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NAALADase inhibition protects motor neurons against chronic glutamate toxicity

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Abstract

Glutamate toxicity is implicated in the pathogenesis of amyotrophic lateral sclerosis. The neuropeptide *N*-acetyl-aspartyl glutamate (NAAG) appears to function both as a storage form for glutamate and as a neuromodulator at glutamatergic synapses. *N*-acetylated-α-linked acidic dipeptidase (NAALADase; also termed glutamate carboxypeptidase II) yields *N*-acetyl aspartate (NAA) and glutamate. Prior studies have demonstrated NAALADase upregulation in motor cortex and increased NAAG, NAA and glutamate in cerebrospinal fluid from amyotrophic lateral sclerosis patients. The potent NAALADase inhibitor, 2-(phosphonomethyl)-pentanedioic acid (2-PMPA), was tested in an in vitro model of chronic glutamate-mediated motor neuron degeneration. Neuroprotection was determined (1) biochemically, by measuring choline acetyltransferase activity, (2) immunohistochemically, by counting neurofilament-H-positive motor neurons and (3) morphologically, with phase contrast microscopy. 2-PMPA (10 μM) had significant neuroprotective effects on motor neurons as evidenced by increased choline acetyltransferase activity, decreased motor neuron loss and improved gross morphology. Results suggest that NAALADase inhibitors protect against chronic glutamate-mediated motor neuron degeneration and may prove therapeutic towards amyotrophic lateral sclerosis.

Keywords: Amyotrophic lateral sclerosis; GCP II; Glutamate; NAAG; NAALADase; Neuroprotection; Motor neuron disorder

1. Introduction

In the central nervous system, the concentration of the neuropeptide *N*-acetyl-aspartyl-glutamate (NAAG) is highest in the spinal cord, specifically in the ventral horn (2 mM; Koller and Coyle, 1984; Miyake et al., 1981). This abundant neuropeptide appears to function both as a proposed precursor of neurotransmitter glutamate and as a neuromodulator at glutamatergic synapses (Moffett et al., 1990; Mori-Okamoto et al., 1987; Tsai et al., 1990). NAAG is hydrolyzed by the enzyme *N*-acetylated-α-linked acidic dipeptidase (NAALADase; also called glutamate carboxypeptidase II, EC 3.4.17.21), located on neuronal and glial surfaces (Cassidy and Neale, 1993; Neale et al., 2000), to yield *N*-acetylaspartate (NAA) and glutamate (Robinson et al., 1987). Prior studies have demonstrated an upregulation of NAALADase in motor cortex in amyotrophic lateral sclerosis tissue and increased levels of NAAG, NAA and glutamate in the cerebrospinal fluid of amyotrophic lateral sclerosis patients (Tsai et al., 1991). Chronic glutamate toxicity, implicated in the pathogenesis of motor neuron degeneration in amyotrophic lateral sclerosis (Rothstein, 1995a), may be a consequence of the hydrolysis of NAAG by NAALADase.

Detailed analysis of the role of NAALADase has recently become possible with the discovery of a potent and selective NAALADase inhibitor, 2-(phosphonomethyl)-pentanedioic acid (2-PMPA, $K_i = 280$ pM; Jackson et al., 1996). Using organotypic rat spinal cord cultures prepared from postnatal rats, we first examined constitutive effects of 2-PMPA on relatively mature motor neurons. Chronic glutamate toxicity was induced by D,L-threo-hydroxyaspartate (THA; Rothstein et al., 1990). THA selectively inhibits glutamate transport, raising extracellular glutamate concentrations leading to motor neuron degeneration. This in vitro model of toxicity has proven predictive success of Rilutek and

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insulin-like growth factor (IGF-I), and failure of ciliary neurotrophic factor (CNTF) and brain derived neurotrophic factor (BDNF) in clinical trials in amyotrophic lateral sclerosis. In this model, we tested whether inhibition of NAALADase by 2-PMPA could protect motor neurons from chronic glutamate-mediated excitotoxicity, presumably by reducing extracellular glutamate.

Our data herein strongly suggest that NAALADase inhibition with 2-PMPA protects motor neurons against chronic glutamate-mediated toxicity based on: (1) motor neuron cell counts and (2) choline acetyltransferase (EC 2.3.1.6) activity. Thus, NAALADase inhibitors may provide a novel therapeutic approach against motor neuron disorders such as amyotrophic lateral sclerosis.

2. Materials and methods

All procedures reported here have been carried out in accordance with the Declaration of Helsinki and/or with the Guide for the Care and Use of Laboratory Animals as adopted and put forth by the National Institutes of Health of the United States.

2.1. Organotypic spinal cord cultures

Organotypic spinal cord cultures were prepared from the lumbar cord of postnatal 8-day-old rat pups (Corse and Rothstein, 1995). The pups were decapitated and the spinal cords rapidly removed and placed in 35-mm petri dishes containing sterile Gey's balanced salt solution (GBSS) with glucose (3.2 g/500 ml). The meninges surrounding the cords were removed and the nerve roots transected under magnification. The spinal cords were then collected under sterile conditions and sectioned transversely, at 350-μm intervals, using a McIlwain tissue chopper. Sections were transferred to new petri dishes containing GBSS and each individual section gently separated. Slices were then carefully placed on 30-mm semipermeable Millipore Millicell membranes (five slices per membrane) in a 6-well plate. Each well

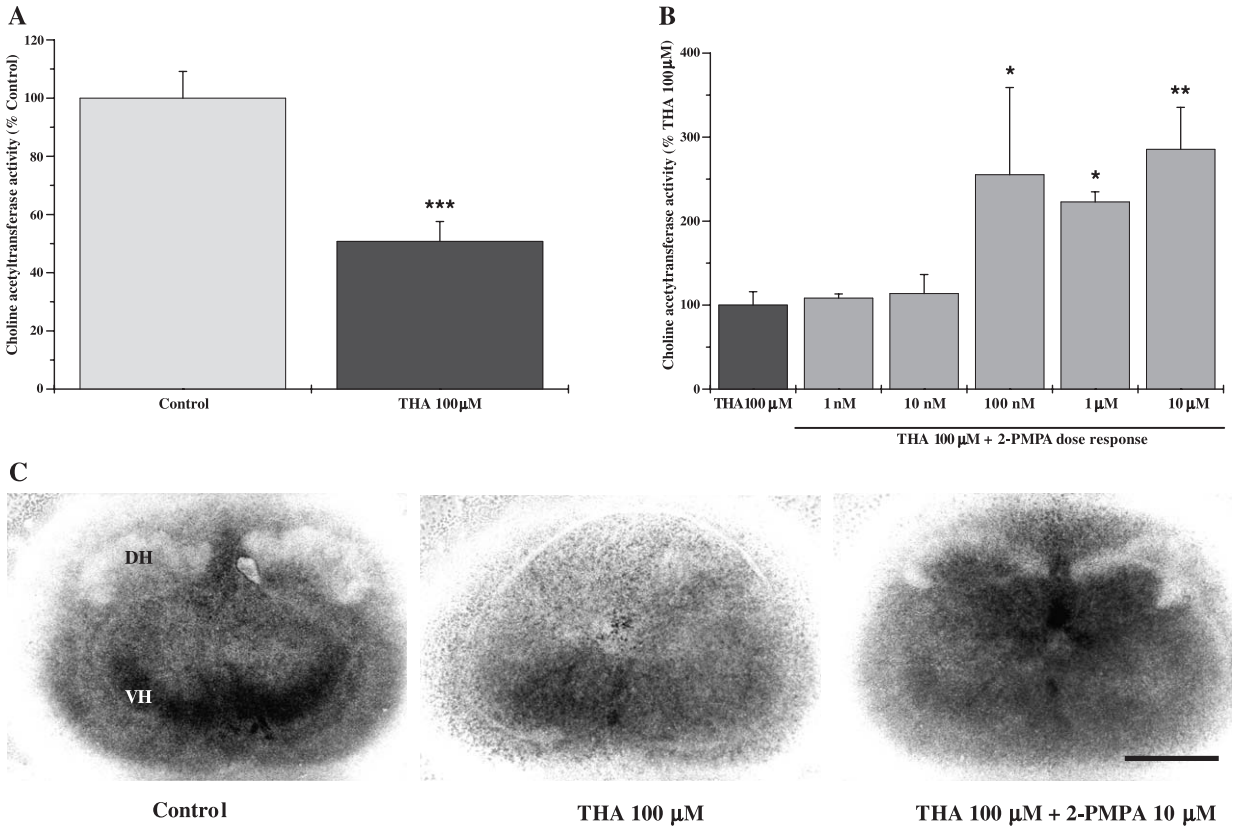

Fig. 1. NAALADase inhibition protects against chronic glutamate toxicity: choline acetyltransferase activity. (A) Choline acetyltransferase activity is reduced by 50% in cultures treated with 100-μM THA, from 0.038 ± 0.005 to 0.018 ± 0.004 nmol/mg/40 min. Twenty-four wells were used for the untreated control group and 20 for THA-treated group. Data is presented as a percentage of choline acetyltransferase activity of untreated controls. Asterisk indicates statistical significance from untreated control cultures, ***$P < 0.001$. (B) 2-PMPA dramatically prevents the reduction of choline acetyltransferase activity in a dose-dependent manner after more than 21 days in culture. Data is presented as a percentage of choline acetyltransferase activity of THA-treated controls. Asterisk indicates significant differences from THA-treated group; *$P < 0.05$, **$P < 0.01$. (C) Phase contrast microscopy: This 31-day-old control spinal cord culture shows intact gross morphologic features. Cultures treated with 100-μM THA appear thin, grainy and shrunken. Gross morphology is strikingly preserved in cultures co-treated with 100-μM THA and 10-μM 2-PMPA. VH = ventral horn; DH = dorsal horn. Scale bar = 500 μm.

contained 1 ml of incubation medium (50% phosphate-free minimum essential media, 25% heat-inactivated horse serum, 25% phosphate-free Hanks' balanced salt solution supplemented with glucose (12.8 g/500 ml) and 2 mM L-glutamine) at pH 7.2. Cultures were maintained in a humidified environment at 37 °C, in a 5% CO_2 incubator. Medium was changed every 3 days along with any pharmacological agents.

2.2. Experimental design

Cultures were allowed an 8-day recovery period, after initial preparation, prior to initiation of treatment. Chronic toxicity was produced by inhibiting the uptake of glutamate using the glutamate transport blocker, THA. THA and/or neuroprotectants were replenished with every culture medium change. At the end of the experimental time period, the viability of motor neurons was determined by (i) biochemical analysis of tissue choline acetyltransferase activity and (ii) immunohistochemical staining of neurofilaments within the motor neurons. Whereas choline acetyltransferase activity studies were conducted on cultures that were 21–43 days old and treated with 100-μM THA, immunohistochemical studies were conducted on cultures that were 17–19 days old and treated with 200-μM THA. Six to twenty-four culture wells were used for choline acetyltransferase activity determinations and 15–50 immunostained sections, per treatment group, were counted. Statistical analyses were performed using a two-population *t*-test.

2.3. Choline acetyltransferase assay

Choline acetyltransferase activity, which is largely restricted to motor neurons, was determined radiometrically using [^{3}H]acetyl-coenzyme A (Fonnum, 1975). In brief, five spinal cord sections from each well were pooled and frozen, at − 80 °C, until the time of the assay. Each well represented one time point or drug concentration. Pooled sections were homogenized in cold phosphate buffer and an aliquot of the sample incubated in equal volumes of choline acetyltransferase assay mix (100 mM acetyl-coenzyme A, 12.5 mM choline chloride, 300 mM sodium chloride, 35 mM EDTA, 0.2 mM eserine and 2.5 μCi/ml [^{3}H]acetyl-coenzyme A in 50 mM phosphate buffer at pH 7.4) for 40 min at 37 °C. The

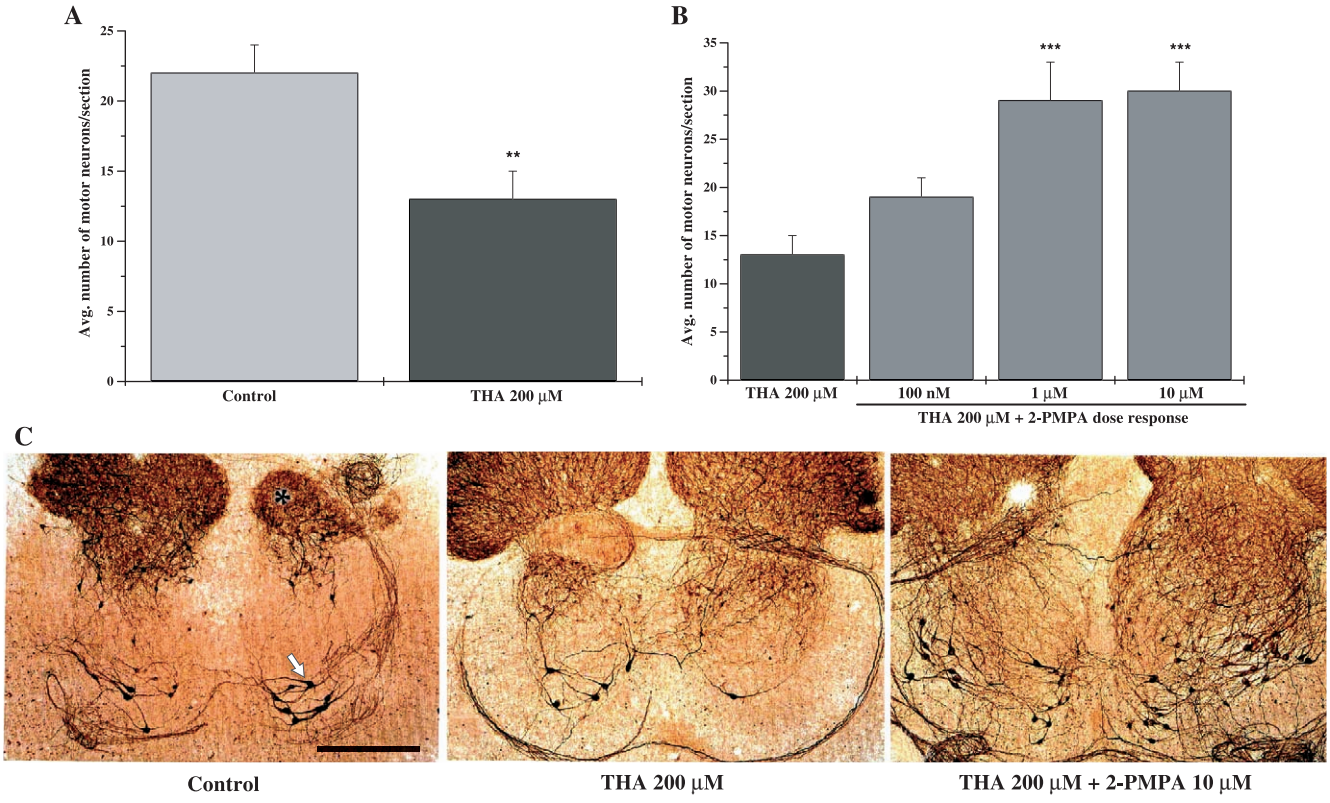

Fig. 2. NAALADase inhibition protects against motor neuron loss. (A) Approximately 40% of motor neurons are lost due to chronic glutamate toxicity in cultures treated with 200-μM THA. Asterisk indicates statistical difference from untreated control; $^{**}P<0.01$. (B) 2-PMPA prevents motor neuron loss, in a dose-dependent manner, after more than 17 days in culture. Asterisk indicates statistical differences from THA-treated group; $^{***}P<0.001$. (C) Neurofilament-H Immunohistochemistry: Neurofilament immunostaining in a 17-day control culture shows large motor neurons in the ventral horn (arrow) with plentiful neurite extensions. The dorsal horn neuropil (*) also stains intensely. THA-treated cultures (200 μM) show reduced number of motor neurons with reduced neurite outgrowth. Motor neuron numbers are preserved in cultures co-treated with 200-μM THA and 10-μM 2-PMPA. Neurite extension has not been quantified. Scale bar = 500 μm.

reaction was terminated using ice-cold scintillation cocktail mix (10 mM phosphate buffer at pH 7.4, scintillant consisting of toluene and liquiflour and 0.015 mM tetraphenylboron in acetonitrile). The resultant aqueous/organic mixture was separated upon centrifugation (4000 × *g* for 5 min at 5 °C). Labelled acetylcholine was extracted into the organic scintillation phase and counted, whereas unreacted acetyl-CoA remained in the aqueous phase and was not counted since the aqueous phase does not function as a scintillation solvent. Results of each biochemical assay were normalized according to authentic choline acetyltransferase enzyme standards (two concentrations) included in each assay. Statistical analyses were performed using two-population *t*-test.

2.4. Immunohistochemistry

Cultures were fixed with 4% phosphate-buffered paraformaldehyde (pH 7.4) for 20 min, rinsed with 0.1 M phosphate buffer and then permeabilized with cold methanol for 10 min. The methanol was washed off with 0.05 M Tris-buffered saline (TBS; 1.5% sodium chloride) and the cultures incubated in blocking media (10% horse serum in TBS) for 1 h at room temperature. Subsequently, the cultures were incubated for 24 h at 4 °C with SMI-32 monoclonal antibody, at 1:8000 dilution in TBS containing 2% horse serum, directed against the 200-kDa subunit of non-phosphorylated epitopes in neurofilament-H. Following primary antibody incubation, cultures were incubated with biotinylated rat-adsorbed horse anti-mouse immunoglobulin (1:200 dilution in TBS containing 2% horse serum) for 1 h at room temperature. The reaction products were visualized with the Vectastain *Elite* ABC kit using 1.4 mM 3,3′-diaminobenzidine tetrahydrochloride and 0.0075% hydrogen peroxide. Cultures were thoroughly rinsed, mounted on slides and air dried prior to dehydration with ethanol. The cultures were then defatted with a xylene run and permanently cover slipped using permount. The survival of motor neurons, operationally defined as large cells within the ventral horn region of the spinal cord, was quantified by manually counting the same using light microscopy.

2.5. Materials

[^{3}H] Acetyl-coenzyme A and liquiflour were obtained from NEN™ Life Science Products (Boston, MA), SMI-32 monoclonal antibody from Sternberger Monoclonals (Lutherville, MD), biotinylated rat-adsorbed horse anti-mouse immunoglobulin and the Vectastain *Elite* ABC kit from Vector Laboratories (Burlingame, CA), 3,3′-diaminobenzidine tetrahydrochloride from Polysciences (Warrington, PA) and hydrogen peroxide from J.T. Baker (Phillipsburg, NJ). 2-PMPA and 2-(phosphonomethyl)succinic acid (2-PMSA) were synthesized by SRI International (Menlo Park, CA) and phosphate-free Hanks' balanced salt solution was custom made by Paragon Biotech (Baltimore, MD). GBSS, minimum essential media and the horse serum were purchased from GIBCO (Rockville, MD). All other drugs and chemicals were obtained from Sigma (St. Louis, MO).

3. Results

3.1. NAALADase inhibition with 2-PMPA prevents the reduction of choline acetyltransferase activity due to chronic glutamate-mediated toxicity in the spinal cord

To examine the potential usefulness of NAALADase inhibitors in amyotrophic lateral sclerosis, spinal cord cultures were co-treated with the glutamate transport blocker, THA and with the NAALADase inhibitor, 2-PMPA. Whereas choline acetyltransferase activity was significantly reduced in cultures treated with THA alone (Fig. 1A, 50% reduction compared to untreated cultures, $P < 0.001$), 2-PMPA dramatically prevented the reduction of choline acetyltransferase activity induced by either 100-μM (Fig. 1B) or 200-μM THA (data not shown). The effects of 2-PMPA were dose-dependent. At the highest dose, 2-PMPA (10 μM) had significant neuroprotective effects on motor neurons as evidenced by higher choline acetyltransferase activity-185% compared to 100-μM THA-control. The gross morphology, as observed by phase contrast microscopy, was reflective of these results (Fig. 1C). Control spinal cord cultures showed intact gross morphologic features

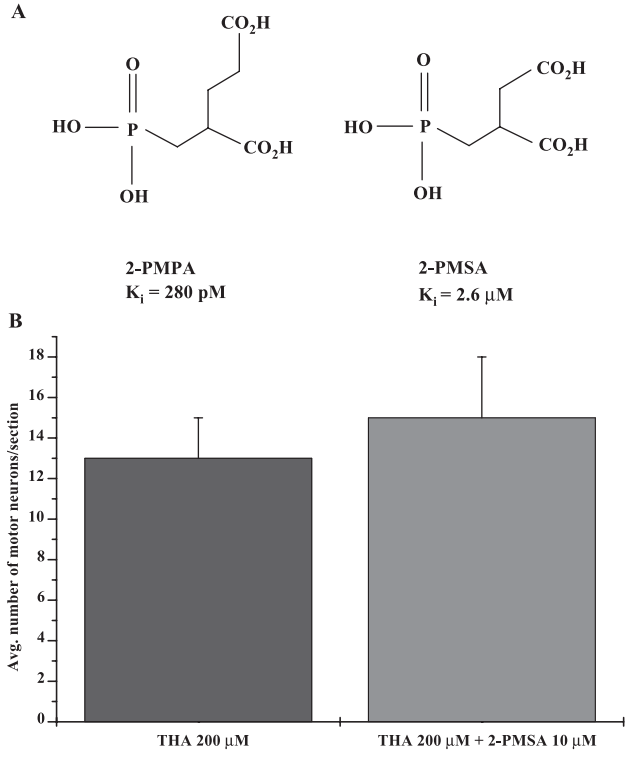

Fig. 3. Neuroprotection correlates with potency of NAALADase inhibition. (A) Structure of the NAALADase inhibitor 2-PMPA and its inactive analog 2-PMSA. (B) 2-PMSA (10 μM) does not protect motor neurons against THA toxicity, $P = 0.39$.

while cultures treated with 100-μM THA appeared thin, grainy and shrunken. By contrast, cultures co-treated with 100-μM THA and 10-μM 2-PMPA had strikingly well-preserved gross morphology.

3.2. NAALADase inhibition with 2-PMPA protects motor neurons from chronic glutamate-mediated cell death

Neuroprotection with NAALADase inhibition was confirmed immunohistochemically, by counting the number of neurofilament-H-positive motor neurons. Whereas approximately 40% of motor neurons were lost due to chronic glutamate toxicity in cultures treated with 200-μM THA (Fig. 2A, $P < 0.01$), 2-PMPA prevented motor neuron loss, in a dose-dependent manner, after more than 18 days in culture (Fig. 2B). Light microscopic analyses show neurofilament-stained control cultures with large motor neurons in the ventral horn (arrow), THA-treated cultures (200 μM) with reduced number of motor neurons and THA (200 μM) and 2-PMPA (10 μM) co-treated cultures with well-preserved motor neurons (Fig. 2C). Conversely, equimolar concentrations of a structurally similar but inactive analog of 2-PMPA, 2-(phosphonomethyl)succinic acid (2-PMSA, $K_i = 2.6$ μM, Fig. 3A; Slusher et al., 1999), did not protect motor neurons against THA toxicity (Fig. 3B, $P = 0.39$).

4. Discussion

There are four main hypotheses regarding the cause of amyotrophic lateral sclerosis including excitotoxicity linked to glutamate receptor over activation, mutation of the superoxide dismutase gene, production of autoantibodies to calcium channels and neurofilament accumulation (Hugon, 1996). Our results show that NAALADase inhibition reduces the death of motor neurons in a model of amyotrophic lateral sclerosis.

NAAG is highly concentrated in the motor neurons of the brainstem, in the ventral horn of the spinal cord and in a number of putative glutamatergic neuronal systems (Blakely and Coyle, 1988; Tsai et al., 1991). In patients with amyotrophic lateral sclerosis, NAAG levels decrease in all regions of the spinal cord except in the posterior column (Tsai et al., 1991). Glutamate, a product of NAAG hydrolysis, is significantly elevated in the plasma of amyotrophic lateral sclerosis patients as compared to those of healthy and disease controls (Plaitakis, 1990; Rothstein et al., 1990). Concomitantly, NAALADase activity is also significantly upregulated in the ventral column of patients with amyotrophic lateral sclerosis (Tsai et al., 1991). The co-localization of NAALADase with NAAG suggests the possibility that NAAG could act as a glutamate precursor and that the glutamate liberated from NAAG could be involved in the pathogenesis of amyotrophic lateral sclerosis. Our data are consistent with NAALADase-mediated chronic glutamate toxicity and subsequent motor neuron degeneration. Spinal cord sections co-treated with THA and 2-PMPA had significantly higher choline acetyltransferase activities (Fig. 1) and greater number of neurofilament-H-positive motor neurons (Fig. 2) than those treated with THA alone. In contrast, 2-PMSA, a structurally similar analog of 2-PMPA with 10,000-fold less potency for inhibiting NAALADase had no effect (Fig. 3).

Synaptic concentrations of glutamate are regulated by high-affinity glutamate transporters. A dramatic loss of glutamate transport has been observed in the brain and spinal cord of patients with sporadic amyotrophic lateral sclerosis (Rothstein and Kuncl, 1995; Rothstein et al., 1992). Excitotoxicity, due to the loss of glutamate transport function, has been implicated in the pathogenesis of the disease. However, it is unlikely that 2-PMPA-mediated neuroprotection is a result of altered glutamate transport since 2-PMPA is a potent and highly selective inhibitor of NAALADase. In fact, even at concentrations as high as 10 μM, 2-PMPA is inactive in more than 100 different receptor, transporter, ion channel and enzyme assays including several glutamatergic sites such as NMDA, AMPA and glutamate transporters (Slusher et al., 1999).

The selective vulnerability of the motor neurons to glutamate toxicity via the AMPA/kainate receptors is also well documented (Carriedo et al., 1996; Rothstein, 1995b; Saroff et al., 2000; Van Den Bosch et al., 2000). It is improbable that neuroprotection mediated by 2-PMPA, against THA toxicity, is a consequence of blocked AMPA/kainate receptors since 2-PMPA is not known to have any effects at the AMPA/kainate receptors (Slusher et al., 1999).

We had previously demonstrated 2-PMPA to be neuroprotective in both cell culture and animal models of cerebral ischemia (Slusher et al., 1999). We observed that neuroprotection via NAALADase inhibition was affected not only through decreased glutamate but also through increased extracellular NAAG following middle cerebral artery occlusion. While decreased glutamate is neuroprotective in many disease models including amyotrophic lateral sclerosis, by limiting glutamate receptor overactivation (Meldrum, 1990; Rothman and Olney, 1986; Rothstein et al., 1993), the role of NAAG in neuroprotective mechanisms mediated by NAALADase inhibition against THA toxicity is unclear. Nevertheless, the abundance of spinal NAAG suggests a potential role for the dipeptide in the pathophysiology of amyotrophic lateral sclerosis.

Several reports have demonstrated effects of NAAG as a partial antagonist/agonist at the NMDA receptor (Puttfarcken et al., 1993; Sekiguchi et al., 1989; Valivullah et al., 1994). At micromolar concentrations, NAAG acts as a NMDA receptor antagonist. At higher concentrations, the antagonism is lost as NAAG gains agonist properties (Thomas et al., 2000). Early studies have shown that NMDA receptor antagonists do not protect against THA toxicity (Rothstein et al., 1993). It is, therefore, unlikely that the neuroprotection mediated by 2-PMPA against THA toxicity is a consequence of NAAG antagonism at the

NMDA receptors. Previous reports have also demonstrated that direct activation of NMDA receptors do not induce motor neuron toxicity (Rothstein et al., 1993). The lack of toxicity with 2-PMPA is consistent with earlier observations wherein NAALADase inhibitors did not cause NMDA receptor-mediated toxicity through the accumulation of NAAG (Slusher et al., 1999). Additionally, though AMPA/kainate receptor activation has been shown to mediate motor neuron injury, early binding studies indicate that NAAG has no activity at these receptors (Valivullah et al., 1994; Westbrook et al., 1986).

NAAG is also an endogenous agonist at the group-II metabotropic glutamate receptors with specificity for the $mGlu_3$ receptors (Wroblewska et al., 1997). Recently, NAAG has been shown to be neuroprotective via activation of metabotropic glutamate receptors (Bruno et al., 1998b; Thomas et al., 2000). Evidence of $mGlu_3$ receptors on motor neurons and in the surrounding white matter of the spinal cord, from antibody and immunoreactivity studies (Aronica et al., 2001; Ohishi et al., 1993; Petralia et al., 1996; Silva et al., 1999; Tomiyama et al., 2001), suggests the possibility that neuroprotection against THA toxicity, via NAALADase inhibition, may be mediated by the activation of $mGlu_3$ receptors. In fact, Pizzi et al. (2000) recently demonstrated that activation of metabotropic glutamate receptors can protect against kainate-induced degeneration of motor neurons in spinal cord slices from adult rats. Several mechanisms that underlie the neuroprotection afforded by the activation of metabotropic glutamate receptors have been postulated including reduced glutamate release through presynaptic receptor activation (Sanchez-Prieto et al., 1996), inhibition of cAMP formation (Buisson and Choi, 1995) and inactivation of voltage-sensitive calcium channels (Schoepp and Conn, 1993). Neuroprotection by 2-PMPA may be mediated by anyone or a combination of these mechanisms through NAAG's activation of $mGlu_3$ receptors.

NAAG's activation of glial $mGlu_3$ receptors has been shown to protect against neurotoxicity also via the de novo synthesis of transforming growth factor (TGF-β; Bruno et al., 1997, 1998a,b). Many neurotrophic factors, including TGF-β-like trophic factors, have been shown to modulate motor axon growth, enhance survival of motor neurons or affect their response to injury (Bilak et al., 1999; Corse et al., 1999; Ho et al., 2000). Moreover, in a similar tissue culture system, exogenous application of TGF-β1 was shown to protect motor neurons against THA-induced excitotoxicity (Ho et al., 2000). We had previously demonstrated a role for TGF-βs in neuroprotective mechanisms mediated by NAALADase inhibition in both in vitro and in vivo models of cerebral ischemia (Thomas et al., 2001). It is possible that neuroprotection afforded by 2-PMPA, against THA toxicity, may also be mediated by endogenous TGF-β release, possibly via NAAG's activation of $mGlu_3$ receptors.

Regardless of the precise mechanisms involved, overwhelming evidence suggests that glutamate plays an important role in motor neuron death associated with amyotrophic lateral sclerosis. Therefore, any agent that negatively modulates glutamate could potentially prolong motor neuron survival and thereby, alter the clinical course of the disease. The effects of glutamate can be regulated by decreasing glutamate release, blocking post-synaptic receptors, decreasing glutamate synthesis or increasing glutamate transport (Rothstein, 1995a). However, a large number of randomized controlled trials involving anti-glutamate drugs, such as lamotrigine (decreases glutamate release; Eisen et al., 1993), dextromethorphan (post-synaptic receptor blocker; Askmark et al., 1993; Hollander et al., 1994), gabapentin (pre-synaptic inhibitor of glutamate biosynthesis; Brigell and Taylor, 2001; Miller et al., 2001), as well as other growth factors and cytokines such as CNTF (Miller et al., 1996), IGF-1 (Lai et al., 1997) and BDNF (Bradley et al., 1999) have failed to show benefit for the treatment of amyotrophic lateral sclerosis.

Riluzole mitigates excitotoxic effects by blocking presynaptic release of glutamate (Gelinas, 2000). In an in vivo model of amyotrophic lateral sclerosis, riluzole treatment increased survival of superoxide dismutase-1 transgenic mice by 13 days compared to the untreated group. However, riluzole had no significant effect on the delay in onset of clinical symptoms (Gurney et al., 1996). Nonetheless, of all the drugs that have been tested in the clinic, riluzole has shown modest increase in the survival of amyotrophic lateral sclerosis patients in two separate double-blinded, placebo-controlled trials (Bensimon et al., 1994; Lacomblez et al., 1996; Riviere et al., 1998). In a similar superoxide dismutase-1 transgenic model of amyotrophic lateral sclerosis, two structurally different NAALADase inhibitors, administered via two different routes, orally and intraperitoneally, increased survival by almost 29 days (Slusher et al., 2000). Additionally, NAALADase inhibition delayed the onset of clinical symptoms, such as impaired gait and righting reflex, by at least 17 days. In the absence of successful therapies, NAALADase inhibition may provide an exciting therapeutic approach to the devastating disease, amyotrophic lateral sclerosis.

Furthermore, NAALADase inhibition offers potential benefits over existing strategies. NAALADase inhibition represents an 'upstream' mechanism of glutamate regulation that could reduce transmission at all glutamatergic receptors (Slusher et al., 1999). This feature allows for good tolerability. Unlike NMDA or AMPA receptor antagonists, which have been known to cause sedation, impairment of synaptic plasticity, ataxia or psychotomimetic effects, 2-PMPA does not cause similar acute behavioral effects even at high doses (Slusher et al., 1999). Additionally, NAALADase inhibition offers neuroprotection via a combination of mechanisms including pre-synaptic metabotropic receptor activation and through TGF-β release via post-synaptic glial metabotropic receptor activation (Thomas et al., 2000, 2001). Taken together, NAALADase inhibitors present a novel and promising approach to the treatment of amyotrophic lateral sclerosis.

References

Aronica, E., Catania, M.V., Geurts, J., Yankaya, B., Troost, D., 2001. Immunohistochemical localization of group I and II metabotropic glutamate receptors in control and amyotrophic lateral sclerosis human spinal cord: upregulation in reactive astrocytes. Neuroscience 105, 509–520.

Askmark, H., Aquilonius, S.M., Gillberg, P.G., Liedholm, L.J., Stalberg, E., Wuopio, R., 1993. A pilot trial of dextromethorphan in amyotrophic lateral sclerosis. J. Neurol. Neurosurg. Psychiatry 56, 197–200.

Bensimon, G., Lacomblez, L., Meininger, V., 1994. A controlled trial of riluzole in amyotrophic lateral sclerosis. ALS/Riluzole Study Group. N. Engl. J. Med. 330, 585–591.

Bilak, M.M., Shifrin, D.A., Corse, A.M., Bilak, S.R., Kuncl, R.W., 1999. Neuroprotective utility and neurotrophic action of neurturin in postnatal motor neurons: comparison with GDNF and persephin. Mol. Cell. Neurosci. 13, 326–336.

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

Bradley, W., Miller III, R., T.B.S.G.P., 1999. A controlled trial of recombinant methionyl human BDNF in ALS: The BDNF Study Group (Phase III). Neurology 52, 1427–1433.

Brigell, M.G., Taylor, C.P., 2001. ALS defeats gabapentin: reflections on another failed treatment. Neurology 57, 1524–1525.

Bruno, V., Sureda, F.X., Storto, M., Casabona, G., Caruso, A., Knopfel, T., Kuhn, R., Nicoletti, F., 1997. The neuroprotective activity of group-II metabotropic glutamate receptors requires new protein synthesis and involves a glial–neuronal signaling. J. Neurosci. 17, 1891–1897.

Bruno, V., Battaglia, G., Casabona, G., Copani, A., Caciagli, F., Nicoletti, F., 1998a. Neuroprotection by glial metabotropic glutamate receptors is mediated by transforming growth factor-beta. J. Neurosci. 18, 9594–9600.

Bruno, V., Wroblewska, B., Wroblewski, J.T., Fiore, L., Nicoletti, F., 1998b. Neuroprotective activity of *N*-acetylaspartylglutamate in cultured cortical cells. Neuroscience 85, 751–757.

Buisson, A., Choi, D.W., 1995. The inhibitory mGluR agonist, *S*-4-carboxy-3-hydroxy-phenylglycine selectively attenuates NMDA neurotoxicity and oxygen–glucose deprivation-induced neuronal death. Neuropharmacology 34, 1081–1087.

Carriedo, S.G., Yin, H.Z., Weiss, J.H., 1996. Motor neurons are selectively vulnerable to AMPA/kainate receptor-mediated injury in vitro. J. Neurosci. 16, 4069–4079.

Cassidy, M., Neale, J.H., 1993. *N*-acetylaspartylglutamate catabolism is achieved by an enzyme on the cell surface of neurons and glia. Neuropeptides 24, 271–278.

Corse, A.M., Rothstein, J.D., 1995. Organotypic spinal cord cultures and a model of chronic glutamate-mediated motor neuron degeneration. In: Ohnishi, S., Ohnishi, T. (Eds.), Central Nervous System Trauma: Research Techniques. CRC Press, Boca Raton, FL, pp. 341–351.

Corse, A.M., Bilak, M.M., Bilak, S.R., Lehar, M., Rothstein, J.D., Kuncl, R.W., 1999. Preclinical testing of neuroprotective neurotrophic factors in a model of chronic motor neuron degeneration. Neurobiol. Dis. 6, 335–346.

Eisen, A., Stewart, H., Schulzer, M., Cameron, D., 1993. Anti-glutamate therapy in amyotrophic lateral sclerosis: a trial using lamotrigine. Can. J. Neurol. Sci. 20, 297–301.

Fonnum, F., 1975. A rapid radiochemical method for the determination of choline acetyltransferase. J. Neurochem. 24, 407–409.

Gelinas, D.F., 2000. Riluzole. Amyotroph. Lateral Scler. Other Mot. Neuron Disord. 1 (Suppl. 4), 3–4.

Gurney, M.E., Cutting, F.B., Zhai, P., Doble, A., Taylor, C.P., Andrus, P.K., Hall, E.D., 1996. Benefit of vitamin E, riluzole, and gabapentin in a transgenic model of familial amyotrophic lateral sclerosis. Ann. Neurol. 39, 147–157.

Ho, T.W., Bristol, L.A., Coccia, C., Li, Y., Milbrandt, J., Johnson, E., Jin, L., Bar-Peled, O., Griffin, J.W., Rothstein, J.D., 2000. TGFbeta trophic factors differentially modulate motor axon outgrowth and protection from excitotoxicity. Exp. Neurol. 161, 664–675.

Hollander, D., Pradas, J., Kaplan, R., McLeod, H.L., Evans, W.E., Munsat, T.L., 1994. High-dose dextromethorphan in amyotrophic lateral sclerosis: phase I safety and pharmacokinetic studies. Ann. Neurol. 36, 920–924.

Hugon, J., 1996. ALS therapy: targets for the future. Neurology 47, S251–S254.

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

Koller, K.J., Coyle, J.T., 1984. Ontogenesis of *N*-acetyl-aspartate and *N*-acetyl-aspartyl-glutamate in rat brain. Brain Res. 317, 137–140.

Lacomblez, L., Bensimon, G., Leigh, P.N., Guillet, P., Meininger, V., 1996. Dose-ranging study of riluzole in amyotrophic lateral sclerosis. Amyotrophic Lateral Sclerosis/Riluzole Study Group II. Lancet 347, 1425–1431.

Lai, E.C., Felice, K.J., Festoff, B.W., Gawel, M.J., Gelinas, D.F., Kratz, R., Murphy, M.F., Natter, H.M., Norris, F.H., Rudnicki, S.A., 1997. Effect of recombinant human insulin-like growth factor-I on progression of ALS. A placebo-controlled study. The North America ALS/IGF-I Study Group. Neurology 49, 1621–1630.

Meldrum, B., 1990. Protection against ischaemic neuronal damage by drugs acting on excitatory neurotransmission. Cerebrovasc. Brain Metab. Rev. 2, 27–57.

Miller, R.G., Petajan, J.H., Bryan, W.W., Armon, C., Barohn, R.J., Goodpasture, J.C., Hoagland, R.J., Parry, G.J., Ross, M.A., Stromatt, S.C., 1996. A placebo-controlled trial of recombinant human ciliary neurotrophic (rhCNTF) factor in amyotrophic lateral sclerosis. rhCNTF ALS Study Group. Ann. Neurol. 39, 256–260.

Miller, R.G., Moore II, D.H., Gelinas, D.F., Dronsky, V., Mendoza, M., Barohn, R.J., Bryan, W., Ravits, J., Yuen, E., Neville, H., Ringel, S., Bromberg, M., Petajan, J., Amato, A.A., Jackson, C., Johnson, W., Mandler, R., Bosch, P., Smith, B., Graves, M., Ross, M., Sorenson, E.J., Kelkar, P., Parry, G., Olney, R., 2001. Phase III randomized trial of gabapentin in patients with amyotrophic lateral sclerosis. Neurology 56, 843–848.

Miyake, M., Kakimoto, Y., Sorimachi, M., 1981. A gas chromatographic method for the determination of *N*-acetyl-L-aspartic acid, *N*-acetyl-alpha-aspartylglutamic acid and beta-citryl-L-glutamic acid and their distributions in the brain and other organs of various species of animals. J. Neurochem. 36, 804–810.

Moffett, J.R., Williamson, L., Palkovits, M., Namboodiri, M.A., 1990. *N*-acetylaspartylglutamate: a transmitter candidate for the retinohypothalamic tract. Proc. Natl. Acad. Sci. 87, 8065–8069.

Mori-Okamoto, J., Okamoto, K., Sekiguchi, M., 1987. Electrophysiological and pharmacological actions of *N*-acetylaspartylglutamate intracellularly studied in cultured chick cerebellar neurons. Brain Res. 401, 60–67.

Neale, J.H., Bzdega, T., Wroblewska, B., 2000. *N*-acetylaspartylglutamate: the most abundant peptide neurotransmitter in the mammalian central nervous system. J. Neurochem. 75, 443–452.

Ohishi, H., Shigemoto, R., Nakanishi, S., Mizuno, N., 1993. Distribution of the mRNA for a metabotropic glutamate receptor (mGluR3) in the rat brain: an in situ hybridization study. J. Comp. Neurol. 335, 252–266.

Petralia, R.S., Wang, Y.X., Niedzielski, A.S., Wenthold, R.J., 1996. The metabotropic glutamate receptors, mGluR2 and mGluR3, show unique postsynaptic, presynaptic and glial localizations. Neuroscience 71, 949–976.

Pizzi, M., Benarese, M., Boroni, F., Goffi, F., Valerio, A., Spano, P.F., 2000. Neuroprotection by metabotropic glutamate receptor agonists on kainate-induced degeneration of motor neurons in spinal cord slices from adult rat. Neuropharmacology 39, 903–910.

Plaitakis, A., 1990. Glutamate dysfunction and selective motor neuron degeneration in amyotrophic lateral sclerosis: a hypothesis. Ann. Neurol. 28, 3–8.

Puttfarcken, P.S., Handen, J.S., Montgomery, D.T., Coyle, J.T., Werling, L.L., 1993. *N*-acetyl-aspartylglutamate modulation of *N*-methyl-D-as-

partate-stimulated [3H]norepinephrine release from rat hippocampal slices. J. Pharmacol. Exp. Ther. 266, 796–803.

Riviere, M., Meininger, V., Zeisser, P., Munsat, T., 1998. An analysis of extended survival in patients with amyotrophic lateral sclerosis treated with riluzole. Arch. Neurol. 55, 526–528.

Robinson, M.B., Blakely, R.D., Couto, R., Coyle, J.T., 1987. Hydrolysis of the brain dipeptide *N*-acetyl-L-aspartyl-L-glutamate. Identification and characterization of a novel *N*-acetylated alpha-linked acidic dipeptidase activity from rat brain. J. Biol. Chem. 262, 14498–14506.

Rothman, S.M., Olney, J.W., 1986. Glutamate and the pathophysiology of hypoxic–ischemic brain damage. Ann. Neurol. 19, 105–111.

Rothstein, J.D., 1995a. Excitotoxic mechanisms in the pathogenesis of amyotrophic lateral sclerosis. Adv. Neurol. 68, 7–20.

Rothstein, J.D., 1995b. Excitotoxicity and neurodegeneration in amyotrophic lateral sclerosis. Clin. Neurosci. 3, 348–359.

Rothstein, J.D., Kuncl, R.W., 1995. Neuroprotective strategies in a model of chronic glutamate-mediated motor neuron toxicity. J. Neurochem. 65, 643–651.

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. Abnormal excitatory amino acid metabolism in amyotrophic lateral sclerosis. Ann. Neurol. 28, 18–25.

Rothstein, J.D., Martin, L.J., Kuncl, R.W., 1992. Decreased glutamate transport by the brain and spinal cord in amyotrophic lateral sclerosis. N. Engl. J. Med. 326, 1464–1468.

Rothstein, J.D., Jin, L., Dykes-Hoberg, M., Kuncl, R.W., 1993. Chronic inhibition of glutamate uptake produces a model of slow neurotoxicity. Proc. Natl. Acad. Sci. 90, 6591–6595.

Sanchez-Prieto, J., Budd, D.C., Herrero, I., Vazquez, E., Nicholls, D.G., 1996. Presynaptic receptors and the control of glutamate exocytosis. Trends Neurosci. 19, 235–239.

Saroff, D., Delfs, J., Kuznetsov, D., Geula, C., 2000. Selective vulnerability of spinal cord motor neurons to non-NMDA toxicity. NeuroReport 11, 1117–1121.

Schoepp, D.D., Conn, P.J., 1993. Metabotropic glutamate receptors in brain function and pathology. Trends Pharmacol. Sci. 14, 13–20.

Sekiguchi, M., Okamoto, K., Sakai, Y., 1989. Low-concentration *N*-acetylaspartylglutamate suppresses the climbing fiber response of Purkinje cells in guinea pig cerebellar slices and the responses to excitatory amino acids of *Xenopus laevis* oocytes injected with cerebellar mRNA. Brain Res. 482, 87–96.

Silva, G.A., Theriault, E., Mills, L.R., Pennefather, P.S., Feeney, C.J., 1999. Group I and II metabotropic glutamate receptor expression in cultured rat spinal cord astrocytes. Neurosci. Lett. 263, 117–120.

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., Tortella, F.C., Wozniak, K.M., Yudkoff, M., Potter, B.M., Jackson, P.F., 1999. Selective inhibition of NAALADase, which converts NAAG to glutamate, reduces ischemic brain injury. Nat. Med. 5, 1396–1402.

Slusher, B., Wozniak, K., Hartman, T., Jada, P., Chandran, M., DalCanto, M., 2000. NAALADase Inhibition Increases Survival and Delays Clinical Symptoms in SOD Transgenic Model of ALS. Society for Neuroscience, New Orleans, LA.

Thomas, A.G., Vornov, J.J., Olkowski, J.L., Merion, A.T., Slusher, B.S., 2000. *N*-acetylated alpha-linked acidic dipeptidase converts *N*-acetylaspartylglutamate from a neuroprotectant to a neurotoxin. J. Pharmacol. Exp. Ther. 295, 16–22.

Thomas, A.G., Liu, W., Olkowski, J.L., Tang, Z., Lin, Q., Lu, X.C., Slusher, B.S., 2001. Neuroprotection mediated by glutamate carboxypeptidase II (NAALADase) inhibition requires TGF-beta. Eur. J. Pharmacol. 430, 33–40.

Tomiyama, M., Kimura, T., Maeda, T., Tanaka, H., Furusawa, K., Kurahashi, K., Matsunaga, M., 2001. Expression of metabotropic glutamate receptor mRNAs in the human spinal cord: implications for selective vulnerability of spinal motor neurons in amyotrophic lateral sclerosis. J. Neurol. Sci. 189, 65–69.

Tsai, G., Stauch, B.L., Vornov, J.J., Deshpande, J.K., Coyle, J.T., 1990. Selective release of *N*-acetylaspartylglutamate from rat optic nerve terminals in vivo. Brain Res. 518, 313–316.

Tsai, G.C., Stauch-Slusher, B., Sim, L., Hedreen, J.C., Rothstein, J.D., Kuncl, R., Coyle, J.T., 1991. Reductions in acidic amino acids and *N*-acetylaspartylglutamate in amyotrophic lateral sclerosis CNS. Brain Res. 556, 151–156.

Valivullah, H.M., Lancaster, J., Sweetnam, P.M., Neale, J.H., 1994. Interactions between *N*-acetylaspartylglutamate and AMPA, kainate, and NMDA binding sites. J. Neurochem. 63, 1714–1719.

Van Den Bosch, L., Vandenberghe, W., Klaassen, H., Van Houtte, E., Robberecht, W., 2000. Ca(2+)-permeable AMPA receptors and selective vulnerability of motor neurons. J. Neurol. Sci. 180, 29–34.

Westbrook, G.L., Mayer, M.L., Namboodiri, M.A., Neale, J.H., 1986. High concentrations of *N*-acetylaspartylglutamate (NAAG) selectively activate NMDA receptors on mouse spinal cord neurons in cell culture. J. Neurosci. 6, 3385–3392.

Wroblewska, B., Wroblewski, J.T., Pshenichkin, S., Surin, A., Sullivan, S.E., Neale, J.H., 1997. *N*-acetylaspartylglutamate selectively activates mGluR3 receptors in transfected cells. J. Neurochem. 69, 174–181.