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N-acetylaspartylglutamate protects against transient focal cerebral ischemia in rats

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Abstract

The inhibition of *N*-acetylated α-linked acidic dipeptidase (NAALADase: glutamate carboxypeptidase II) has been previously shown to protect against ischemic injury presumably through mechanisms of decreasing glutamate and increasing *N*-acetylaspartylglutamate (NAAG). Preventing excessive glutamate release is known to be neuroprotective. However, the role of increased NAAG is not clear. We used a middle cerebral artery occlusion model in rats to investigate the neuroprotective effect of NAAG via its action as a metabotropic glutamate (mGlu) receptor agonist. Rats received intracerebral injections of NAAG (1, 2, or 4 μmol), or a co-injection of NAAG (2 μmol) and the non-selective mGlu receptor antagonist, (*R,S*)-α-methyl-4-carboxyphenylglycine, (MCPG, 2 μmol). Immediately after the treatment, the animals received 2 h of middle cerebral artery occlusion followed by 22 h of reperfusion. Treatment with 1 or 2 μmol of NAAG significantly reduced total infarct volume. Treatment with MCPG partially attenuated the neuroprotective effect of NAAG, indicating that the protective effect of NAAG against ischemic injury may be in part mediated via activation of mGlu receptors.

Keywords: NAAG (*N*-acetylaspartylglutamate); mGlu receptor; MCPG ((*R,S*)-α-methyl-4-carboxyphenylglycine); Ischemia; NAALADase (*N*-acetylated α-linked acidic dipeptidase)

1. Introduction

The abundant endogenous neuropeptide, *N*-acetylaspartylglutamate (NAAG), has been recognized to possess two possible roles in neuronal transmission. It is thought to act either as a functional neurotransmitter or as a precursor of glutamate (Blakely and Coyle, 1988; Coyle, 1997; Slusher et al., 1999). NAAG is located in synaptic vesicles of neurons in various regions of the brain, including putative glutamate pathways. Its release is calcium-dependent in response to neuronal depolarization (Tsai et al., 1990). As a neurotransmitter, NAAG targets both ionotropic glutamate receptors and metabotropic glutamate (mGlu) receptors. NAAG acts as a mixed agonist/antagonist of the *N*-methyl-D-aspartic acid (NMDA) receptor (Puttfarcken et al., 1993; Sekiguchi et al., 1992) and as an agonist at the group II mGlu receptor (Wroblewska et al., 1997). NAAG is readily hydrolyzed to liberate glutamate and *N*-acetyl-aspartate by a synaptically located enzyme, *N*-acetylated α-linked acidic dipeptidase (NAALADase) (Robinson et al., 1987) and, thus, has also been considered to be a potential storage supply for glutamate. In this respect, it may partially contribute to the excessive release of glutamate during neurological diseases, such as stroke and brain trauma.

A recent study demonstrated that inhibition of NAALADase significantly reduces cerebral ischemic injury, presumably through preventing NAAG from being hydrolyzed to glutamate and consequently reducing excessive glutamate induced excitotoxicity (Slusher et al., 1999). In support of this hypothesis, in vivo microdialysis studies demonstrate that NAALADase inhibition indeed causes significant decreases in extracellular glutamate and increases in extracellular NAAG in the rat brain during middle cerebral artery occlusion and reperfusion (Slusher et al., 1999). While the reduction of glutamate release is recognized as a putative strategy against neuronal excitotoxicity, one question raised from this study was to what extent the increased NAAG level contributed to the neuro-

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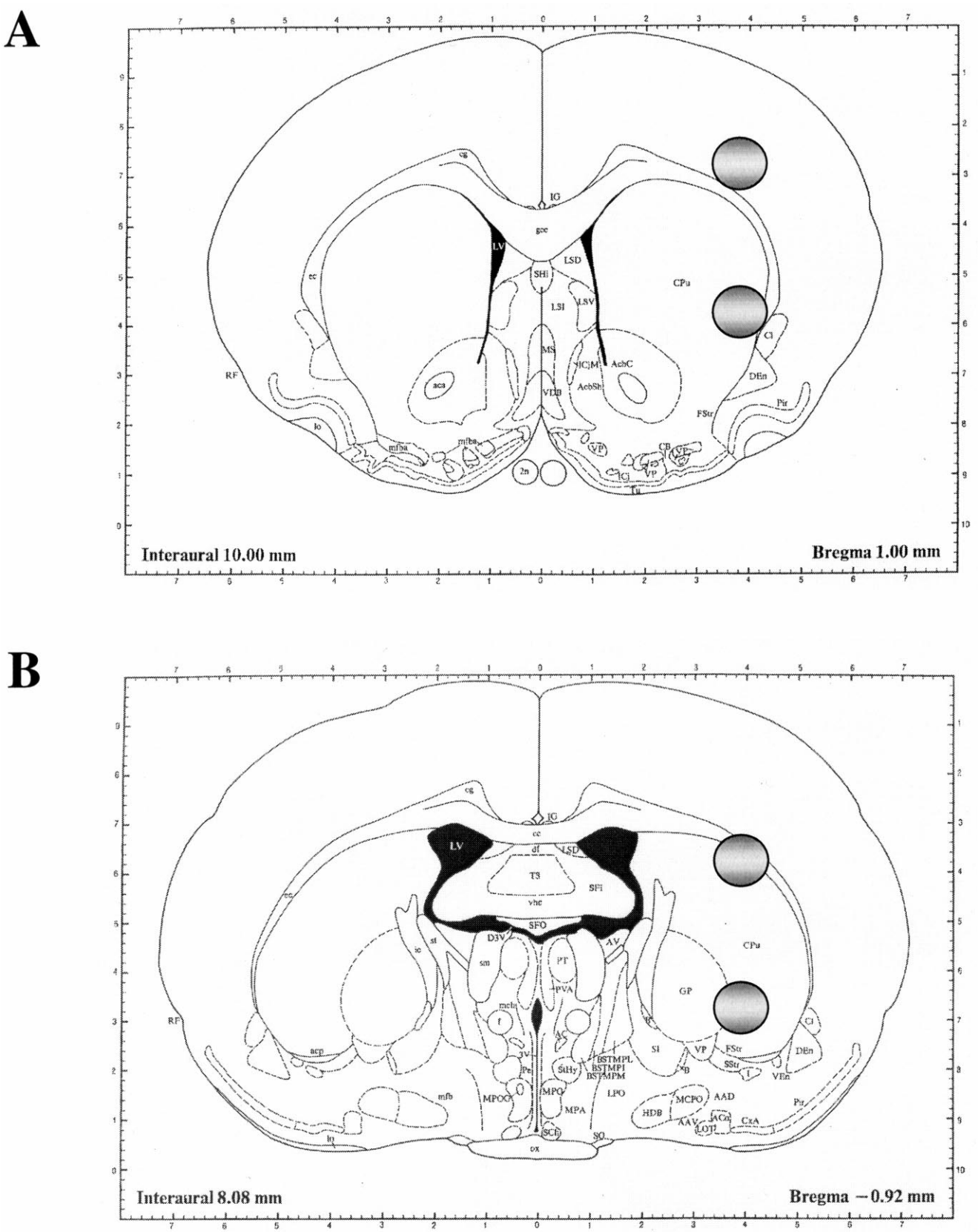

Fig. 1. Two coronal sections of a rat brain with the coordinates of injection sites shown as shaded circles. All coordinates are described in relation to Bregma. The anterior injection sites (Panel A): AP: +1.0, ML: −4.0, DV: −6.0 and −3.0. The posterior injection sites (Panel B): AP: −1.0, ML: −4.0, DV: −6.0 and −3.0. The injection volume was 2 μl/site.

protection. NAAG has been demonstrated to be neuroprotective against excitotoxicity in both in vivo and in vitro studies. In one study, Orlando et al. (1997) reported that intact NAAG, when co-injected with quinolinic acid into the rat striatum, significantly reduced quinolinic acid-induced lesions. Based on the recent identification of NAAG as an $mGlu_3$ receptor agonist, Bruno et al. (1998b) further demonstrated that, in vitro, NAAG protected neurons from NMDA induced toxicity via activation of astrocyte group II mGlu receptors. To further elucidate the mechanism of NAAG as a potential neuroprotective agent, in this study, we examined the effect of NAAG in an animal model of transient middle cerebral artery occlusion with a particular interest in its role as an mGlu receptor agonist.

2. Materials and methods

All procedures were performed in accordance with Guide for the Care and Use of Laboratory Animals established by the National Institute of Health of the United States.

2.1. Materials

Male Sprague–Dawley rats were obtained from Charles River Laboratory (Cambridge, MA, USA). These animals were housed individually in a well-ventilated vivarium. Free access to food and water was allowed, providing that the body weights were maintained at 260–290 g.

NAAG, (*R*,*S*)-α-methyl-4-carboxyphenylglycine (MCPG), and poly-L-lysine were obtained from Sigma (St. Louis, MO, USA). Artificial cerebral spinal fluid (ACSF) was obtained from Paragon (Baltimore, MD, USA. Monofilament suture was obtained from J.A. Webster (Sterling, MA, USA). Halothane was obtained from Penn Veterinary Supply (Lancaster, PA, USA).

2.2. Treatments

Five treatment groups were randomly assigned prior to the experiment. The treatments included intracerebral injections of ACSF ($n = 12$), three concentrations of NAAG (1 μmol, $n = 8$; 2 μmol, $n = 9$; or 4 μmol, $n = 9$), and NAAG (2 μmol) co-injected with an mGlu receptor antagonist, MCPG (2 μmol, $n = 8$). For each rat, the total dose of the treatment was equally delivered at four stereotaxic sites in a volume of 2 μl/site. The injection sites were aimed at the striatal and cortical regions supplied by the right middle cerebral artery (Fig. 1). Two striatal injections were made at the coronal levels of 1.0 mm anterior and 1.0 mm posterior to Bregma (AP: ±1.0; ML: −4.0; DV: −6.0). Two cortical injections were made at the same coronal levels, but more superficially (AP: ±1.0; ML: −4.0; DV: −3.0). NAAG and MCPG were dissolved in 0.1 M phosphate buffer and the pH was adjusted to 7.4 before administration.

2.3. Middle cerebral artery occlusion surgery

Immediately after intracerebral injections, each rat received transient middle cerebral artery occlusion for 2 h via the intraluminal filament technique. Briefly, the common carotid artery was exposed at the level of external and internal carotid artery bifurcation. The external carotid artery and its branches were cauterized and cut at its lingual and maxillary artery branches. A piece of 3-0 monofilament nylon suture with a blunted tip, coated with 0.1% poly-L-lysine, was introduced into the internal carotid artery via the proximal end of the external carotid artery stump. It was advanced through the carotid canal to the origin of the middle cerebral artery and consequently blocked the blood flow to its territory. At the end of the 2 h occlusion period, the suture was carefully pulled out to allow reperfusion.

During the surgical procedures, the rat was anesthetized with 1.5% halothane in 30% oxygen and 70% nitrous oxide and the body temperature was maintained at 37.0°C by a homeothermic heating blanket. Each rat was allowed to wake up during the 2 h occlusion time.

2.4. Data analysis

At 22 h of reperfusion, the rats were sacrificed. The brains were cut into seven 2-mm thick coronal slices, stained with 1% 2,3,5-triphenytetrazolium chloride (TTC), and subsequently imaged using a computer-assisted digital imaging analysis system. The ischemic injury was quanti-

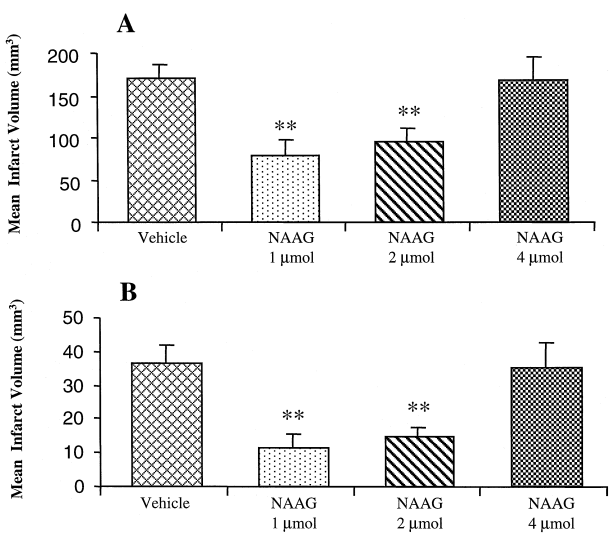

Fig. 2. Histograms showing the dose response of mean cortical (panel A) and subcortical (panel B) infarct volumes (mean ± S.E.M.) of three NAAG treated groups in comparison with the vehicle treated group. ** Indicates $P < 0.005$ in comparison with the vehicle treatment.

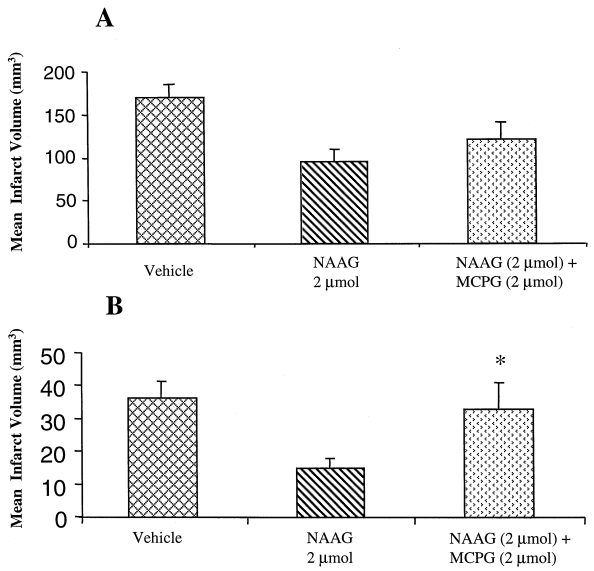

Fig. 3. Histograms showing the mean infarct volumes (mean ± S.E.M.) in the cortical (panel A) and subcortical (panel B) regions of the rat brain treated with Vehicle, NAAG (2 μmol) alone, and NAAG (2 μmol) + MCPG (2 μmol). * Indicates $P < 0.05$ in comparison with NAAG alone treatment.

fied based on the volume of the infarct tissue completely lacking TTC staining. The mean total infarct volume and the infarct volumes in the cortical and subcortical regions of each group were used for statistical analysis. One-way analysis of variance test was used for comparison of treatment effects. Values were considered statistically significant at $P < 0.05$.

3. Results

3.1. Neuroprotective effect of NAAG

Intracerebral injections of 1 or 2 μmol of NAAG significantly reduced the total infarct volume by 57% ($P < 0.005$) or 46% ($P < 0.005$), respectively, in rats subjected to 2 h of middle cerebral artery occlusion and 22 h of reperfusion, compared to vehicle-treated control rats. The reduction of the total infarct volume was reflected more in the subcortical area of the brain than in the cortical area in each group. The treatment with 1 μmol of NAAG reduced cortical infarct volume by 54% ($P < 0.005$) and subcortical infarct volume by 68% ($P < 0.005$). The treatment with 2 μmol of NAAG reduced cortical infarct volume by 44% ($P < 0.005$) and subcortical infarct volume by 59% ($P < 0.005$). The treatment of the higher dose of NAAG (4 μmol) had no effect on the infarct volume compared to the vehicle treatment. The reduction of ischemic damage of this group was within 5% of the control group on each of the parameters (total, cortical, or subcortical infarct volume) used in the study. Fig. 2 presents the dose response effect of NAAG on the cortical and subcortical infarct volumes.

3.2. Attenuation of NAAG's neuroprotection with an mGlu receptor antagonist

Based on the above results that NAAG protected against middle cerebral artery occlusion-induced ischemic damage in a dose response manner, we selected a moderate dose of

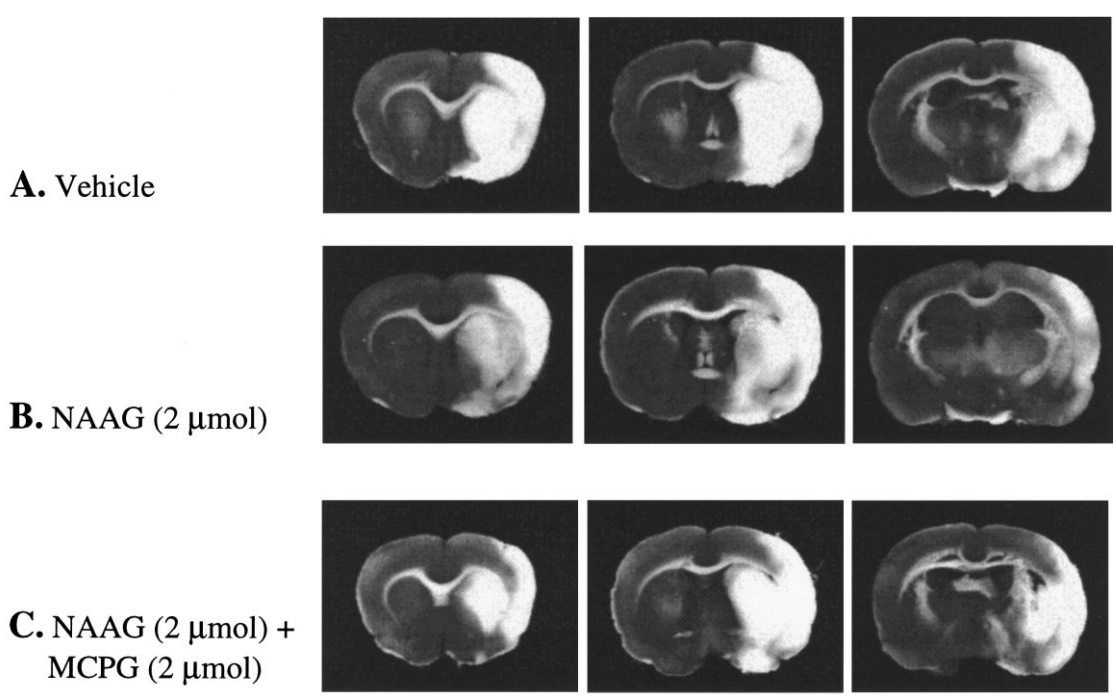

Fig. 4. Three images of TTC stained coronal sections around the injection sites of a representative rat brain selected from groups treated with (A) vehicle; (B) NAAG (2 μmol) alone; (C) NAAG (2 μmol) plus MCPG (2 μmol). The white area represents brain infarction.

NAAG to further investigate its action as an mGlu receptor agonist. In the second part of this experiment, MCPG (2 μmol), an mGlu receptor antagonist, was co-injected with NAAG (2 μmol) at the same cortical and subcortical regions as described above. While 2 μmol of NAAG alone produced 59% reduction of the subcortical infarct volume compared with vehicle treatment, MCPG significantly attenuated this neuroprotective effect by decreasing the subcortical protection to 9% when co-injected with equal amount of NAAG, which constituted an 84% reduction of the protective effect compared to NAAG treatment alone ($P < 0.05$, Fig. 3, panel B). In the cortical region, co-injection of MCPG and NAAG also partially decreased NAAG's protection from 44% to 29%, although this trend of reversal did not achieve statistical significance ($P > 0.05$, Fig. 3, panel A).

Fig. 4 presents brain slice images of the injection sites from selected animals in the vehicle, NAAG (2 μmol) alone, and NAAG (2 μmol) + MCPG (2 μmol) treated groups.

4. Discussion

The results of this study demonstrate that direct application of NAAG (1–2 μmol) into the rat brain reduces infarct volumes in a rat model of transient middle cerebral artery occlusion. The neuroprotective properties of NAAG were mediated, in part, via activation of mGlu receptors, since co-injection of an mGlu receptor antagonist, MCPG, partially attenuated the NAAG-mediated neuroprotective effect. It is well recognized that mGlu receptors have differential activities depending upon the properties of their subtypes. A family of eight mGlu receptor subtypes has been identified and classified into three groups based on their sequence similarity and pharmacological profiles. NAAG has been identified as a selective agonist for $mGlu_3$ receptor, one of the subtypes of group II mGlu receptors (Wroblewska et al., 1997). Therefore, its neuroprotective effect observed in this study is presumably mediated by its activation on the group II mGlu receptors.

The involvement of group II mGlu receptors in neuroprotection has been demonstrated in various neuronal injury models. A series of cyclopropyl and phenyl–glycine derivatives, such as (2*s*, 1′*r*,2′*r*,3′*r*)-2-(2,3-dicarboxycyclopropyl)-glycine, and *s*-4-carboxy-3-hydroxyphenylglycine have been classified as selective agonists of group II mGlu receptors and shown to protect cultured neurons against NMDA- or kainate-induced neuronal degeneration (Nicoletti et al., 1996; Bruno et al., 1995). A group of newly developed group II mGlu receptor agonists, namely LY354740 or LY379268, has been reported to attenuate various neuronal injuries, such as traumatic neuronal injury (Allen et al., 1999) or global ischemic injury (Bond et al., 1999). Two mechanisms of the neuroprotective action of the group II mGlu receptors have been proposed. First, activation of group II mGlu receptors by selective agonists attenuates glutamate release (Bruno et al., 1995; Allen et al., 1999). Second, the activation of group II mGlu receptors of glia cells causes a release of neurotrophic factors, such as transforming growth factor-β (TGF-β), from astrocytes, which, in turn, protects neighboring neurons from excitotoxicity (Bruno et al., 1998a).

In this in vivo study of focal cerebral ischemia, we did not examine the effect of NAAG on TGF-β release. However, in separate studies performed in our laboratory, we observed that treatment of primary cortical cultures with NAAG under the condition of metabolic inhibition, which mimics cell ischemia, increased TGF-β release in the culture media and reduced neuronal death. This neuroprotective effect was completely reversed by treatment with MCPG (Thomas et al., 2000). Bruno et al. also reported that culture media of pure cortical astrocytes, after a transient exposure of NAAG, was neuroprotective when subsequently applied to sister mixed cell cultures challenged with NMDA toxicity. This form of neuroprotection was attenuated by an mGlu receptor antagonist, but not by an NMDA receptor antagonist (Bruno et al., 1998b). They suggested that NAAG was activating glia mGlu receptors and caused glial release of neurotrophic agents, which protected surrounding neurons. In an in vivo experiment previously performed in our laboratory, we demonstrated that inhibition of NAALADase by intravenous administration of a potent and selective NAALADase inhibitor, 2-(phosphonomethyl)pentanedioic acid, reduced ischemic injury in an animal model of middle cerebral artery occlusion. This neuroprotective effect of NAALADase was attenuated with concomitant intracerebral injections of TGF-β neutralizing antibody (Lu et al., 1999). These data suggest that preserved NAAG due to NAALADase inhibition might activate glial mGlu receptors in the brain and cause release of TFG-β, which protect neurons from ischemic injury. Collectively, this evidence strongly indicates that NAAG exhibits neuroprotective properties, in part, by activating group II mGlu receptor and potentially promoting neurotrophic factors.

In this study, NAAG exerted stronger protection against ischemic injury in the subcortical regions than in the cortical regions, and MCPG caused stronger reversal of NAAG's protection in the subcortical regions (84%) compared to the cortical regions (34%). The subcortical regions supplied by the middle cerebral artery consist primarily of the corpus striatum of the basal ganglia and were the immediate targets of our NAAG injections. The cortical regions affected by the middle cerebral artery occlusion involve a larger extent of the anterior and posterior regions of the cortex, which is beyond the immediate injection sites and therefore might be less affected by the intended treatment. The significant reversal of NAAG's effect by MCPG in the striatum may also be partially related to the differential distributions of $mGlu_3$ receptors in the brain. Testa et al. (1994) have reported that in the rat striatum,

strong $mGlu_3$ receptor expression was observed in glia as well as in neurons, whereas, in the cortex, high level of labeling of $mGlu_3$ receptors was located in pyramidal cells. Since, as discussed above, glial mGlu receptors are primarily responsible for promoting TGF-β release upon activation, the antagonism of glial mGlu receptor by MCPG presumably accounted for the greater blockade of NAAG's effect on the TGF-β release in the striatum than in the cortex, and consequently significantly reduced NAAG protection.

It has been well established that NAALADase hydrolyzes NAAG to liberate glutamate and *N*-acetyl-aspartate. Therefore, the pharmacological effect of NAAG depends upon the activity of NAALADase. A previous study reported by our group (Thomas et al., 2000) has clearly demonstrated that when NAALADase is active, NAAG acts as a neurotoxin, similar to a NMDA receptor agonist, because the liberated glutamate from hydrolyzed NAAG becomes the active agent. However, when NAALADase activity is inhibited, the intact NAAG serves as a neuroprotectant by acting on mGlu receptors. In the present study, care was taken to prevent exogenously applied NAAG from being hydrolyzed by NAALADase. NAAG was dissolved in 0.1 M phosphate buffer, as phosphate is a known NAALADase inhibitor ($IC_{50} = 100–200$ μM) (Stauch et al., 1989). In a previous in vivo study, Orlando et al. (1997) also demonstrated that the neuroprotective effect of NAAG against quinolinic acid lesion was not hampered by its hydrolysis when exogenously applied in phosphate buffer.

Unlike other mGlu receptor agonists, NAAG possesses the unique characteristic of being both an mGlu receptor agonist and a mixed NMDA receptor agonist/antagonist. In this regard, its neuroprotective effect against middle cerebral artery occlusion-induced brain injury may involve its interaction with NMDA receptors as well. Although no direct evidence was obtained in this study to determine NAAG's role as an NMDA receptor antagonist or agonist, an incomplete reversal of NAAG's protection by MCPG suggested that at least part of its neuroprotection be mediated by non-mGlu activities.

The neuroprotective effect of NAAG appeared to be dose sensitive. Only moderate doses of NAAG exhibited protective effect, while the higher dose had no effect. This phenomenon could be linked to its mixed agonist/antagonist activity at the NMDA receptor. The ineffectiveness of the high dose of NAAG has been repeatedly observed in several studies. Thomas et al. (2000) from our laboratory reported a U-shaped dose response curve of NAAG against an in vitro model of neuronal death induced by metabolic inhibition. Bruno et al. also reported the failure of high dose of NAAG in protecting against NMDA-induced excitotoxicity (Bruno et al., 1998b). In an in vivo study, Orlando et al. (1997) found slight toxicity with a single injection of 2 μmol of NAAG into the rat striatum.

The neuroprotective effect of NAAG described in this study provides a strong support for the newly proposed mechanism of NAALADase inhibition in reducing ischemic neuronal damage (Slusher et al., 1999). Evidence from microdialysis studies shows that inhibition of NAALADase decreases glutamate and increases NAAG levels under ischemic conditions. The present study specifically investigated the role of NAAG as a neuroprotectant in an animal model of transient focal cerebral ischemia and demonstrated that its neuroprotective effect was mediated, in part, via activation of mGlu receptors. In this regard, regulating endogenous NAAG level by inhibiting the enzymatic activity of NAALADase represents a multi-faceted mechanism against neuroexcitotoxicity. Although to what extent the neuroprotective effect of the exogenously injected NAAG can be applied to the endogenously increased NAAG due to NAALADase inhibition remains unclear, the co-existence of NAAG and glial $mGlu_3$ receptors within the territory of middle cerebral artery provides a substrate for their interaction. Based on these results in connection with our early observations that NAALADase inhibition provides similar protection against ischemic injury, we suggest that regulating endogenous NAAG level in the brain by NAALADase inhibition may provide a new therapeutic target for stroke.

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