ELSEVIER

European Journal of Pharmacology 402 (2000) 31–37

ejp

www.elsevier.nl/locate/ejphar

Neuroprotection produced by the NAALADase inhibitor 2-PMPA in rat cerebellar neurons

Frank C. Tortella [a,*], Yu Lin [a], Haresh Ved [b], Barbara S. Slusher [c], Jitendra R. Dave [a]

[a] *Division of Neurosciences, Walter Reed Army Institute of Research, Silver Spring, MD 20910-7500, USA*
[b] *Division of Biochemistry, Walter Reed Army Institute of Research, Silver Spring, MD 20910-7500, USA*
[c] *Guilford Pharmaceuticals, Department of Research, Baltimore, MD 21224, USA*

Received 6 March 2000; received in revised form 11 July 2000; accepted 17 July 2000

Abstract

The present study examined the neuroprotective actions of the *N*-acetylated-α-linked-acidic dipeptidase (NAALADase) inhibitor 2-(phosphonomethyl)pentanedioic acid (2-PMPA) in four in vitro models of neurotoxicity. Using neuron-enriched primary cultures derived from rat embryo (E15) cerebellum, 2-PMPA afforded 100% neuroprotection from injuries induced by hypoxia (EC_{50} = 8.4 μM). In contrast, against glutamate or *N*-methyl-D-aspartate (NMDA) injury, 2-PMPA was less potent and its efficacy limited to a maximum of 46% and 16%, respectively. 2-PMPA was not effective against veratridine-induced injury. Also, the less potent analog of 2-PMPA, 2-[phosphonomethyl]succinic acid (2-PMSA), was ineffective. Unlike 2-PMPA, the endogenous NAALADase substrate and $mGlu_3$ receptor agonist *N*-acetyl-aspartyl-glutamate (NAAG) was neuroprotective against all four injury mechanisms and compared to 2-PMPA, exhibited a different "phosphate effect" on neuroprotection. These results confirm the superior efficacy of 2-PMPA to protect against injury caused by cellular anoxia, and are discussed relative to upstream modulation of hyperglutamatergic activity vs. downstream modulation of metabotropic receptors as possible targets for ischemia/stroke therapy.

Keywords: NAALADase inhibition (*N*-acetylated-α-linked-acidic dipeptidase); Stroke; Neuroprotection; (Rat)

1. Introduction

Glutamate is the major excitatory neurotransmitter in mammalian brain. Excessive activation of cellular glutamatergic processes (i.e. receptors, ion channels, transcription proteins, etc.) is believed to represent the seminal cellular mechanism promoting neurodegeneration leading to neuron dysfunction and cell death. Regardless of etiology, or subsequent clinical event (i.e. stroke, brain trauma, progressive neurodegenerative diseases, chemical/biological terrorism, etc.), the arrest of hyperglutamatergic activity and subsequent attenuation of the excitotoxic cascade remains a dynamic challenge to drug development and experimental therapeutics, and one of significant clinical potential.

Much research has focused on the two primary classes of glutamate receptors (ionotropic and metabotropic) and the role of intracellular calcium and other downstream mechanisms representing the excitotoxic "secondary signaling cascade". Alternatively, upstream modulation of glutamate by interfering with its neuropeptide precursor *N*-acetyl-aspartyl-glutamate (NAAG) (Coyle, 1997), via inhibition of the hydrolyzing enzyme *N*-acetylated-α-linked-acidic dipeptidase (NAALADase), has recently received attention as a novel strategy to suppress excitotoxic glutamate neurotransmission pathogenic to several brain disorders (Slusher et al., 1999).

The purpose of this study was to examine the neuroprotective properties of an inhibitor of NAALADase, 2-(phosphonomethyl)pentanedioic acid (2-PMPA) (Jackson et al., 1996), in different in vitro neuronal culture models of cell death. Using cerebellar neurons, neuroprotection was studied following hypoxia/hypoglycemia, *N*-methyl-D-aspar-

* Corresponding author. Department of Neuropharmacology and Molecular Biology, Division of Neuroscience, Walter Reed Army Institute of Research, Bldg. 503, Robert Grant Ave., Forest Glen Annex, Silver Spring, MD 20910, USA. Tel.: +1-301-319-9461; fax: +1-301-319-9905.

E-mail address: frank.tortella@na.amedd.army.mil (F.C. Tortella).

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00519-7

tate (NMDA), glutamate, or veratridine-induced neuronal injury.

2. Materials and methods

2.1. Cerebellum cultures

Neuronal cultures were prepared from prenatal day 15 Sprague–Dawley rat embryo cerebellum (whole cerebellum). The harvested neurons were plated in 48-well culture plates (5×10^5 cells/well) pre-coated with poly-L-lysine. The cultures were maintained in a medium containing equal parts of Eagle's basal media (without glutamine) and Ham's F12K media supplemented with 10% heat-inactivated horse serum, 10% fetal bovine serum, glucose (600 μg/ml), glutamine (100 μg/ml), penicillin (50 units/ml), and streptomycin (50 μg/ml). After 48 h in culture, cytosine arabinoside (5×10^{-5} M) was added to inhibit non-neuronal cell division. Cells prepared by this method were used in experiments after 7–10 days in culture and consisted of approximately 80% neurons and 10% glia (Dave et al., 1997). Additional characterization of the 80% neuronal population in these cultures has determined that while greater than 50% are glutamatergic/cholinergic, approximately 35% are GABAergic or dopaminergic with the remaining 10–15% representing mixed populations of neurons (unpublished observation). Furthermore, the specific activity for NAALADase in our cerebellar cultures is 0.019 ± 0.002 pmol/min/mg protein (unpublished observations).

2.2. Neurotoxicity models and assessment

Neurotoxicity was produced by 2 h of hypoxia/hypoglycemia, or a 45-min exposure to 80 μM glutamate, or 100 μM NMDA, or 20 μM veratridine. Hypoxia/hypoglycemia was induced by incubating the cells in a humidified air-tight chamber saturated with 95% nitrogen:5% CO_2 gas for 2 h in Locke's solution without added glucose. In the first series of experiments, cells were pretreated with either vehicle or 2-PMPA 2 h prior to hypoxia/hypoglycemia, or 45 min prior to neurotoxin exposure. In addition, in separate hypoxia/hypoglycemia experiments, treatment with 2-PMPA was delayed for 1, 2, or 4 h after hypoxia/hypoglycemia. Critically, all of the 2-PMPA (and 2-[phosphonomethyl]succinic acid (2-PMSA)) experiments were performed in a phosphate-free media throughout the 24-h period since phosphate itself is a potent inhibitor of NAALADase activity (Slusher et al., 1990). In a second series of experiments, the effects of NAAG, β-NAAG (the nonhydrolizable form of NAAG) and 2-PMSA, a less potent structural analog of 2-PMPA, were examined in "phosphate-free" and "phosphate-containing" media.

At 24 h post-injury, neuronal viability was qualitatively assessed by morphological evaluations and quantified using colorimetric assay. For morphological evaluation, the neuronal cultures were viewed under bright-field phase contrast ($80\times$) light microscopy. Quantification of cell viability was made using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, this dye was added to each well (final concentration of 1.5 mg/ml) and cells were incubated with MTT for 1 h at 37°C. When the assay was terminated, the dye was solubilized by acidified isopropanol (0.1 N HCl in isopropanol) and the absorbance intensity (540 nm) of each sample measured in a 96-well plate reader. Values are expressed relative to vehicle-treated control cells that were maintained on each plate (normoxic conditions) and the percent change in cell viability is calculated. Using the following equation (Ved et al., 1997), percent neuroprotection is defined as:

$$\%\text{Neuroprotection} = \frac{\text{Survival}_{(\text{insult}+\text{PMPA})} - \text{Survival}_{(\text{insult})}}{\text{Survival}_{(\text{Vehicle})} - \text{Survival}_{(\text{insult})}} \times 100.$$

Differences in the cell viability among treatment groups were determined using one-way analysis of variance and the Newman–Keuls test.

3. Results

3.1. Neurotoxicity

Exposure of normal cerebellar neurons (Fig. 1A) to hypoxia/hypoglycemia (Fig. 1B), glutamate (Fig. 1C), NMDA (Fig. 1D), or veratridine (Fig. 1E), was highly neurotoxic. Following hypoxia/hypoglycemia, neuronal cell death in untreated neurons approached 70% ($65 \pm 4\%$). Neuronal cell death following glutamate, NMDA or veratridine exposures were $75 \pm 5\%$, $70 \pm 5\%$ and $60 \pm 4\%$, respectively.

3.2. Effect of 2-PMPA

Co-treatment with 2-PMPA was neuroprotective (Fig. 1, F–I), and the degree of neuroprotection dependent upon the mechanism of cell death. Against hypoxia/hypoglycemia, the 2-PMPA neuroprotection (Fig. 1F) was concentration-dependent ($EC_{50} = 8.4$ μM) and 100% effective (Fig. 2). In contrast, against glutamate (Fig. 1G) or NMDA (Fig. 1H) neurotoxicity, the protective effects of 2-PMPA were limited. Maximal neuroprotection obtained was only 46% and 16%, respectively, at doses of 2-PMPA as high as 1000 μM (Fig. 2). Against veratridine-induced neuronal injury, concentrations of 2-PMPA as high as 1000 μM

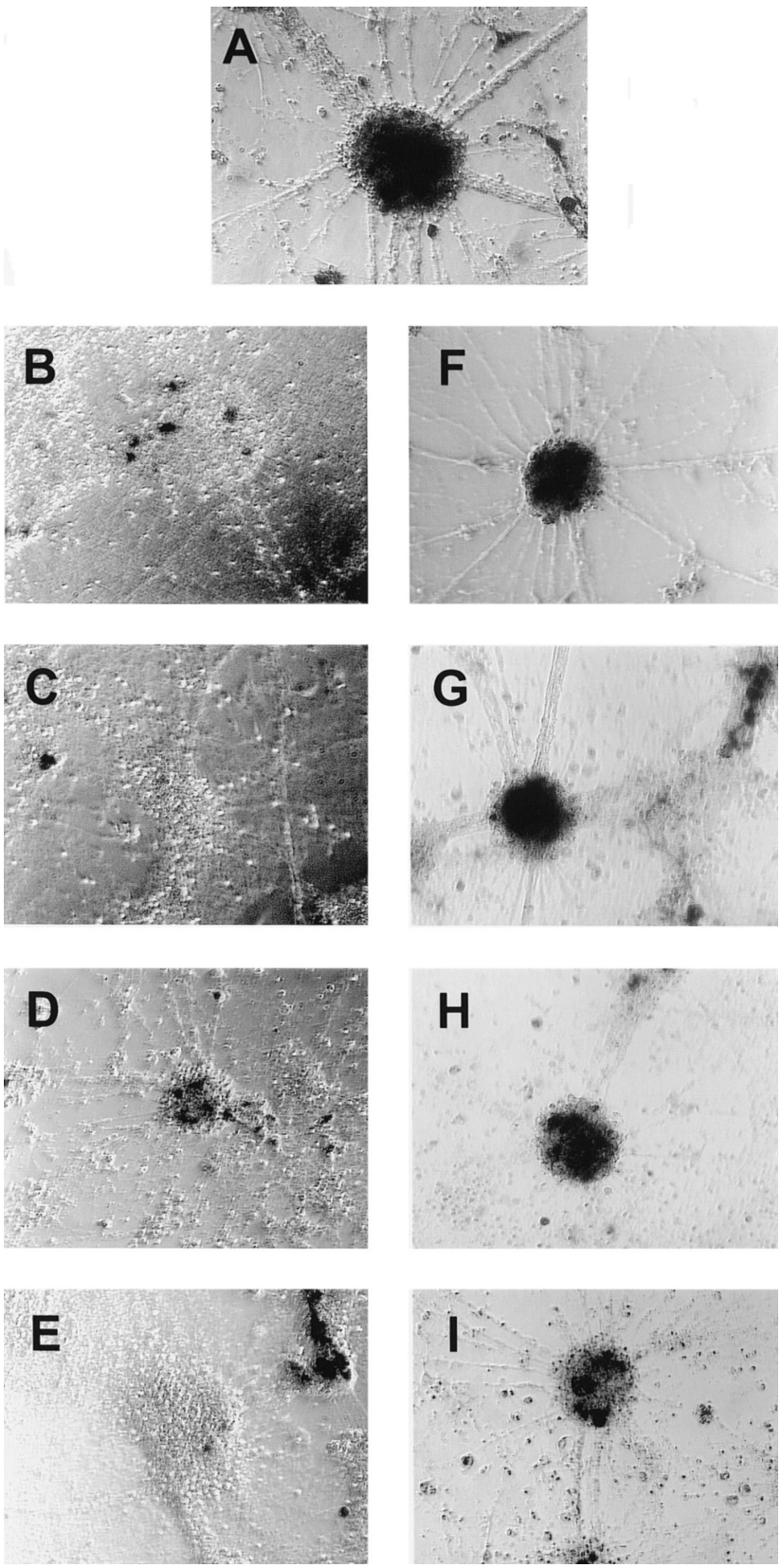

Fig. 1. Representative bright-field micrographs (80 ×) of cultured cerebellar neurons stained with MTT stain after either vehicle treatment (panel A) or hypoxia/hypoglycemia, glutamate (80 μM), NMDA (100 μM) or veratridine (20 μM) treatment (panels B, C, D and E, respectively). Panels F, G, H and I: neurons treated with 500 μM 2-PMPA and subjected to hypoxia/hypoglycemia, glutamate, NMDA or veratridine treatments, respectively.

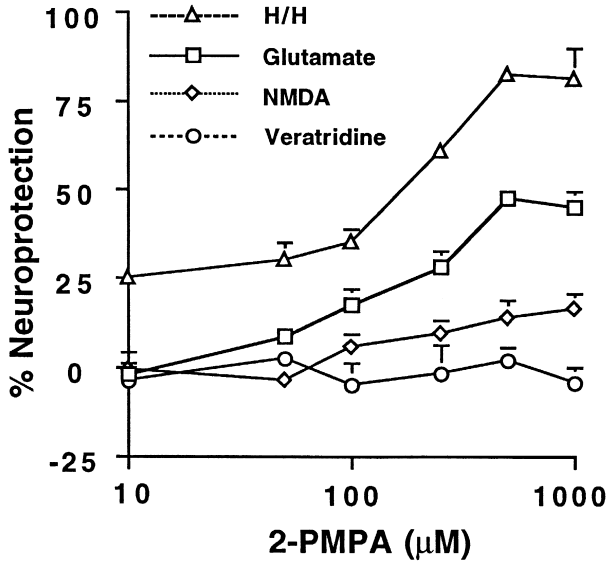

Fig. 2. Neuroprotection dose–response effect of 2-PMPA against hypoxia/hypoglycemia, glutamate, NMDA or veratridine induced neurotoxicity. Values are mean ± S.E. of four independent determinations obtained from two separate experiments ($n = 8$/dose).

failed to protect against injury and cell death (Figs. 1I and 2). Importantly, in the hypoxia/hypoglycemia injury model, delaying 2-PMPA treatment for 1 or 2 h, but not 4 h, was also neuroprotective (Fig. 3).

3.3. Effect of 2-PMSA

In contrast to 2-PMPA, treatment of neurons with 2-PMSA (at concentrations up to 1000 μM) had only a marginal neuroprotective effect (18 ± 6%) against hypoxia/hypoglycemia-induced injury. Furthermore, no protection was measured against neurotoxic insults produced by glutamate, NMDA, or veratridine.

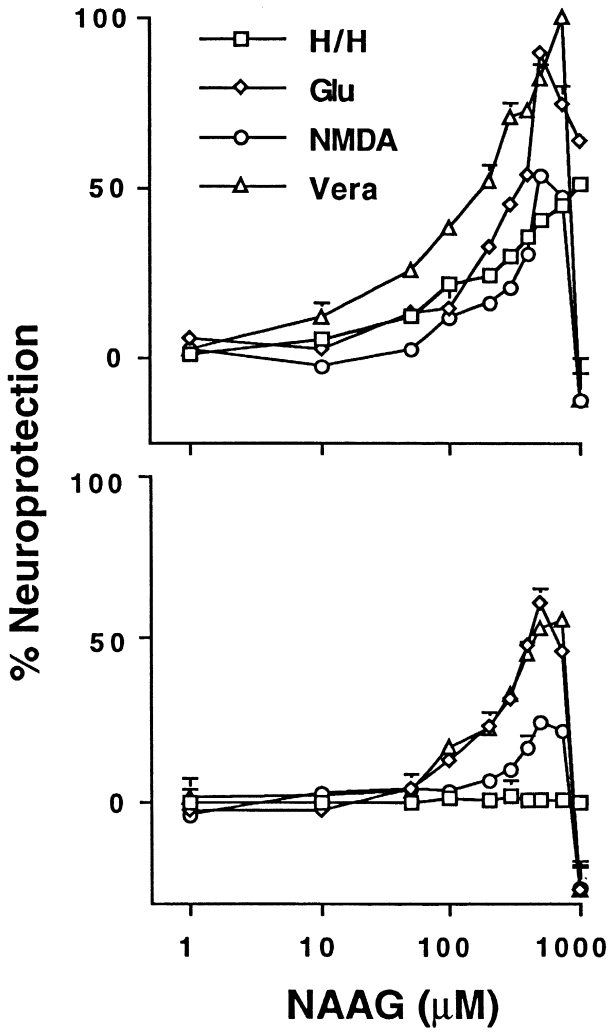

Fig. 4. Neuroprotection dose–response effect of NAAG [in the presence (top panel) or absence (bottom panel) of phosphate] against hypoxia/hypoglycemia, glutamate, NMDA or veratridine toxicity. Values are the same as described for Fig. 2.

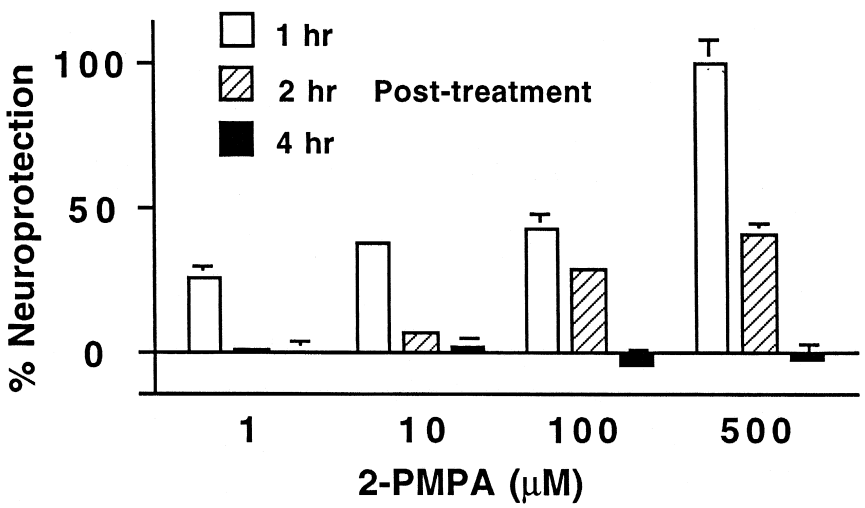

Fig. 3. Post-injury time- and dose-dependent neuroprotection effect of 2-PMPA against hypoxia/hypoglycemia induced injury. Different concentrations of 2-PMPA were added at 1 h (1 h after 2 h of hypoxia/hypoglycemia treatment), 2 h (at the end of hypoxia/hypoglycemia treatment), or 4 h (2 h after hypoxia/hypoglycemia treatment) time intervals. Values are the same as described for Fig. 2.

3.4. Effect of NAAG and β-NAAG

Co-treatment of cerebellar neurons with NAAG was highly neuroprotective against all four injury models. Unlike 2-PMPA, the optimal effects of NAAG were seen in phosphate-containing media (Fig. 4, top) where it was maximally (90–100%) neuroprotective against glutamate and veratridine injuries, with moderate efficacy (approximately 50% neuroprotection) measured against hypoxia/hypoglycemia and NMDA-induced injuries. Also, unlike 2-PMPA, in the absence of phosphate NAAG neuroprotection was markedly reduced. Only limited neuroprotection was seen against glutamate and veratridine induced injuries, with no protective effect measured in neurons injured by hypoxia/hypoglycemia (Fig. 4, bottom).

A concentration dependent neuroprotection was also demonstrated when injured neurons were treated with β-NAAG. Like NAAG, the potency and efficacy (100% neuroprotection) of β-NAAG-induced protection was greatest in the presence of phosphate (Fig. 5, top) and reduced, by 40–50%, in the absence of phosphate (Fig. 5, bottom). However, unlike 2-PMPA and NAAG, the neuroprotective effects of β-NAAG occurred independent of the type of injury.

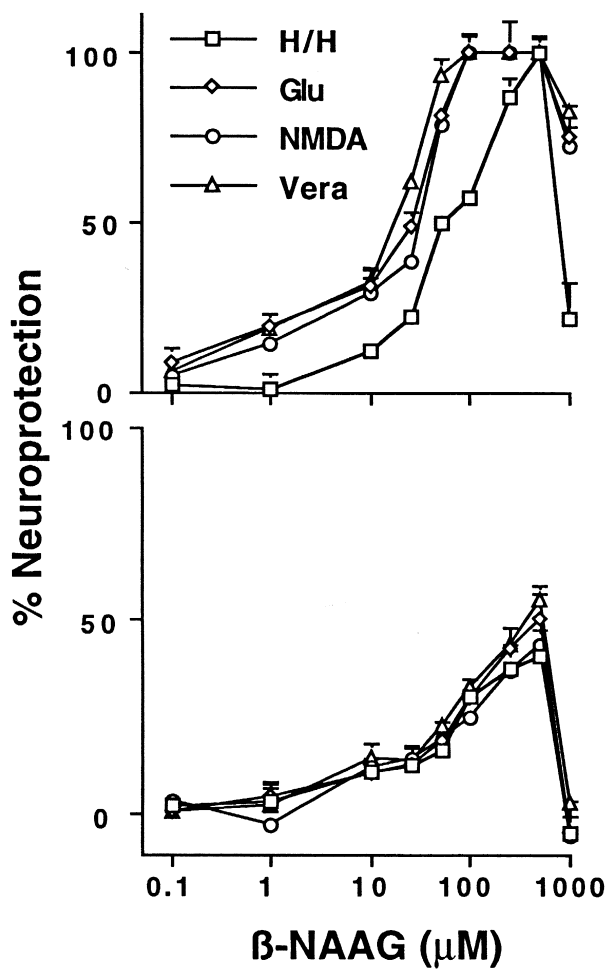

Fig. 5. Neuroprotection dose–response effect of β-NAAG [in the presence (top panel) or absence (bottom panel) of phosphate] against hypoxia/hypoglycemia, glutamate, NMDA or veratridine toxicity. Values are the same as described for Fig. 2.

3.5. High dose neurotoxicity

All the compounds were examined for possible high-dose neurotoxicity in normal cultured neurons. At concentrations as high as 2 mM, 2-PMPA or 2-PMSA failed to produce evidence of neurotoxicity. In contrast, 1 mM of NAAG or β-NAAG was cytotoxic, and at 2 mM concentrations neuronal cell death approached 80%.

4. Discussion

Consistent with our recent report describing neuroprotective effects of 2-PMPA in experimental models of anoxia/ischemia mediated neuronal death (Slusher et al., 1999), the results of the present study confirmed that 2-PMPA protects neurons from injury induced by cellular anoxia (i.e. hypoxia/hypoglycemia deprivation) and that delayed treatment with 2-PMPA is also neuroprotective, at least when the injury is caused by anoxia (i.e. hypoxia/hypoglycemia model). Also, it has been demonstrated here that 2-PMPA is much less effective against neuronal injuries induced by downstream activation of specific glutamate receptors and/or voltage-gated ion channels. Furthermore, while the neuroprotection mechanism of action of 2-PMPA may, in part, involve actions consistent with a metabotropic receptor activation, the differences seen between NAAG, β-NAAG and 2-PMPA in identical injury models suggest other possibilities.

In experimental conditions modeling pathologic anoxia/ischemia, such as the hypoxia/hypoglycemia injury used in the present study or brain ischemia, one possible effect of NAALADase inhibition would be a reduction in the hydrolysis of NAAG, the neuropeptide precursor and endogenous source for cellular glutamate (Blakely and Coyle, 1988), to glutamate. This would be expected to lead to a decrease in the ischemia-induced glutamate release with consequential increases in extracellular NAAG. Consistent with this, it has been shown in experimental stroke in rats that 2-PMPA increases NAAG and decreases the rise in extracellular glutamate at neuroprotective doses significantly reducing cerebral infarction (Slusher et al., 1999).

By increasing NAAG, a second possible effect of NAALADase inhibition synergistic to the decrease in glutamate would be neuroprotection possibly via stimulation of $mGlu_3$ receptors since NAAG has been described as a selective agonist for the $mGlu_3$ site (Orlando et al., 1997; Wroblewska et al., 1998). In contrast to 2-PMPA, where at concentrations as high as 10 μM it fails to bind to

numerous neuronal receptor targets (including various glutamatergic, ion channel, enzyme and transporter sites) (Slusher et al., 1999), NAAG has been reported to display full agonist properties at $mGlu_3$ receptor sites ($EC_{50} = 26$ μM) (Hess et al., 1999). However, the relative importance of the role of $mGlu_3$ receptors to the neuroprotection pharmacology of NAAG (and indirectly 2-PMPA) is unclear since it has been recently reported that NAAG-induced neuroprotection at least in spinal neurons is not blocked by $mGlu_3$ receptor antagonists (Yourick et al., 1999). In addition, since glutamate stimulates both ionotropic and metabotropic receptors, and NMDA (which was relatively unresponsive to 2-PMPA) is coupled primarily to ionotropic ligand-gated channels and has no effect on cloned metabotropic glutamate receptors (Pin and Duvoisin, 1995), it is unlikely that the neuroprotection elicited by 2-PMPA in our studies can be attributable to ionotropic mechanisms. Additional experiments are underway to resolve this issue, including determining whether 2-PMPA neuroprotection can be modulated by $mGlu_3$ receptor ligands, or modulators of ionotropic receptor activity. Finally, an important role for voltage-gated, pre-synaptic or post-synaptic glutamate release mechanisms, or voltage-gated mechanisms triggering abnormal depolarization-induced excitotoxicity, also seems unlikely since 2-PMPA failed to attenuate neurotoxicity caused by the voltage-gated, sodium channel activator veratridine.

What has been established is that 2-PMPA is a potent and selective inhibitor of NAALADase with a $K_i = 280$ ρM (Slusher et al., 1999), and rat brain binding studies have described 2-site kinetics for 2-PMPA inhibition of this enzyme (high affinity $K_d = 2.45$ nM and low affinity $K_d = 190$ nM) (Cai et al., 1998). If the mechanism of action of 2-PMPA involves inhibition of NAALADase then, given its ρM affinity for the enzyme, a similar potency might be predicted for its neuroprotection actions. Although in the present study the neuroprotection potency for 2-PMPA ($EC_{50} = 8.4$ μM) was approximately 13 times lower than that reported in earlier studies (i.e. $EC_{50} = 617$ ρM; Slusher et al., 1999) our data are consistent with those described in a dissociated cortical cell anoxia injury model where the EC_{50} for 2-PMPA was likewise shown to be ~ 7 μM (Olkowski et al., 1998). We believe this apparent discrepancy between enzyme affinity and neuroprotection potency relates to the fact that in both studies, neuronal culture models of injury were used where glial proliferation was highly suppressed. While NAALADase is present in both neurons and glia, it has been demonstrated that at least 90% of NAALADase-expressing cells are glia (Luthi-Carter et al., 1998). Consistent with this, we have examined our neuronal enriched cell cultures for NAALADase activity and determined it to be 0.019 ± 0.002 ρmol/min/mg protein. This is approximately 1/10 the activity reported for glia enriched cultures (NAALADase activity = 0.2 pmol/min/mg; B. Slusher, personal communication) and is consistent with the 10% glia concentration present in our cultures, and the 10–13-fold decrease in 2-PMPA potency described above in our injury model and others (Olkowski et al., 1998) as compared to its potency reported in glia enriched cultures (Slusher et.al, 1999). Collectively, these results confirm and underscore the importance of glia, which are increased in central nervous system (CNS) tissue following injury (Norton et al., 1992), in neuroprotection mechanisms possibly involving upstream modulation of NAALADase.

Although the precise neuroprotection mechanism of action of 2-PMPA is uncertain and the importance of NAALADase inhibition remains unresolved, there is convincing evidence that its inhibitory effects on NAALADase and subsequent neuroprotection are highly selective. For example, we demonstrated here that 2-PMSA, the relatively inactive analog of 2-PMPA possessing a K_i for NAALADase inhibition of 2.6 μM, at concentrations up to 1000 μM was ineffective (or only weakly effective) in all four of the neuronal injury models studied. Similar results have been reported for 2-PMSA and other structurally related NAALADase inhibitors in a cyanide/2-deoxyglucose model of cellular anoxia (Slusher et al., 1999). The results of the experiments carried out in the presence and absence of phosphate are also relevant. Phosphate itself has a potent inhibitory effect on cellular NAALADase activity and experiments with 2-PMPA require a phosphate-free medium for maximal responsiveness (Slusher et al., 1990). However, unlike 2-PMPA the neuroprotective effects of NAAG were not suppressed by phosphate, and actually were greater when phosphate was present in the culture media. As might be expected, compared to 2-PMPA or NAAG the phosphate effect on the non-hydrolyzable analog β-NAAG, where the role of NAALADase would be expected to be less relevant, was even less pronounced. Furthermore, unlike 2-PMPA (or NAAG), β-NAAG induced neuroprotection was shown to occur independent of the injury mechanism/model tested.

Collectively, the various in vitro and in vivo studies reported to date, including the results described here, strongly support a mechanism of action for 2-PMPA linked directly to an upstream modulation of glutamate hyper-excitability, possibly via inhibition of the hydrolyzing enzyme NAALADase. Inhibition of NAALADase represents a novel upstream mechanism aimed at modulating glutamate neurotransmission with limited potential for causing adverse effects (Slusher et al., 1999; Tortella et al., 1999) and 2-PMPA and related compounds represent a new class of therapeutic agents for advanced evaluation in neurodegenerative diseases of the CNS.

References

Blakely, R., Coyle, J., 1988. The neurobiology of *N*-acetylaspartylglutamate. Int. Rev. Neurobiol. 30, 39–100.

Cai, N.S., Olkowaski, J.L., Jackson, P.F., Slusher, B.S., 1998. Binding of

the NAALADase inhibitor 2-PMPA to brain membranes exhibiting two-site kinetics. Soc. Neurosci. Abstr. 24, 582.

Coyle, J.T., 1997. The nagging question of the function of *N*-acetylaspartylglutamate. Neurobiol. Dis. 4 (304), 231–238.

Dave, J.R., Koenig, M.L., Tortella, F.C., Pieringer, R.A., Doctor, B.P., Ved, H.S., 1997. Dodecylglycerol provides partial protection against glutamate toxicity in neuronal cultures derived from different regions of embryonic rat brain. Mol. Chem. Neuropathol. 30, 1–13.

Hess, S.D., Pasieczny, R., Rao, S.P., Jachee, C., Varney, M.A., Johnson, E.C., 1999. Activity of *N*-acetylaspartylglutamate at human recombinant glutamate receptors. Soc. Neurosci. Abstr. 25, 975.

Jackson, P.F., Cole, D.C., Slusher, B.S., Stetz, S.L., Ross, L.E., Donznati, B.A., Trainor, D.A., 1996. Design, synthesis, and biological activity of a potent inhibitor of the neuropeptide *N*-acetyl-α-linked acidic dipeptidase. J. Med. Chem. 39, 619–622.

Luthi-Carter, R., Berger, U.V., Barczak, A.K., Enna, M., Coyle, J.T., 1998. Isolation and expression of a rat brain cDNA encoding glutamate carboxypeptidase II. Proc. Natl. Acad. Sci. U. S. A. 95, 3215–3220.

Norton, W.T., Aquino, D.A., Hozumi, I., Chiu, F.C., Brosnan, C.F., 1992. Quantitative aspects of reactive gliosis: a review. Neurochem. Res. 17, 877–885.

Olkowski, J.L., Thomas, A.G., Slusher, B.S., 1998. Neuroprotection afforded by NAALADase inhibition requires glial cells. Soc. Neurosci. Abstr. 24, 582.

Orlando, L.R., Luthi-Carter, R., Standaert, D.G., Coyle, J.T., Penney, J.B., Young, A.B., 1997. *N*-acetylaspartylglutamate (NAAG) protects against rat striatal quinolinic acid lesions in vivo. Neurosci. Lett. 236, 91–94.

Pin, J.P., Duvoisin, R., 1995. Review: neurotransmitter receptors I: the metabotropic glutamate receptors: structure and functions. Neuropharmacology 34, 1–26.

Slusher, B.S., Robinson, M.B., Tsai, G., Simmons, M.L., Richards, S.S., Coyle, J.T., 1990. Rat brain *N*-acetylated alpha-linked acidic dipeptidase activity: purification and immunologic characterization. J. Biol. Chem. 265 (34), 21297–21301.

Slusher, B., Varnov, J., Thomas, A., Hurn, P., Harukuni, I., Bhardwaj, A., Traystman, R., Robinson, M., Britton, P., May Lu, X.-C., Tortella, F., Wozniak, K., Yudkoff, M., Jackson, P., 1999. Reduction of extracellular glutamate via NAALADase inhibition is neuroprotective. Nat. Med. 5 (12), 1396–1402.

Tortella, F., Williams, A., Slusher, B., Lu, M., 1999. EEG studies on 2-PMPA, an upstream modulator of hyperglutamatergic activity and neuroprotectant, in rats. Soc. Neurosci. Abstr. 25, 2230.

Wroblewska, B., Neale, J.H., Bzdega, T., Lea, P.M., Sarvey, J.M., 1998. Antagonist effects of β-NAAG on mGluR3 receptor. Soc. Neurosci. Abstr. 24, 585.

Yourick, D.L., Koenig, M., Long, J.B., 1999. Metabotropic glutamate receptors do not mediate *N*-acetylaspartylglutamate (NAAG) protection of spinal cord cultures from NMDA-induced cell death. Soc. Neurosci. Abstr. 25, 841.

Ved, H.S., Koenig, M.L., Dave, J.R., Doctor, B.P., 1997. Huperzine A, a potential therapeutic agent for dementia, reduces neuronal cell death caused by glutamate. NeuroReport 8 (4), 963–968.