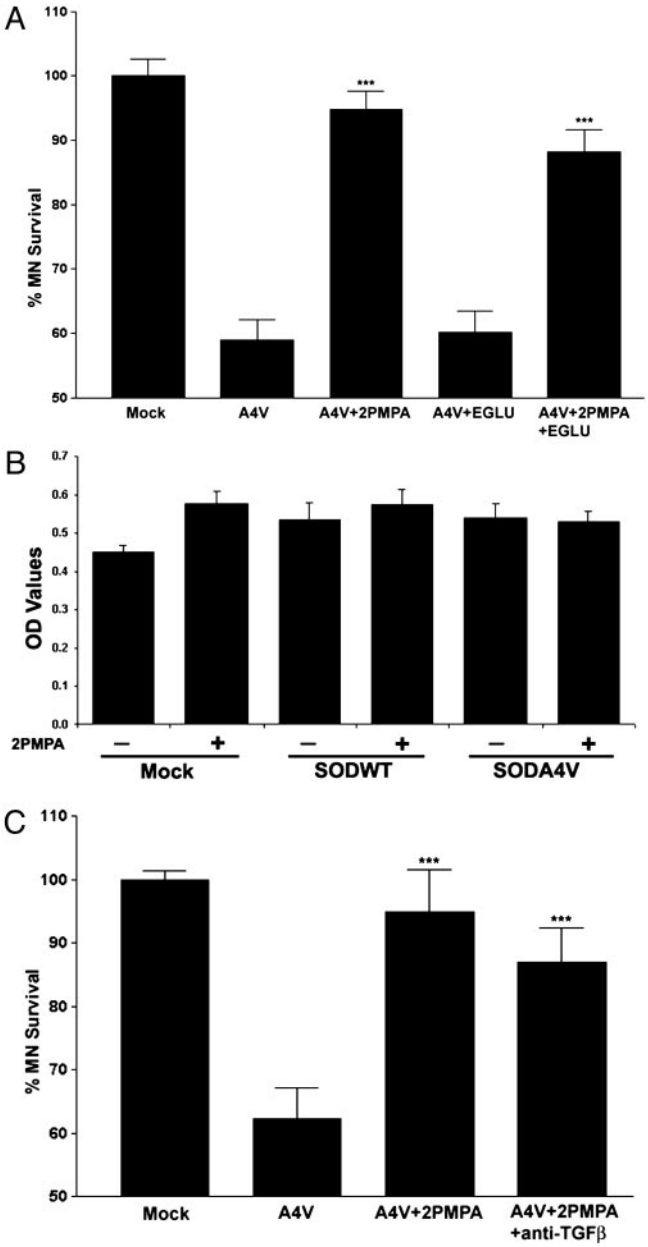


Fig. 2. Protection of MNs from MT SOD1-induced cell death by 2-PMPA does not involve group II mGluRs and TGFβ. (*A*) MN survival 3 days after transduction of AdSODA4V and treatment with 2-PMPA in the presence or absence of EGLU, an antagonist of group II mGluRs. The ability of 2-PMPA to protect MNs from MT SOD1-induced cell death was not significantly altered in the presence of 100 μM EGLU (***, $P < 0.001$ for AdSODA4V versus 2-PMPA with or without EGLU). (*B*) The level of TGFβ, as measured by ELISA, after transduction with AdSODWT or AdSODA4V in the presence and absence of 2-PMPA. The level of TGFβ was similar after 2-PMPA treatments of MNs and/or transduction with AdSODA4V, suggesting that protective effect of 2-PMPA was not associated with the release of TGFβ. (*C*) MN survival 4 days after transduction of AdSODA4V and treatment with 2-PMPA in the presence and absence of anti-TGFβ. Anti-TGFβ antibody failed to significantly reverse the neuroprotective effect of 2-PMPA against MT SOD1-induced cell death (***, $P < 0.001$ for AdSODA4V versus 2-PMPA with or without anti-TGFβ), indicating that the protective effect of 2-PMPA is not mediated by mGluR3 activation and TGFβ release.

To validate this activity in the MN/glia cultures, we used radiolabeled NAAG to test whether 2-PMPA added to the cultures altered glutamate and NAAG levels. The percent of

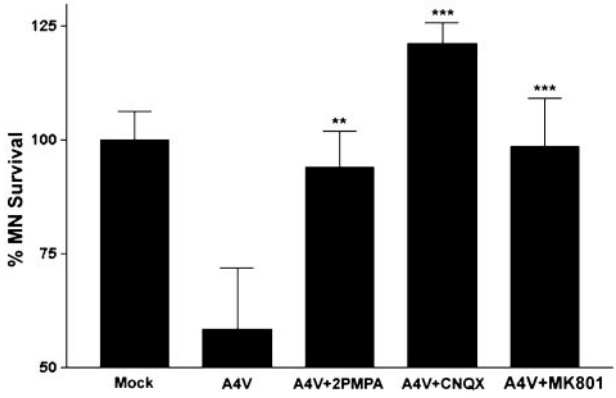


Fig. 3. CNQX and MK801 increase MN survival after transduction with AdSODA4V. Treatment with CNQX, an AMPA receptor antagonist, or MK801, an NMDA receptor antagonist, protected MNs from MT SOD1-induced cell death. Shown are results from a representative experiment from a total of two. ***, $P < 0.001$ for AdSODA4V without versus treatment with CNQX or MK801; **, $P < 0.01$ for AdSODA4V without versus treatment with 2-PMPA; and $P > 0.05$ for mock-transduced versus AdSODA4V plus CNQX.

glutamate and glutamate metabolites derived from exogenously added radiolabeled NAAG decreased in cultures after administration of 2-PMPA, whereas the percent of NAAG remaining unhydrolyzed significantly increased (Table 1). These findings confirmed the inhibitory effect of 2-PMPA on GCPII in our cultures.

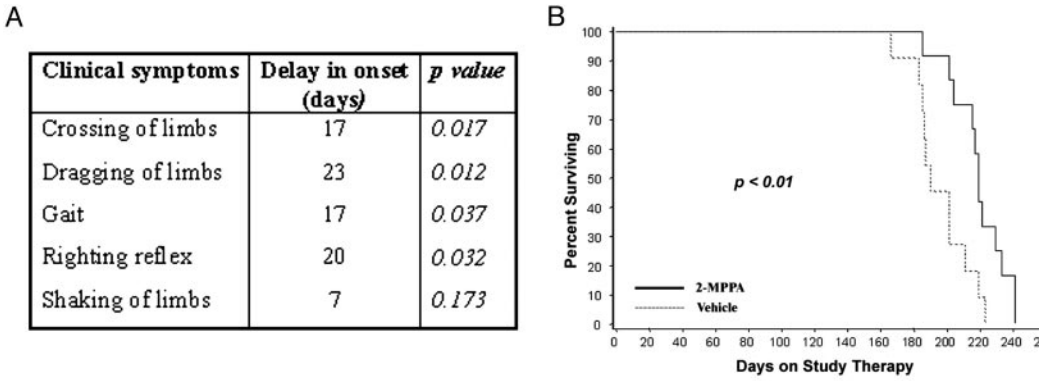
GCPII-Mediated Protection of MN/Glia Cultures from MT SOD1-Induced Cell Death Is Not Mediated by the Group II mGluR and Does Not Involve TGFβ. The administration of 2-PMPA is predicted to increase levels of NAAG, which can act as an mGluR3 agonist. To determine whether this action of 2-PMPA is responsible for its neuroprotection, we tested whether administration of EGLU, an antagonist of group II mGluRs, decreased the rescue seen with 2-PMPA. We found that 100 μM EGLU did not alter the ability of 2-PMPA to protect MNs from MT SOD1-induced cell death (Fig. 2*A*).

To further assess the possible role of NAAG in protecting against MT SOD1-induced cell death, we investigated the role of TGFβ in the rescue, because activation of mGluR3 protects neurons against *N*-methyl-D-aspartate (NMDA) toxicity through a release of TGFβ. This issue was of special interest because the neuroprotective effect of 2-PMPA against *in vitro* and *in vivo* ischemic injury was found to involve TGFβ (28). We measured the level of TGFβ 3 days after transduction with AdSODA4V and treatment with 2-PMPA. There was no significant change in the level of TGFβ in MN/glia cultures in any of the conditions (Fig. 2*B*). In addition, treatment of MNs with antibody directed against TGFβ (anti-TGFβ) after transduction with MT SOD1 failed to significantly reverse the neuroprotective effect of 2-PMPA (Fig. 2*C*). These results demonstrated that the rescue by 2-PMPA of MNs from MT SOD1-induced cell death was not mediated by TGFβ.

MT SOD1-Induced Cell Death Is Decreased by Blocking Glutamate Excitotoxicity. 2-PMPA blocks the hydrolysis of NAAG into *N*-acetylaspartate and glutamate. A 2-PMPA-mediated decrease in glutamate would be expected to decrease excitotoxicity at the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and/or NMDA receptor. We therefore tested whether antagonists of glutamate receptors affected MT SOD1-induced MN death (Fig. 3). We found that treatment with 100 μM CNQX, an antagonist at AMPA receptors, or 0.2 μM MK801, an NMDA receptor antagonist, protected MNs from MT SOD1-

A

Clinical symptoms	Delay in onset (days)	*p value*
Crossing of limbs	17	*0.017*
Dragging of limbs	23	*0.012*
Gait	17	*0.037*
Righting reflex	20	*0.032*
Shaking of limbs	7	*0.173*



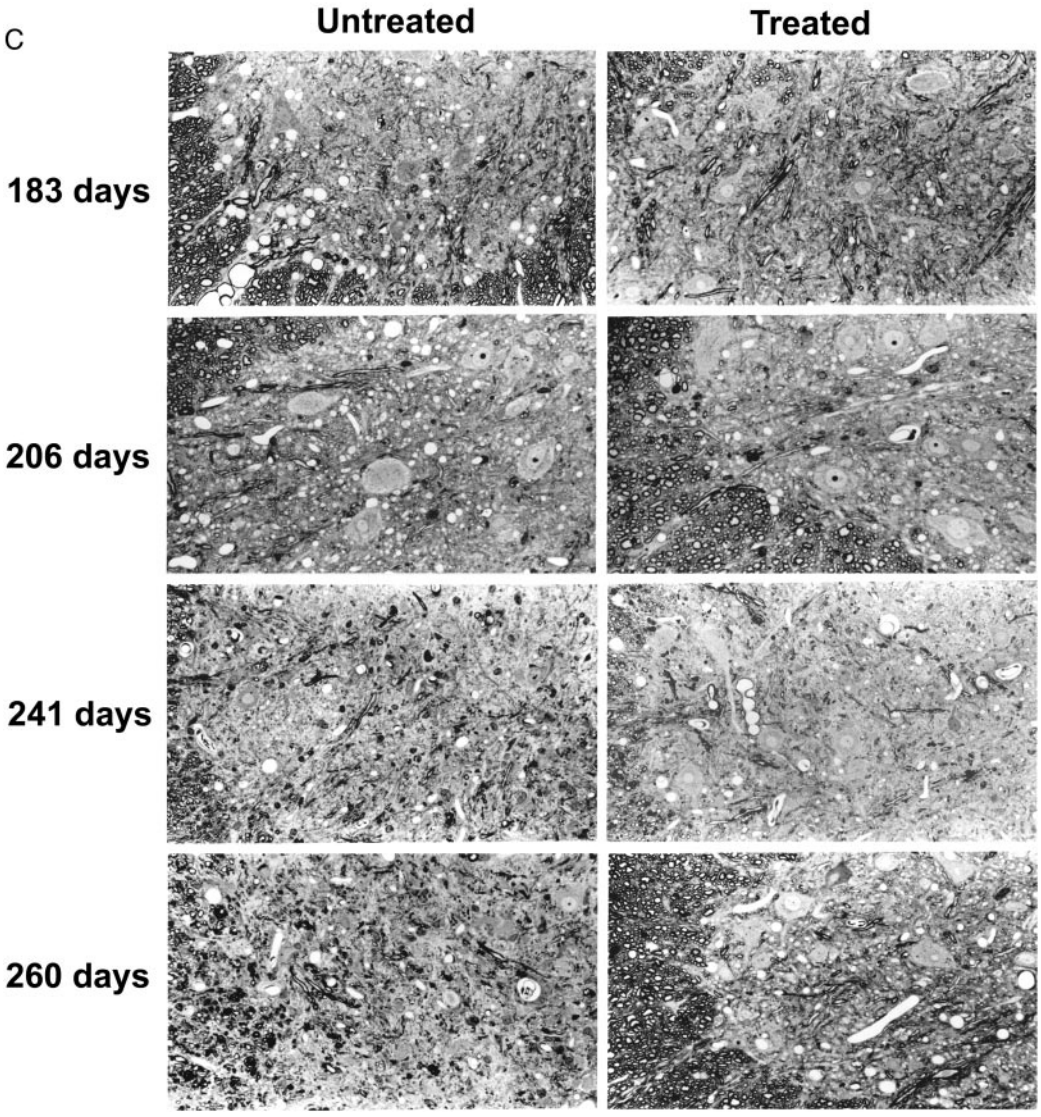


Fig. 4. 2-MPPA treatment delays clinical symptoms, prolongs life, and protects against histological abnormalities in SODG93A transgenic mice. (*A*) Delay in onset of clinical signs in mice treated with 2-MPPA versus vehicle. The following clinical signs were monitored over time by a rater who was blinded to the treatment: shaking of limbs when suspended (rated 0, absent; 1, present), crossing of limbs (rated 0, absent, to 3, worst affected), gait abnormality (assessed by observation of animals walking on a flat surface and rated 0, normal, to 3, most affected), dragging of limbs (rated 0, absent, to 3, most affected), and righting reflex (corresponding to time in seconds that animal takes to right itself). (*B*) Kaplan–Meier survival curve of mice treated with 2-MPPA versus vehicle, $P < 0.01$. (*C*) Histopathological findings in the spinal cord of FALS transgenic mice treated with vehicle or 2-MPPA and killed at the times noted. (Magnification: ×200.)

induced cell death ($P < 0.001$, $n = 2$; Fig. 3). These results suggested that cell death induced by MT SOD1 was at least partly mediated by glutamate excitotoxicity through either AMPA or NMDA receptors. The data support the hypothesis that 2-PMPA is neuroprotective by decreasing the level of glutamate available to act at these receptors.

GCPII Inhibition Delays Onset and Slows the Progression of Disease in FALS Transgenic Mice. To further assess the efficacy of GCPII inhibitors in altering FALS, we administered 2-MPPA, which has an enhanced oral bioavailability compared with 2-PMPA. SODG93A transgenic mice received either 2-MPPA or vehicle on a daily basis and clinical signs were measured weekly. In mice treated with 2-MPPA there were statistically significant delays in onset for all of the clinical endpoints measured except for shaking of limbs (Fig. 4*A*). Additionally, there was a statistically significant prolongation in median survival, from 190 days in the vehicle-treated group to 219 days in the 2-MPPA-treated group ($\Delta = 29$ days, a 15% increase in the mean life span; $P = 0.0059$; Fig. 4*B*).

FALS Transgenic Mice Treated with a GCPII Inhibitor Have Few Neuropathological Abnormalities. In a separate experiment looking at pathological endpoints, one animal from each treatment group was killed on days 164, 183, 206, 241, and 260 (Fig. 4*C*). No abnormalities were seen on day 164 in either group (data not shown). On day 183, minimal changes were seen in the treated animal, with a few small vacuoles in the spinal cord anterior horns. At the same time point, changes in the untreated animal, although mild, were definitely more noticeable, because vacuoles were more numerous and larger, especially in axons projecting to the anterior root zone. At day 206, both control and treated animals showed mild spongy changes in the anterior horns; however, the untreated animal showed more severe changes. At day 241, differences between treated and nontreated animals were more striking. The untreated animal showed severe depletion of neurons in the anterior horns with reactive astrocytosis. Anterior and lateral columns also showed mild to moderate axonal degeneration. The treated animal, on the other hand, showed a normal population of large MNs and mild alterations in the neuropil with mild vacuolar changes. At day 260, differences between treated and untreated animals were very pronounced. The untreated animal again showed severe neuronal depletion and astrocytosis in the anterior horns. In addition, white matter changes consisting of axonal degeneration and accumulation of myelin debris in anterior and lateral columns were severe, as previously described (29), whereas the treated animal showed only modest vacuolar changes in the neuropil with normal neuronal populations.

Discussion

A role for glutamate excitotoxicity in MN death has been proposed in both sporadic ALS and FALS (8). Abnormalities of the EAAT2 transporter have been described in sporadic ALS (7) as well as FALS transgenic mice (9) and rats (4). Elevated levels of glutamate have been reported in the plasma and cerebrospinal fluid of ALS patients (5, 6). The FALS transgenic mouse has a greater elevation in levels of glutamate than WT mice after treatment with a glutamate reuptake inhibitor and NMDA (10). In addition, riluzole, a drug that interferes with glutamate neurotransmission, has been shown to increase survival of FALS transgenic mice and ALS patients (12, 13, 30). Despite these known relationships, it is not clear how the etiology of ALS is linked to abnormalities in the glutamate system that lead to neuronal degeneration.

NAAG is one of the most abundant neuropeptides in the mammalian nervous system (14) and is highly concentrated in the spinal cord neurons, including MNs and interneurons as well as spinal sensory neurons (31). Neuronally released NAAG is hydrolyzed to liberate free glutamate by the enzyme GCPII, which is located on the external surface of glial cells (18, 32). In ALS patients, NAAG levels are significantly elevated in cerebrospinal fluid and decreased in spinal cord tissue (33). In addition, GCPII activity is selectively upregulated in motor cortex and the ventral column of the spinal cord in ALS (31). These data raise the possibility that increased NAAG release and subsequent cleavage by elevated GCPII could be involved in the generation of excitotoxic glutamate in ALS. If this possibility is true, this excitotoxic state should be amenable to treatment by GCPII inhibition. Inhibiting the liberation of glutamate from NAAG should be protective, especially given the evidence of abnormal glutamate uptake (7, 8). In addition, GCPII inhibitors would also increase the level of NAAG. Increasing NAAG levels by blocking hydrolysis may be neuroprotective by activating group II mGluR3 (16). Therefore, GCPII inhibitors may be neuroprotective by means of two distinct mechanisms: by decreasing local glutamate levels and through the increase in NAAG (19). In support of these hypotheses, GCPII inhibitors have been found effective in animal models of a variety of insults in which glutamate excitotoxicity has been implicated, including ischemia, diabetic neuropathy, chronic pain, and drug abuse (19, 21, 23, 28).

Recent studies employing mice in which the GCPII gene has been deleted (34) reveal a small but significant residual NAAG-hydrolyzing activity. In our laboratory, we have found that this residual NAAG-hydrolyzing activity is also inhibited by nanomolar concentrations of 2-PMPA and 2-MPPA. Therefore it is possible that the effects of the GCPII inhibitors used in these studies could be through inhibition of other NAAG-hydrolyzing enzymes in addition to GCPII.

GCPII inhibition is especially attractive as a therapeutic target because the effects occur only during excessive glutamate stimulation. Lieberman and colleagues (35) have shown that GCPII-mediated hydrolysis of NAAG is constitutively quiescent and is induced selectively by nerve stimulation. Using microdialysis, we have shown that GCPII inhibition does not affect basal glutamate but selectively decreases the rise in extracellular glutamate after a pathological insult, e.g., stroke (19). If this selectivity for affecting excess glutamate holds true in clinical situations, GCPII inhibition may represent a neuroprotective mechanism devoid of glutamate receptor antagonist side effects. GCPII inhibitors have, in fact, been shown to be generally free of behavioral effects in normal animals even at doses many times those that are effective in animal models of disease (19).

In the studies described, we show dramatic neuroprotection with GCPII inhibitors after expression of MT SOD1 both in FALS transgenic mice as well as cultured primary MNs. The incubation period of the FALS transgenic mice treated with 2-MPPA was prolonged compared with untreated mice. In addition, 2-MPPA delayed mortality and pathological abnormalities, with as robust an effect as has been reported in other studies (36, 37). Similar results were obtained in a preliminary study with a structurally distinct GCPII inhibitor termed GPI 5232,** providing additional evidence that the mechanism for neuroprotection involved an inhibition of GCPII activity.

We also found that the potent and selective GCPII inhibitor, 2-PMPA, significantly decreased death induced by MT SOD1 in MN/glia cultures at concentrations comparable to its K_i for GCPII inhibition. The effect of 2-PMPA was not mediated by the increased NAAG levels acting at the group II mGluR because the effect did not decrease in the presence of the mGluR group II antagonist EGLU. In addition, there was no evidence that TGFβ played a role in the neuroprotection because there was no change in the level of this cytokine during administration of 2-PMPA and because there was no effect on the protection mediated by 2-PMPA after administration of

Slusher, B. S., Wozniak, K. M., Hartman, T., Jada, P., Chadran, M. & Dal Canto, M. (2000) *Soc. Neurosci. Abstr.* **26, 110.

anti-TGFβ antibody; this finding contrasts with that reported for the action of 2-PMPA in an ischemia model (28). NAAG is already elevated in the cerebrospinal fluid of patients with ALS, so that the primary neuroprotective mechanism of 2-PMPA related to MT SOD1-induced MN death may be one involving limiting glutamate when glutamate uptake is abnormal.

A role for the AMPA receptor rather than the NMDA receptor in mediating glutamate excitotoxicity of MNs has been stressed (8, 38–41). Our results showing protection of MNs from MT SOD1-induced cell death through treatment with either CNQX, an antagonist of the AMPA/kainate receptor, or MK801, an antagonist of the NMDA receptor, suggest that glutamate excitotoxicity plays a role in MT SOD1-induced MN death and that this excitotoxicity is mediated by both the AMPA and NMDA receptors.

In summary, our studies demonstrate the efficacy and mechanism of action of GCPII inhibition in MT SOD1-induced MN death. Our findings provide support for glutamate excitotoxicity as playing a role in this cell death and identify GCPII inhibitors as attractive candidates for clinical treatment trials in ALS.

This study is dedicated to the memory of Amos Bodner, whose enthusiasm and imagination were critical to its inception and completion. Mr. Jada Prasad provided excellent technical assistance. This study was supported by Guilford Pharmaceuticals and National Institutes of Health Grant 5PO1NS21442-18.

1. Cleveland, D. W. & Rothstein, J. D. (2001) *Nat. Rev. Neurosci.* **2,** 806–819.
2. Gurney, M. E., Pu, H., Chiu, A. Y., Dal Canto, M. C., Polchow, C. Y., Alexander, D. D., Caliendo, J., Hentati, A., Kwon, Y. W., Deng, H. X., *et al.* (1994) *Science* **264,** 1772–1775.
3. Nagai, M., Aoki, M., Miyoshi, I., Kato, M., Pasinelli, P., Kasai, N., Brown, R. H., Jr., & Itoyama, Y. (2001) *J. Neurosci.* **21,** 9246–9254.
4. Howland, D. S., Liu, J., She, Y., Goad, B., Maragakis, N. J., Kim, B., Erickson, J., Kulik, J., DeVito, L., Psaltis, G., *et al.* (2002) *Proc. Natl. Acad. Sci. USA* **99,** 1604–1609.
5. Shaw, P. J., Forrest, V., Ince, P. G., Richardson, J. P. & Wastell, H. J. (1995) *Neurodegeneration* **4,** 209–216.
6. Spreux-Varoquaux, O., Bensimon, G., Lacomblez, L., Salachas, F., Pradat, P. F., Le Forestier, N., Marouan, A., Dib, M. & Meininger, V. (2002) *J. Neurol. Sci.* **193,** 73–78.
7. Rothstein, J. D., Martin, L. J. & Kuncl, R. W. (1992) *N. Engl. J. Med.* **326,** 1464–1468.
8. Rothstein, J. D. (1995) *Adv. Neurol.* **68,** 7–20.
9. Warita, H., Manabe, Y., Murakami, T., Shiote, M., Shiro, Y., Hayashi, T., Nagano, I., Shoji, M. & Abe, K. (2002) *Neurol. Res.* **24,** 577–581.
10. Andreassen, O. A., Jenkins, B. G., Dedeoglu, A., Ferrante, K. L., Bogdanov, M. B., Kaddurah-Daouk, R. & Beal, M. F. (2001) *J. Neurochem.* **77,** 383–390.
11. Gurney, M. E., Fleck, T. J., Himes, C. S. & Hall, E. D. (1998) *Neurology* **50,** 62–66.
12. Bensimon, G., Lacomblez, L. & Meininger, V. (1994) *N. Engl. J. Med.* **330,** 585–591.
13. Lacomblez, L., Bensimon, G., Leigh, P. N., Guillet, P. & Meininger, V. (1996) *Lancet* **347,** 1425–1431.
14. Neale, J. H., Bzdega, T. & Wroblewska, B. (2000) *J. Neurochem.* **75,** 443–452.
15. Xi, Z. X., Baker, D. A., Shen, H., Carson, D. S. & Kalivas, P. W. (2002) *J. Pharmacol. Exp. Ther.* **300,** 162–171.
16. Bruno, V., Battaglia, G., Casabona, G., Copani, A., Caciagli, F. & Nicoletti, F. (1998) *J. Neurosci.* **18,** 9594–9600.
17. Robinson, M. B., Blakely, R. D., Couto, R. & Coyle, J. T. (1987) *J. Biol. Chem.* **262,** 14498–14506.
18. Cassidy, M. & Neale, J. H. (1993) *Neuropeptides* **24,** 271–278.
19. Slusher, B. S., Vornov, J. J., Thomas, A. G., Hurn, P. D., Harukuni, I., Bhardwaj, A., Traystman, R. J., Robinson, M. B., Britton, P., Lu, X. C., *et al.* (1999) *Nat. Med.* **5,** 1396–1402.
20. Jackson, P. F., Cole, D. C., Slusher, B. S., Stetz, S. L., Ross, L. E., Donzanti, B. A. & Trainor, D. A. (1996) *J. Med. Chem.* **39,** 619–622.
21. Zhang, W., Slusher, B. S., Murakawa, Y., Wozniak, K. M., Tsukamoto, T., Jackson, P. F. & Sima, A. A. (2002) *J. Neurol. Sci.* **194,** 21–28.
22. Shippenberg, T. S., Rea, W. & Slusher, B. S. (2000) *Synapse* **38,** 161–166.
23. Slusher, B. S., Thomas, A., Paul, M., Schad, C. A. & Ashby, C. R., Jr. (2001) *Synapse* **41,** 22–28.
24. Majer, P., Jackson, P. F., Delahanty, G., Grella, B. S., Ko, Y. S., Li, W., Liu, Q., Maclin, K. M., Polakova, J., Shaffer, K. A., *et al.* (2003) *J. Med. Chem.* **46,** 1989–1996.
25. Vandenberghe, W., Ihle, E. C., Patneau, D. K., Robberecht, W. & Brorson, J. R. (2000) *J. Neurosci.* **20,** 7158–7166.
26. Ghadge, G. D., Lee, J. P., Bindokas, V. P., Jordan, J., Ma, L., Miller, R. J. & Roos, R. P. (1997) *J. Neurosci.* **17,** 8756–8766.
27. Koller, K. J., Zaczek, R. & Coyle, J. T. (1984) *J. Neurochem.* **43,** 1136–1142.
28. Thomas, A. G., Liu, W., Olkowski, J. L., Tang, Z., Lin, Q., Lu, X. C. & Slusher, B. S. (2001) *Eur. J. Pharmacol.* **430,** 33–40.
29. Dal Canto, M. C. & Gurney, M. E. (1997) *Acta Neuropathol.* **93,** 537–550.
30. Gurney, M. E., Cutting, F. B., Zhai, P., Doble, A., Taylor, C. P., Andrus, P. K. & Hall, E. D. (1996) *Ann. Neurol.* **39,** 147–157.
31. Tsai, G. C., Stauch-Slusher, B., Sim, L., Hedreen, J. C., Rothstein, J. D., Kuncl, R. & Coyle, J. T. (1991) *Brain Res.* **556,** 151–156.
32. Berger, U. V., Luthi-Carter, R., Passani, L. A., Elkabes, S., Black, I., Konradi, C. & Coyle, J. T. (1999) *J. Comp. Neurol.* **415,** 52–64.
33. Rothstein, J. D., Tsai, G., Kuncl, R. W., Clawson, L., Cornblath, D. R., Drachman, D. B., Pestronk, A., Stauch, B. L. & Coyle, J. T. (1990) *Ann. Neurol.* **28,** 18–25.
34. Bacich, D. J., Ramadan, E., O'Keefe, D. S., Bukhari, N., Wegorzewska, I., Ojeifo, O., Olszewski, R., Wrenn, C. C., Bzdega, T., Wroblewska, B., *et al.* (2002) *J. Neurochem.* **83,** 20–29.
35. Urazaev, A. K., Buttram, J. G., Jr., Deen, J. P., Gafurov, B. S., Slusher, B. S., Grossfeld, R. M. & Lieberman, E. M. (2001) *Neuroscience* **107,** 697–703.
36. Li, M., Ona, V. O., Guegan, C., Chen, M., Jackson-Lewis, V., Andrews, L. J., Olszewski, A. J., Stieg, P. E., Lee, J. P., Przedborski, S. & Friedlander, R. M. (2000) *Science* **288,** 335–339.
37. Zhu, S., Stavrovskaya, I. G., Drozda, M., Kim, B. Y., Ona, V., Li, M., Sarang, S., Liu, A. S., Hartley, D. M., Wu, D. C., *et al.* (2002) *Nature* **417,** 74–78.
38. Roy, J., Minotti, S., Dong, L., Figlewicz, D. A. & Durham, H. D. (1998) *J. Neurosci.* **18,** 9673–9684.
39. Saroff, D., Delfs, J., Kuznetsov, D. & Geula, C. (2000) *NeuroReport* **11,** 1117–1121.
40. Carriedo, S. G., Yin, H. Z. & Weiss, J. H. (1996) *J. Neurosci.* **16,** 4069–4079.
41. Van Den Bosch, L., Vandenberghe, W., Klaassen, H., Van Houtte, E. & Robberecht, W. (2000) *J. Neurol. Sci.* **180,** 29–34.