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Neuroprotective effects of *N*-acetylaspartylglutamate in a neonatal rat model of hypoxia-ischemia

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Abstract

Neuroprotective effects of *N*-acetylaspartylglutamate (NAAG), the precursor of glutamate and a selective agonist at the Group II metabotropic glutamate (mGlu) receptor, against hypoxic-ischemic brain injury were examined in a neonatal rat model of cerebral hypoxia-ischemia. The neonatal hypoxia-ischemia procedure (unilateral carotid artery ligation followed by exposure to an 8% oxygen hypoxic condition for 1.5 h) was performed in 7-day-old rat pups. Following unilateral carotid artery ligation, NAAG (0.5 to 20 mg/kg, i.p.) was administered before or after the hypoxic exposure. Brain injury was examined 1-week later by weight reduction in the ipsilateral brain and by neuron density in the hippocampal CA1 area. In the saline-treated rat, neonatal hypoxia-ischemia resulted in severe brain injury as indicated by a 24% reduction in the ipsilateral brain weight. Low doses of NAAG (2–10 mg/kg, but not 0.5 mg/kg), administered before or even if 1 h after the hypoxic exposure, greatly reduced hypoxia-ischemia-induced brain injury (3.8–14.2% reduction in the ipsilateral brain weight). A high dose of NAAG (20 mg/kg) was ineffective. While L(+)-2-Amino-4-phosphonobutyric acid (L-AP4) and *trans*-[1S,3R]-1-Amino-cyclopentane-1, 3-dicarboxylic acid (*t*-ACPD) were unable to provide protection against hypoxic-ischemic brain injury, 2-(phosphonomethyl) pentanedioic acid (2-PMPA), an inhibitor of *N*-acetylated alpha-linked acidic dipeptidase (NAALADase), which hydrolyzes endogenous NAAG into *N*-acetyl-aspartate and glutamate, significantly reduced neonatal hypoxia-ischemia-induced brain injury. (αS)-α-Amino-α-[(1S, 2S)-2-carboxycyclopropyl]-9H-xanthine-9-propanoic acid (LY341495), a selective antagonist at the $mGlu_{2/3}$ receptor, prevented the neuroprotective effect of NAAG. Neuron density data measured in the hippocampal CA1 area confirmed that ipsilateral brain weight reduction was a valid measure for hypoxic-ischemic brain injury. Neonatal hypoxia-ischemia stimulated an elevation of cyclic AMP (cAMP) concentration in the saline-treated rat brain. NAAG, L-AP4 and *t*-ACPD all significantly decreased hypoxia-ischemia-induced elevation of cAMP. LY341495 blocked the effect of NAAG, but not of L-AP4 or *t*-ACPD, on hypoxia-ischemia-stimulated cAMP elevation. The overall results suggest that the neuroprotective effect of NAAG is largely associated with activation of $mGlu_{2/3}$ receptor.

Keywords: NAAG (*N*-acetylaspartylglutamate); Glutamate receptor metabotropic; Neonatal hypoxia-ischemia; cAMP; L-AP4 (L-(+)-2-amino-4-phosphono-butyric acid); *t*-ACPD (*trans*-[1S,3R]-1-Amino-cyclopentane-1,3-dicarboxylic acid)

1. Introduction

Glutamate receptors can be classified into two categories: ionotropic and metabotropic glutamate receptors (mGlu receptors). Excessive activation of glutamate receptors, especially the ionotropic *N*-methyl-D-aspartate (NMDA) receptor and α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) receptor, is one of the most important factors involved in brain injury following cerebral hypoxia-ischemia (Barks and Silverstein, 1992; Gill and Lodge, 1997). Therefore, most studies in seeking therapeutic treatment of hypoxic-ischemic brain injury have focused on ionotropic glutamate receptors. Although antagonists of ionotropic subtypes of glutamate receptors (NMDA and AMPA receptors) have been shown to be highly neuroprotective in animal models of cerebral hypoxia-ischemia (Choi, 1992), side effects of these compounds hinder their clinical use (Hagberg et al., 1994). Recently, the role of mGlu receptors in the hypoxic-ischemic brain injury has received increasing attention (Schoepp and Conn, 1993; Nicoletti et al., 1996; Maiese, 1998). The eight mGlu receptor subtypes are divided into three groups, based on their pharmacological characteristics, amino acid sequences and second messenger coupling. Group I mGlu receptors ($mGlu_1$ and $mGlu_5$ receptors) are associated with stimulation of phosphoinositide

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hydrolysis, Group II ($mGlu_2$ and $mGlu_3$ receptors) and Group III mGlu receptors ($mGlu_4$, $mGlu_6$, $mGlu_7$, and $mGlu_8$ receptors) are negatively coupled to cyclic AMP (cAMP) formation and are thought to act as presynaptic autoreceptors, regulating glutamate transmission. As shown in a number of animal studies (Bond et al., 1998, 1999, 2000; Cai et al., 1999), the neuroprotection against ischemic brain injury afforded by selective Group II mGlu receptors agonists, such as (+)-2-aminobicyclo[3.1.0]-hexane-2,6-dicarboxylic acid (LY345740) and 1R, 4R, 5S, 6R-2-oxa-4-aminobicyclo[3.1.0] hexane-4,6-dicarboxylate (LY379268), indicates that selective activation of Group II mGlu receptors may be a possible target for ischemia/stroke therapy.

N-acetylaspartylglutamate (NAAG) is an abundant peptide neurotransmitter found in millimolar concentrations in the mammalian brain (Neale et al., 2000). NAAG is an agonist at Group II mGlu receptors (Wroblewska et al., 1997, 1998; Neale et al., 2000) and a mixed agonist/antagonist at the NMDA receptor (Puttfarcken et al., 1993). NAAG has been shown to protect against excitotoxic neuronal death in vitro (Battaglia et al., 1998; Bruno et al., 1998b) and in vivo (Orlando et al., 1997). NAAG also protects against hypoxic neuronal injury in vitro (Tortella et al., 2000) and against brain injury induced by transient focal cerebral ischemia in vivo (Lu et al., 2000). The neuroprotective effect of NAAG has been proposed to be attributed to its action as an agonist at Group II mGlu receptors (Lu et al., 2000). However, this hypothesis has not been extensively tested in in vivo animal models.

Perinatal hypoxia-ischemia is a major contributor to newborn infant death and long-term neurological abnormalities. The Rice–Vannucci (Rice et al., 1981) rat model (unilateral common carotid artery ligation in 7-day-old rat pups followed by a period of hypoxia) is a well established and characterized model for perinatal cerebral hypoxia-ischemia studies. It is thought that the developing rat brain at this stage is comparable to that of the full-term newborn human baby. Therefore, investigation of the role of NAAG in the neonatal hypoxic-ischemic rat model may provide important information to the development of therapeutic treatment of perinatal hypoxic-ischemic brain injury. Using the neonatal rat model of hypoxia-ischemia, we have examined whether NAAG offers neuroprotection against neonatal hypoxic-ischemic brain injury and whether its effect is through activation of Group II mGlu receptors.

2. Materials and methods

2.1. Chemicals

Unless otherwise stated, all chemicals used in this study were purchased from Sigma (St. Louis, MO). NAAG was obtained from Alexis (San Diego, CA) and (αS)-α-amino-α-[(1S, 2S)-2-carboxycyclopropyl]-9H-xanthine-9-propanoic acid (LY341495) was purchased from Tocris (Ballwin, MO).

2.2. Neonatal hypoxia-ischemia and animal treatment

The neonatal hypoxia-ischemia procedure was performed as described by Rice et al. (1981). On postnatal day 7, the right common carotid artery of the individual Sprague–Dawley rat pup was exposed, isolated from nerves and veins, and permanently ligated with 4-0 surgical silk under light anesthesia with isoflurane and the wound was sutured with 6-0 surgical silk. After recovery from anesthesia, the pups were returned to their dams for 1–1.5 h. Thereafter, groups of six pups were placed in 1000-ml airtight jars and exposed to a humidified oxygen–nitrogen mixture (8% O_2 and 92% N_2) delivered at 4 to 5 l/min for 1.5 h. The jars were partially submerged in a 37 °C water bath to maintain a constant thermal environment. After the hypoxic exposure, the pups were returned to the dams. NAAG, L-(+)-2-amino-4-phosphonobutyric acid (L-AP4) or *trans*-[1S,3R]-1-Amino-cyclopentane-1,3-dicarboxylic acid (*t*-ACPD) in *N*-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES) saline buffer (pH 7.2) was administrated intraperitoneally (i.p., 10 ml/kg) to the pups after the right common carotid artery ligation surgery and 1 h before or 5, 30 and 60 min after the hypoxic exposure. NAAG was administered at a dosage of 0.5, 2, 5, 10 or 20 mg/kg and the control group received the same volume of HEPES saline buffer. The NAAG dose range chosen here was based on published data (Lu et al., 2000). NAAG protects against focal ischemia brain injury in adult rats (260–290 g), when it is administered intracerebrally at a dose of 1–2 μmol/rat (Lu et al., 2000), which is equivalent to 1.1–2.3 mg/kg. Since intraperitoneal injection is used in our study, a little higher dose range was chosen. LY341495, a selective $mGlu_{2/3}$ receptors antagonist (Kingston et al., 1998), was administered (10 mg/kg, i.p.) 1 h before the hypoxic exposure. The experimental procedure was approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center and was in accordance with the guide of National Institute of Health on the care and use of animals.

2.3. Estimate of brain injury

Brain injury was estimated 7 days after the neonatal hypoxia-ischemia procedure, using the method described by Gidday et al. (1994). Rat pups were sacrificed by decapitation. The right (ipsilateral to carotid artery ligation) and the left (contralateral to carotid artery ligation) cerebral hemispheres (without the cerebellum) were separated and weighed. This hypoxia-ischemia model results in severe brain damage only on the ipsilateral, but not the contralateral side of the brain (Rice et al., 1981). Therefore, the percentage weight reduction of the ipsilateral cerebral hemisphere as compared to the contralateral hemisphere was calculated as a measure of hypoxic-ischemic brain injury, i.e. % weight reduction = (left hemisphere weight − right hemisphere weight)/left hemisphere weight.

To confirm percentage brain weight reduction as an effective measure of brain injury, we fixed brain samples with 4% paraformaldehyde after weighing them. Frozen coronal brain sections (10 μm) were prepared from these samples and stained with hematoxylin and eosin. Under light microscopy, viable neurons in the hippocampal CA1 region at the level of anterior thalamus were counted in a blinded manner and expressed as number of cells/mm of CA1 as previously described (Cai et al., 1999).

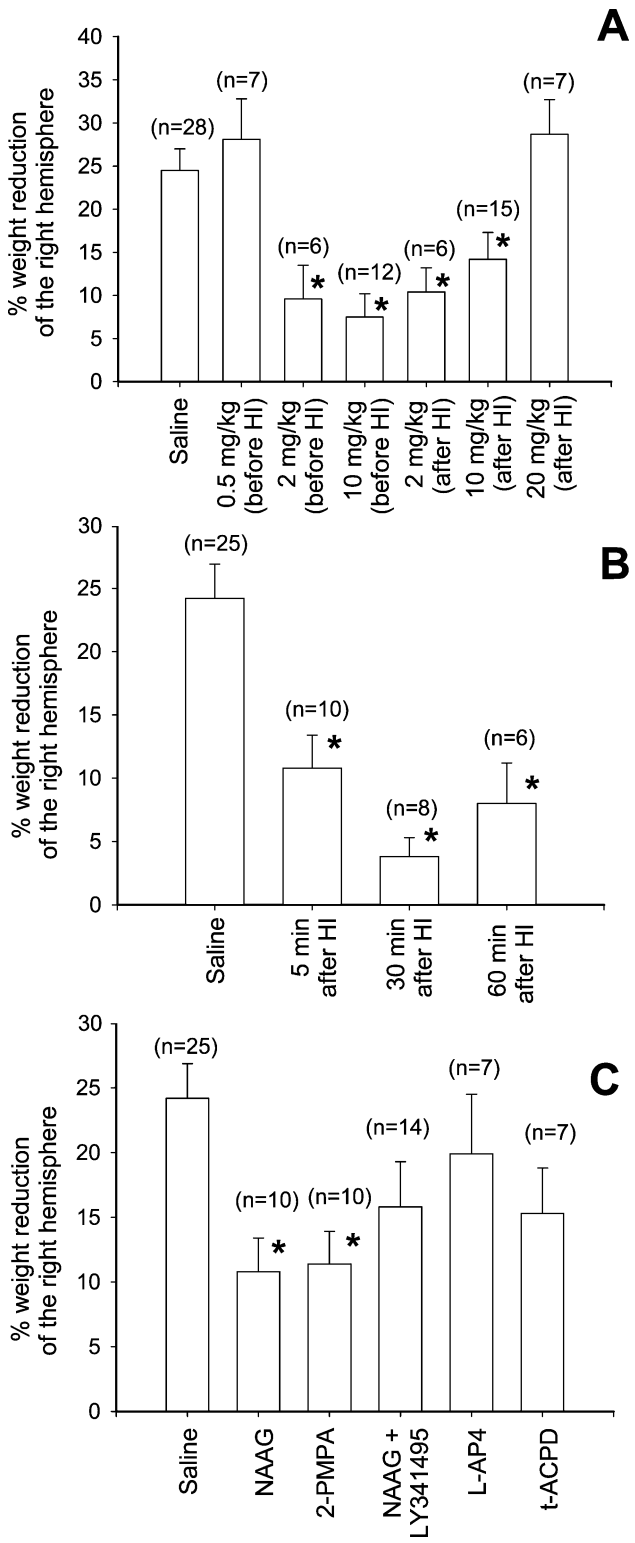

Fig. 1. NAAG significantly reduces neonatal hypoxic–ischemic brain injury. Neonatal hypoxia–ischemia procedure was performed as described in the text. One week later, brain injury was estimated by % weight reduction in the ipsilateral hemisphere as defined in the Materials and methods. (A) Hypoxic–ischemic brain injury in rat pups treated with various doses of NAAG before or after the hypoxic exposure as labeled in the figure. Rats received HEPES saline was taken as the control. (B) Hypoxic–ischemic brain injury in rat pups treated with 5 mg/kg NAAG at various times after the hypoxic exposure. (C) Hypoxic–ischemic brain injury in rat pups treated with NAAG (5 mg/kg), 2-PMPA (10 mg/kg), or other mGlu receptor agonists (10 mg/kg) 5 min after the hypoxic exposure. LY341495 (10 mg/kg, i.p.) was injected 1 h before the hypoxic exposure. Each bar represents the mean ± S.E.M. of the number of samples indicated in the parenthesis. * $P<0.05$ from the value for the saline-treated group.

2.4. cAMP measurement

Activation of Group II mGlu receptors is known to be associated with inhibition of cAMP formation (Tanabe et al., 1992). In the current study, cAMP concentration in the neonatal rat brain following hypoxia-ischemia was determined as a measure of activation of Group II mGlu receptors, using a direct cAMP immunoassay kit (Assay Designs, MI). One hour after hypoxia-ischemia exposure, rat pups were sacrificed by decapitation. The brain was dissected on ice, snap frozen in liquid nitrogen and stored at -70 °C until use. For assay sample preparation, the right rat brain (ipsilateral to the ligation side) was homogenized in 10 volumes (w/v) of 0.1 M HCl. The homogenates were centrifuged at $600 \times g$ for 20 min at room temperature and the supernatants were collected for assay. cAMP concentration in the ipsilateral rat brain was determined colorimetrically at a wavelength of 405 nm, following the manufacturer's instructions for a non-acetylated procedure. The results were expressed as the increase in cAMP concentrations (pmol per mg of protein) above the cAMP level measured from the naive neonatal rat brain at the same age.

2.5. Statistics

The neuron density data, brain weight reduction data and cAMP data were analyzed by one-way Analysis of Variation (ANOVA) followed by Student–Newman–Keuls test. The significance level was set at $P<0.05$.

3. Results

3.1. Neuroprotective effects of NAAG

The neonatal hypoxia-ischemia procedure resulted in severe brain tissue damage in the ipsilateral hemisphere as indicated by a 24% brain weight reduction in the saline-treated group (Fig. 1A). NAAG (2 or 10 mg/kg, but not 0.5

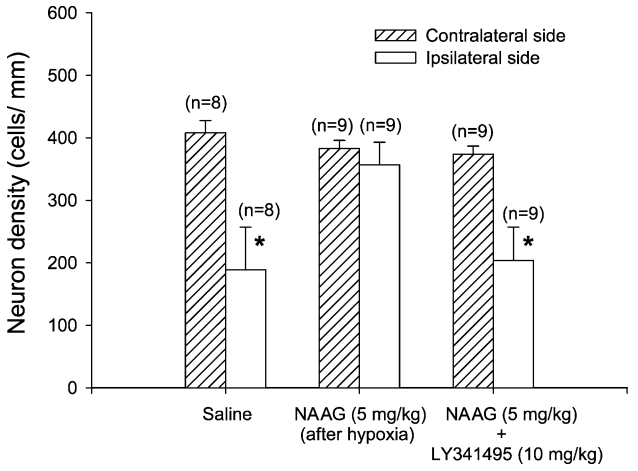

Fig. 2. Neuron density (cells/mm) in the hippocampal CA1 area. The neonatal hypoxia–ischemia procedure was performed on postnatal day 7 and brain sections were prepared as described in the text. NAAG or saline was administered i.p. 5 min after the hypoxic exposure. LY341495 (10 mg/kg, i.p.) was administered 1 h before the hypoxic exposure. Number of viable neurons at the hippocampal CA1 region in both the contralateral and the ipsilateral hemispheres was counted under a microscope by a person blinded to the treatment with the Cell Analysis Systems (CAS) from Becton Dickinson (Elmhurst, IL). Results are expressed as the mean ± S.E.M. of the number of samples indicated in the parenthesis. * $P < 0.05$ from the neuron density measured at the contralateral brain in the same treatment group.

mg/kg) administered 1 h prior to the hypoxic exposure provided dose-dependent protection against hypoxic-ischemic brain injury as indicated by a decreased brain weight reduction (9.6% and 7.5%, respectively, Fig. 1A). Significant protective effects of NAAG were also prominent when NAAG was administered at these two doses 5 min after the hypoxic exposure. However, the protection of 10 mg/kg NAAG was less effective than 2 mg/kg NAAG. When the NAAG dose was further increased to 20 mg/kg, NAAG lost its protection or even became toxic, as indicated by an increased ipsilateral brain weight reduction (28.7%). We tested the effect of NAAG (5 mg/kg) administered at various times after the hypoxic exposure. Significant protection of NAAG was observable even if it was administered 1 h after the hypoxic exposure (Fig. 1B). The best protection of NAAG (3.8% in ipsilateral brain weight reduction) was observed when it was administered at this dose 30 min after the hypoxic-ischemic insult. Although NAAG (5 mg/kg, administered 5 min after the hypoxic exposure) decreased ipsilateral brain weight reduction to 10%, pretreatment of LY341495 (10 mg/kg), a selective antagonist at the $mGlu_{2/3}$ receptors, blocked the neuroprotection of NAAG (Fig. 1C). L-AP4 (10 mg/kg), a selective agonist of the Group III mGlu receptors, or *t*-ACPD (10 mg/kg), a non-selective agonist at the Groups I and II mGlu receptors (Schoepp et al., 1999), failed to provide protection against hypoxic-ischemic brain injury (Fig. 1C). On the other hand, 2-(phosphonomethyl) pentanedioic acid (2-PMPA, 10 mg/kg), a selective inhibitor of *N*-acetylated-α-linked-acidic-dipeptidase (NAALADase), which hydrolyses endogenous NAAG to *N*-acetylaspartate and glutamate, was as effective as NAAG in protection of the neonatal rat brain from hypoxic-ischemic injury (Fig. 1C).

As shown in Fig. 2, neuron density in the ipsilateral hippocampal CA1 region in the saline-treated group was significantly reduced as compared to that in the contralateral side (189 vs. 408 neurons/mm). In the NAAG-treated groups (5 mg/kg, 5 min after hypoxic exposure), although neuron density in the ipsilateral hippocampal CA1 region was lower than that of the contralateral side (357 vs. 383 neurons/mm), the difference was not statistically significant. Pretreatment with LY341495 (10 mg/kg) blocked the neuroprotective effect of NAAG and significantly reduced ipsilateral hippocampal CA1 neuron density as compared to the contralateral hippocampus (204 vs. 374 neurons/mm).

3.2. Effect of NAAG on hypoxia-ischemia-induced elevation of cAMP

cAMP concentration in the naive rat pup brain was 27.3 ± 0.7 pmol/mg protein. Neonatal hypoxia-ischemia elevated cAMP concentration in the saline-treated rat brain by 6.5 pmol/mg protein (Fig. 3). Pretreatment with LY341495 (10 mg/kg) did not significantly affect hypoxia-ischemia-induced elevation of cAMP concentration. Administration of NAAG (2 or 5 mg/kg) 5 min after hypoxic exposure significantly reduced hypoxia-ischemia-stimulated increase in cAMP (1.1 and − 0.8 pmol/mg protein, respectively). Pre-

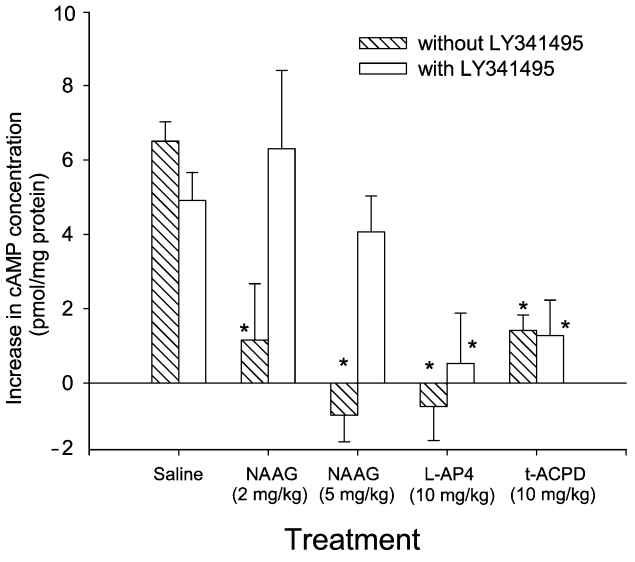

Fig. 3. Neonatal hypoxia–ischemia-induced elevation of cAMP concentration in the rat brain. One hour after the neonatal hypoxia–ischemia procedure, brain samples were collected and cAMP concentrations were determined as described in the text. NAAG or other mGlu receptor agonists (10 mg/kg) was administered 5 min after the hypoxic exposure and LY341495 was injected 1 h before the hypoxic exposure. cAMP concentration in the naive rat pup brain was 27.3 ± 0.7 pmol/mg protein and the results presented here are the increase in cAMP concentrations above this level. Each bar is the mean ± S.E.M. of four to six brain samples. * $P < 0.05$ from the value for the saline-treated, without LY341495 group.

treatment with LY341495 (10 mg/kg) completely blocked the suppressive effects of NAAG on hypoxia-ischemia-stimulated cAMP elevation. L-AP4 or *t*-ACPD (10 mg/kg, respectively) was also capable of preventing the increase of cAMP concentration stimulated by hypoxia-ischemia (Fig. 3). However, pretreatment with LY 341495 failed to block the effects of L-AP4 or *t*-ACPD on suppression of hypoxia-ischemia-stimulated cAMP elevation, suggesting that inhibition of hypoxia-ischemia-stimulated elevation of cAMP concentration by NAAG and by L-AP4 or *t*-ACPD was mediated through different subtypes of mGlu receptors.

4. Discussion

Intracerebral administration of NAAG before middle cerebral artery occlusion or co-injection of NAAG with quinolinic acid has been shown to protect the adult rat brain from transient focal cerebral ischemic injury (Lu et al., 2000) or quinolinic acid excitotoxicity (Orlando et al., 1997). Consistent with results from these reports, we have demonstrated in the current study that NAAG has also neuroprotective effects in the neonatal rat model of hypoxia-ischemia. Furthermore, NAAG was administered systemically in the present study and the significant neuroprotective effect was observed when NAAG was even injected 1 h after the hypoxia-ischemia event. Although the protective dose range of NAAG (2–10 mg/kg) observed in this study is a little higher than the effective dose range of intracerebrally injected NAAG (1–2 μmol/rat, equivalent to 1.1–2.3 mg/kg) previously reported (Lu et al., 2000), no toxic sign was observed following NAAG injection in this study. Intraperitoneal administration is more applicable. This may be of important significance for potential use of NAAG as a therapeutic treatment for perinatal hypoxic-ischemic brain injury.

2-PMPA, a potent inhibitor of NAALADase that hydrolyzes endogenous NAAG into *N*-acetylaspartate and glutamate, has been shown to protect the rat brain or neurons from hypoxic-ischemic or excitotoxic injury (Slusher et al., 1999; Harada et al., 2000; Thomas et al., 2000; Tortella et al., 2000). The neuroprotective effect of 2-PMPA has been attributed to the reduction in glutamate production and the increase in endogenous NAAG, which activates Group II mGlu receptors (Slusher et al., 1999; Vornov et al., 1999). The decreased extracellular glutamate concentration and the increased extracellular NAAG upon application of 2-PMPA have been demonstrated in a rat model of transient middle cerebral artery occlusion (Vornov et al., 1999). While neuroprotection of preventing excessive glutamate release is well documented, the neuroprotective effects of NAAG as a result of activation of Group II mGlu receptors has not been confirmed by in vivo studies. Results from the present study have provided evidence that the neuroprotective effects of NAAG against hypoxic-ischemic brain injury is primarily mediated by the activation of Group II mGlu receptors. NAAG is a selective agonist at Group II mGlu receptors, especially at $mGlu_3$ receptor (Wroblewska et al., 1997; Neale et al., 2000). In the present study, the significant neuroprotection against neonatal hypoxic-ischemic brain injury was only observed following the administration of NAAG, but not following the injection of L-AP4, a selective agonist of the Group III mGlu receptors or *t*-ACPD, a non-selective agonist at the Groups I and II mGlu receptors (Schoepp et al., 1999). LY341495, a highly potent and selective antagonist at Group II mGlu receptors (Kingston et al., 1998), attenuates the neuroprotective effects of NAAG (Fig. 1C). Our cAMP data provide further evidence for the action of NAAG as a result of selective activation of Group II mGlu receptors. Hypoxia-ischemia-induced elevation of cAMP concentration, which may be associated with dopamine release or protein kinase C activation following hypoxia-ischemia (Prado et al., 1992; Domanska-Janik and Pylova, 1992), was observed in the current study. Since both Groups II and III mGlu receptors are negatively coupled with cAMP formation, NAAG, L-AP4 and *t*-ACPD all reduced hypoxia-ischemia-induced elevation of cAMP (Fig. 3). While effects of NAAG on hypoxia-ischemia-induced elevation of cAMP were completely prevented by LY341495, effects of L-AP4 or *t*-ACPD on hypoxia-ischemia-induced cAMP elevation were not affected by LY341495, which has also weak antagonist activity on $mGlu_1$ and $mGlu_5$ receptors (Schoepp et al., 1999). These results consistently indicate that the neuroprotective effect of NAAG on neonatal hypoxia-ischemia-induced brain injury is largely associated with activation of Group II mGlu receptors, although the possibility of NAAG acting as a mixed agonist/antagonist at specific NMDA receptor subtypes cannot be completely excluded (Puttfarcken et al., 1993; Neale et al., 2000).

It has been found that the neuroprotective effect of NAAG in the subcortical region is more prominent than in the cortical region in an adult rat model of focal ischemia (Lu et al., 2000). Hence, the authors found that a Group II mGlu receptors antagonist, (*S*)-α-methyl-4-carboxyphenylglycine ((*S*)-MCPG), is more effective in blocking the protective effect of NAAG in the subcortical area than in the cortical area (Lu et al., 2000). In the present study, the protective effect of NAAG (5 mg/kg) was completely reversed by LY341495 compared to the saline-treated group, when brain injury was estimated by neuron density in the hippocampal CA1 region (Fig. 2). When brain injury was estimated by percentage weight reduction in the ipsilateral hemisphere (Fig. 1C), however, blockade of the protective effect of NAAG by LY341495 appeared not complete. The exact reason for this discrepancy is unclear at this time. Since the neuron density data in the current study specifically reflect the hypoxic-ischemic injury in the hippocampal region while brain weight reduction may represent brain injuries in many brain regions, the above-mentioned discrepancy may be an indication that there might be regional differences in neuroprotective effect of NAAG. It is worthy of further investigation.

NAAG can be hydrolyzed by NAALADase into *N*-acetylaspartate and glutamate, of which an excessive amount is neurotoxic. A recent study has demonstrated that when NAALADase is active in cortical cell cultures, the added NAAG has toxic effects and when NAALADase is inhibited, the added NAAG is neuroprotective (Thomas et al., 2000). Therefore, use of phosphate buffer, which inhibits NAALADase, for NAAG administration has been recommended (Lu et al., 2000). We used a HEPES buffer as the vehicle for NAAG administration in our study and the neuroprotective effect of NAAG was still observable when it is used at low doses. However, data from the current study have shown that NAAG used at high doses was no longer protective and even became toxic. When NAAG was used at 10 mg/kg, its protective effect was less than when used at 2 or 5 mg/kg and when NAAG was used at 20 mg/kg, ipsilateral brain injury was increased rather than decreased (Fig. 1A). The loss of neuroprotective effects of NAAG at higher doses has been reported in other in vitro (Bruno et al., 1998b; Thomas et al., 2000) and in vivo studies (Orlando et al., 1997; Lu et al., 2000). This observation is possibly associated with the dual effects of NAAG: the activator of Group II mGlu receptors and the precursor of glutamate. In the presence of endogenous NAALADase, it appears there is a balance between the dual effects. When exogenous NAAG is administered at low or moderate doses, the effect of NAAG as an activator of Group II mGlu receptors is prevalent and it is neuroprotective. When NAAG is used at high doses, however, its effect as the precursor of glutamate becomes predominant. Excessive exogenous NAAG may lead to more glutamate production, which may offset the effect of NAAG acting on mGlu receptors and result in toxicity. Therefore, results from the present study support the idea that inhibition of endogenous NAALADase should be taken into consideration when exogenous NAAG is going to be used as a therapeutic treatment for hypoxic-ischemic brain injury (Lu et al., 2000).

The involvement of Group II mGlu receptors in neuroprotection has been demonstrated in a number of in vivo and in vitro studies. Recent studies have shown that (*S*)-4-carboxy-3-hydroxyphenylglycine ((*S*)-4C3HPG), a competitive antagonist at $mGlu_1$ receptor and an agonist at $mGlu_{2/3}$ receptor, provides neuroprotection against transient global ischemia in gerbils and focal cerebral ischemia in adult rats (Henrich-Noack et al., 1998; Rauca et al., 1998). Selective $mGlu_{2/3}$ receptor agonists, LY354740 and LY379268, have been shown to attenuate brain injury in a gerbil model of global ischemia (Bond et al., 1998, 1999, 2000) and in a neonatal rat model of cerebral hypoxia-ischemia (Cai et al., 1999). Group II mGlu receptors agonists have also been shown to protect cultured neurons against NMDA- or kainate-induced neuronal degeneration (Nicoletti et al., 1996; Bruno et al., 1995; Battaglia et al., 1998). However, the detailed mechanisms involved in the neuroprotection mediated through activation of Group II mGlu receptors remain unclear. In the present study, while L-AP4 and *t*-ACPD reduced hypoxia-ischmia-induced elevation of cAMP as did NAAG, L-AP4 and *t*-ACPD did not protect the neonatal rat brain from hypoxic-ischemic injury. Therefore, it is unlikely that reduced cAMP elevation is associated with the neuroprotection mediated by activation of Group II mGlu receptors. At the present time, two possible explanations have been proposed. First, activation of Group II mGlu receptors attenuates glutamate release (Bruno et al., 1995). All three groups of mGlu receptors have been implicated in the inhibition of glutamate transmission at specific synapses (Cartmell and Schoepp, 2000). In the current study, the neuroprotective effect was only observed following administration of NAAG, but not L-AP4 or *t*-ACPD. This hypothesis appears difficult to explain the neuroprotection of NAAG observed in this study. However, effects of NAAG on glutamate release have not been directly examined in the current study. Therefore, the possibility that NAAG inhibits glutamate release should not be completely excluded. Second, the activation of Group II mGlu receptors on glial cells causes release of neurotrophic factors, such as transforming growth factor (TGF-β), which protects surrounding neurons from excitotoxicity (Bruno et al., 1998a). This hypothesis is supported by the observation that NAAG treatment to cortical neurons increases TGF-β release in the cultural media and reduces neuronal death induced by cellular ischemia (see Lu et al., 2000). We have not tested this possibility in our present study. A recent study has shown that LY379268, a selective $mGlu_{2/3}$ receptor agonist protects gerbil brains from global ischemic injury induced by bilateral carotid artery occlusion, but fails to alter the expression of TGF-β, brain-derived neurotrophic factor, nerve growth factor and basic fibroblast growth factor (Bond et al., 2000). It appears that the in vivo situation may be more complicated than the in vitro situation. To elucidate mechanisms involved in the neuroprotection mediated by activation of Group II mGlu receptors, further investigations are needed.

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References

Barks, J.D., Silverstein, F.S., 1992. Excitatory amino acids contribute to the pathogenesis of perinatal hypoxic-ischemic brain injury. Brain Pathol. 2, 235–243.

Battaglia, G., Bruno, V., Ngomba, R.T., Grezia, R.D., Copani, A., Nicoletti, F., 1998. Selective activation of group-II metabotropic glutamate receptors is protective against excitotoxic neuronal death. Eur. J. Pharmacol. 356, 271–274.

Bond, A., O'Neill, M.J., Hicks, C.A., Monn, J.A., Lodge, D., 1998. Neuroprotective effects of a systemically active group II metabotropic receptor agonist LY354740 in a gerbil model of global ischemia. NeuroReport 9, 1191–1193.

Bond, A., Ragumoorthy, N., Monn, J.A., Hicks, C.A., Ward, M.A., Lodge, D., O'Neill, M.J., 1999. LY379268, a potent and selective group

II metabotropic glutamate receptor agonist, is neuroprotective in gerbil global cerebral ischemia. Neurosci. Lett. 273, 191–194.

Bond, A., Jones, N.M., Hicks, C.A., Whiffin, G.M., Ward, M.A., O'Neill, M.F., Kingston, A.E., Monn, J.A., Ornstein, P.L., Schoepp, D.D., Lodge, D., O'Neill, M.J., 2000. Neuroprotective effects of LY379268, a selective mGlu2/3 receptor agonist: investigations into possible mechanism of action in vivo. J. Pharmacol. Exp. Ther. 294, 800–809.

Bruno, V., Battaglia, G., Copani, A., Giffard, R.G., Raciti, G., Raffaele, R., Shinozaki, H., Nicoletti, F., 1995. Activation of class II or III metabotropic glutamate receptors protects cultured cortical neurons against excitotoxic degeneration. Eur. J. Neurosci. 7, 1906–1913.

Bruno, V., Battaglia, G., Casabone, G., Copani, A., Caciagli, F., Nicoletti, F., 1998a. Neuroprotection by glia metabotropic glutamate receptors is mediated by transforming growth factor-β. 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.

Cai, Z., Xiao, F., Fratkin, J.D., Rhodes, P.G., 1999. Protection of neonatal rat brain from hypoxic-ischemic injury by LY379268, a group II metabotropic glutamate receptor agonist. NeuroReport 10, 3927–3931.

Cartmell, J., Schoepp, D.D., 2000. Regulation of neurotransmitter release by metabotropic glutamate receptors. J. Neurochem. 75, 889–907.

Choi, D.W., 1992. Bench to bedside: the glutamate connection. Science 258, 241–243.

Domanska-Janik, K., Pylova, S., 1992. Postreceptor modulation of cAMP accumulation in rat brain particulate fraction after ischemia-involvement of protein kinase C. Mol. Chem. Neuropathol. 17, 65–77.

Gidday, J.M., Fitzgibbons, J.C., Shah, A.R., Park, T.S., 1994. Neuroprotection from ischemic brain injury by hypoxic preconditioning in the neonatal rat. Neurosci. Lett. 168, 221–224.

Gill, R., Lodge, D., 1997. Pharmacology of AMPA antagonists and their role in neuroprotection. Int. Rev. Neurobiol. 40, 197–232.

Hagberg, H.K., Gilland, E., Diemer, N.H., Andin, P., 1994. Hypoxia-ischemia in the neonate rat brain: histopathology after post-treatment with NMDA and non-NMDA receptor antagonists. Biol. Neonate 66, 205–213.

Harada, C., Harada, T., Slusher, B.S., Yoshida, K., Matsuda, H., Wada, K., 2000. *N*-acetylated-alpha-linked-acidic dipeptidase inhibitor has a neuroprotective effect on mouse retinal ganglion cells after pressure-induced ischemia. Neurosci. Lett. 292, 134–136.

Henrich-Noack, P., Hatton, C.D., Reymann, K.G., 1998. The mGlu receptor ligand (*S*)-4C3HPG protects neurons after global ischemia in gerbils. NeuroReport 9, 985–988.

Kingston, A.E., Ornstein, P.L., Wright, R.A., Johnson, B.G., Mayne, N.G., Burnett, J.P., Belagaje, R., Wu, S., Schoepp, D.D., 1998. LY341495 is a nanomolar potent and selective antagonist of group II metabotropic glutamate receptors. Neuropharmacology 37, 1–12.

Lu, X.M., Tang, Z., Liu, W., Lin, Q., Slusher, B.S., 2000. *N*-acetylaspartylglutamate protects against transient focal cerebral ischemia. Eur. J. Pharmacol. 408, 233–239.

Maiese, K., 1998. From the bench to the bedside: the molecular management of cerebral ischemia. Clin. Neuropharmacol. 21, 1–7.

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.

Nicoletti, F., Bruno, V., Copani, A., Casabona, G., Knopfel, T., 1996. Metabotropic glutamate receptors: a new target for the therapy of neurodegenerative disorders? Trends Neurosci. 19, 172–267.

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.

Prado, R., Busto, R., Globus, M.Y., 1992. Ischemia-induced changes in extracellular level of striatal cyclic AMP: role of dopamine neurotransmission. J. Neurochem. 59, 1581–1584.

Puttfarcken, P.S., Montgomery, D., Coyle, J.T., Werling, L.L., 1993. *N*-acetyl-L-aspartyl-L glutamate (NAAG) modulation of NMDA stimulated [3H]norepinephrine release from rat hippocampal slices. J. Pharmacol. Exp. Ther. 266, 796–803.

Rauca, C., Henrich-Noack, P., Schafer, K., Hollt, V., Reymann, K.G., 1998. (*S*) 4C3HPG reduces infarct size after focal cerebral ischemia. Neuropharmacology 37, 1649–1652.

Rice, J.E., Vannucci, R.C., Brierley, J.B., 1981. The influence of immaturity on hypoxic-ischemic brain damage in the rat. Ann. Neurol. 9, 131–141.

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

Schoepp, D.D., Jane, D.E., Monn, J.A., 1999. Pharmacological agents acting at subtypes of metabotropic glutamate receptors. Neuropharmacology 38, 1431–1476.

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.M., 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.

Tanabe, Y., Masu, M., Ishii, T., Shigemoto, R., Nakanishi, S., 1992. A family of metabotropic glutamate receptors. Neuron 8, 169–179.

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.

Tortella, F.C., Lin, Y., Ved, H., Slusher, B.S., Dave, J.R., 2000. Neuroprotection produced by the NAALADse inhibitor 2-PMPA in rat cerebellar neurons. Eur. J. Pharmacol. 402, 31–37.

Vornov, J.J., Wozniak, K., Lu, M., Jackson, P., Tsukamoto, T., Wang, E., Slusher, B., 1999. Blockage of NAALADase: a novel neuroprotective strategy based on limiting glutamate and elevating NAAG. Ann. N. Y. Acad. Sci. 890, 400–405.

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.

Wroblewska, B., Santi, M.R., Neale, J.H., 1998. *N*-acetylaspartylglutamate activates cyclic AMP-coupled metabotropic glutamate receptors in cerebellar astrocytes. Glia 24, 172–179.