

β-NAAG Rescues LTP From Blockade by NAAG in Rat Dentate Gyrus via the Type 3 Metabotropic Glutamate Receptor

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Lea, Paul M. IV, Barbara Wroblewska, John M. Sarvey, and Joseph H. Neale. β-NAAG rescues LTP from blockade by NAAG in rat dentate gyrus via the type 3 metabotropic glutamate receptor. J Neurophysiol 85: 1097–1106, 2001. N-Acetylaspartylglutamate (NAAG) is an agonist at the type 3 metabotropic glutamate receptor (mGlur3), which is coupled to a Gi/o protein. When activated, the mGlur3 receptor inhibits adenyl cyclase and reduces the cAMP-mediated second-messenger cascade. Long-term potentiation (LTP) in the medial perforant path (MPP) of the hippocampal dentate gyrus requires increases in cAMP. The presence of mGlur3 receptors and NAAG in neurons of the dentate gyrus suggests that this peptide transmitter may inhibit LTP in the dentate gyrus. High-frequency stimulation (100 Hz; 2 s) of the MPP resulted in LTP of extracellular transmitter release in acute hippocampal slices cultured mammalian cells stably expressing mGlur3 cDNAs (Wroblewska et al. 1993, 1997, 1998). The mGlur3 receptor is a selective agonist at the type 3 metabotropic glutamate receptor (mGlur3) in neurons, glia, and transfected cells, where it has a potency similar to that of glutamate (Wroblewska et al. 1993, 1997, 1998). The peptide is also a low potency agonist at the N-methyl-D-aspartate (NMDA) receptor (Trombley and Westbrook 1990; Valivullah et al. 1994; Westbrook et al. 1986). Additional data suggest that NAAG may act as a partial agonist at this receptor (Grunze et al. 1996; Puttfarken et al. 1993). The inactivation of synthetically released NAAG is achieved by a membrane-bound (Riveros and Orrego 1984; Robinson et al. 1987) peptidase on the extracellular face of glia (Cassidy and Neale 1993). This glutamate carboxypeptidase II (GCP II) was found to be identical to prostate specific membrane antigen in humans (Carter et al. 1996) and has been cloned from rat nervous system libraries (Bzdenga et al. 1997; Luthi-Carter et al. 1998). GCP II activity is inhibited by quisqualate (Kic = 2 μM), phosphate (IC50 = 100 μM), sulfate (IC50 = 1 mM), and β-NAAG (Kf = 1 μM), a synthetic structural analogue of NAAG in which the peptide bond is formed by the β-carboxyl group of aspartate (Robinson et al. 1987; Serval et al. 1990). The distribution of both NAAG and GCP II are altered in human degenerative diseases (see Coyle 1997 for review), although it is not possible from these data to resolve primary from secondary degenerative tissue changes.

Acting at the mGlur3 receptor, NAAG causes a decrease in forskolin-stimulated cAMP levels in neurons, glia, and cultured mammalian cells stably expressing mGlur3 cDNAs (Wroblewska et al. 1993, 1997, 1998). The mGlur3 receptor is a member of the group II metabotropic glutamate receptors, which have been implicated in suppression of voltage-dependent calcium conductance in cerebellar granule cells (Chavis et al. 1994), neocortical neurons (Sayer et al. 1992), and amphibian olfactory neurons (Bischofberger and Schild 1996). The group II agonist, DCG-IV, which also inhibits voltage-dependent calcium currents, has been reported to suppress synaptic transmission to motor neurons in the spinal cord (Ishida et al. 1993), potassium-induced release of GABA in cortical cell cultures (Schaffhauser et al. 1998; Zhao et al. 2001), and transmission at the mossy fiber-CA3 synapse in the hippocampus (Hayashi et al. 1993). In cerebellar

INTRODUCTION

Since its discovery in 1964 (Curatolo 1964), the vertebrate neurometapetide, N-acetylaspartylglutamate (NAAG) has been shown to be widely distributed in neurons throughout the mammalian nervous system (Anderson et al. 1986; Forloni et al. 1987; Moffett and Nambodiri 1995; Moffett et al. 1993, 1994; Renno et al. 1997; Tieman and Tieman 1996; Tieman et al. 1987, 1991; Williamson and Neale 1988). NAAG meets each of the traditional criteria for a neurotransmitter (for review, see Neale et al. 2000). NAAG is a selective agonist at the type 3 metabotropic glutamate receptor (mGlur3) in neurons, glia, and transfected cells, where it has a potency similar to that of glutamate (Wroblewska et al. 1993, 1997, 1998). The peptide is also a low potency agonist at the N-methyl-d-aspartate (NMDA) receptor (Trombley and Westbrook 1990; Valivullah et al. 1994; Westbrook et al. 1986). Additional data suggest that NAAG may act as a partial agonist at this receptor (Grunze et al. 1996; Puttfarken et al. 1993). The inactivation of synthetically released NAAG is achieved by a membrane-bound (Riveros and Orrego 1984; Robinson et al. 1987) peptidase on the extracellular face of glia (Cassidy and Neale 1993). This glutamate carboxypeptidase II (GCP II) was found to be identical to prostate specific membrane antigen in humans (Carter et al. 1996) and has been cloned from rat nervous system libraries (Bzdenga et al. 1997; Luthi-Carter et al. 1998). GCP II activity is inhibited by quisqualate (Kic = 2 μM), phosphate (IC50 = 100 μM), sulfate (IC50 = 1 mM), and β-NAAG (Kf = 1 μM), a synthetic structural analogue of NAAG in which the peptide bond is formed by the β-carboxyl group of aspartate (Robinson et al. 1987; Serval et al. 1990). The distribution of both NAAG and GCP II are altered in human degenerative diseases (see Coyle 1997 for review), although it is not possible from these data to resolve primary from secondary degenerative tissue changes.

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slices, exogenously applied NAAG suppressed the excitatory response of Purkinje cell dendrites to climbing fiber activation (Sekiguchi et al. 1989). These data have led to the hypothesis that one function of NAAG following synaptic release is to activate presynaptic mGluR₃ to inhibit subsequent transmitter release.

The rat hippocampal slice is a preparation richly endowed with ionotropic and metabotropic glutamate receptors, including mGluR₃ (Shigemoto et al. 1997). NAAG is concentrated in hippocampal interneurons (Anderson et al. 1986; Moffett and Namboodiri 1995; Moffett et al. 1993), and GCP II activity is found throughout the hippocampus (Bzdenga et al. 1997; Fuhrman et al. 1994; Luthi-Carter et al. 1998). Group II metabotropic glutamate receptors (mGluR2 and mGluR3) have been localized to the suprapyramidal blade of the dentate gyrus in apparent association with the medial perforant path from entorhinal cortex to the midmolecular layer (Petralia et al. 1996; Testa et al. 1994). Inhibitory interneurons and collaterals, located in the dentate gyrus, modulate responses at the dendritic tree and cell body of the granule cells as well as the presynaptic afferents at the medial perforant path-granule cell synapse (Freund and Buzsaki 1996).

Hippocampal LTP has been shown to require NMDA receptor activation (Burgard et al. 1989; Harris and Cotman 1986; Herron et al. 1986) and an increase in cAMP levels (Blitzer et al. 1995, 1998; Nguyen and Kandel 1996; Stanton and Sarvey 1985b). In the dentate gyrus, LTP also requires β-adrenergic receptor activation (Bramham et al. 1997; Stanton and Sarvey 1985a). Because NAAG decreases forskolin-stimulated cAMP levels via the mGluR3 receptor (Wroblewska et al. 1993, 1997, 1998) and NAAG and mGluR3 are present in the dentate gyrus, we speculate that NAAG may have a modulatory role in synaptic plasticity in the medial perforant path-granule cell (MPP-gc) synapse. Consistent with a role for NAAG and mGluR3 in hippocampal plasticity, activation of group II mGluRs has been reported to decrease excitatory postsynaptic potentials (EPSPs) in the mid-molecular layer (Kilbride et al. 1998; Macek et al. 1996), and the group II mGluR agonist, DCG-IV, blocks induction of LTP in the dentate gyrus (Huang et al. 1997). Additionally, DCG-IV and NAAG have been reported to induce long-lasting depression in the medial perforant path of the disinhibited dentate gyrus (Huang et al. 1999), and NAAG reduces LTP of inhibitory postsynaptic potentials in the recurrent inhibitory circuit following alvear stimulation in CA1 (Grunze et al. 1996). In the course of testing the hypothesis that NAAG affects synaptic plasticity in the hippocampus, we discovered that β-NAA functions as a highly selective mGluR₃ antagonist and that the mGluR3 has a role in LTP.

**METHODS**

Both α- and β-isomers of NAAG and ATP were purchased from Sigma. trans-1-aminocyclopentane-1,3-dicarboxylate (trans-ACPD), (2S,2'R,3'R)-2-(2',3'-dicarbocyclopropylglycine (DCG-IV), ethyl glutamate, L(+)-2-amino-4-phosphonomobutyric acid (L-AP4), MK801, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and CGP-55845 were purchased from Tocris Cookson. Tissue culture reagents were obtained from Gibco and Biofluids. Leupeptin was obtained from Boehringer Mannheim, and QX-314 from Astra Pharmaceuticals.

**Preparation of hippocampal slices**

Male Sprague-Dawley rats (Taconic, Germantown, NY) weighing 80–210 g were anesthetized with ketamine hydrochloride (100 mg/kg ip) and decapitated. Experiments were conducted according to the principles set forth in the “Guide for Care and Use of Laboratory Animals,” Institute of Animal Resources, National Research Council, National Institutes of Health Pub. No. 74-23. Transverse slices (400 μm) of hippocampus were prepared using a McIlwain tissue chopper. Slices were placed in a modified Oslo interface recording chamber at 32–34°C and perfused at a rate of 3 ml/min with artificial cerebrospinal fluid (ACSF) containing (in mM) 26 NaHCO₃, 124 NaCl, 1.75 KCl, 1.25 mM KH₂PO₄, 1.3 MgSO₄, 2.4 CaCl₂, and 10 dextrose; pH was adjusted to 7.4 by bubbling with a 95% CO₂-5% O₂ gas mixture. Slices were allowed to equilibrate for at least 2 h before recordings were initiated.

**Electrophysiology**

Figure 1A is a schematic of the hippocampal slice showing the placement of the stimulating electrode in the medial perforant path (MPP) leading from the entorhinal cortex to the dentate gyrus. Recording electrodes were placed in the MPP and in the granule cell layer located along the suprapyramidal blade of the dentate gyrus. In subsequent figures, the initial negative slope of the EPSP, recorded in the MPP, is plotted as percent of baseline EPSP slope (mean ± SE). Stimuli were delivered to the MPP fibers through a 100-μm-diameter monopolar Teflon-insulated, stainless steel wire electrode, exposed only at the tip. Extracellular recordings were obtained using glass micropipettes filled with 2 M NaCl, 2–6 MΩ resistance. Recording electrodes were positioned a minimum of 500 μm from the stimulating electrodes and lowered to a final depth of 80 μm into the slice. Only slices showing complete abolition of the population spike, recorded in the cell body layer at 20-ms interpulse intervals in a paired-pulse paradigm, were selected for study. Isolation of medial perforant-path responses was confirmed by paired-pulse depression of the EPSP seen at an 80-ms interpulse interval using a current intensity that elicited an EPSP that was just subthreshold for a reflected spike (Bramham et al. 1997; McNaughton 1980). Test stimuli were delivered to the mid-molecular layer of the dentate gyrus every 30 s to evoke subthreshold EPSPs. After establishment of a stable baseline recording, EPSPs and population spikes were recorded extracellularly from the medial perforant path and the granule cell body layer in the dentate gyrus. Application of pharmacological agents was achieved by switching the chamber perfusion solution to ACSF containing the drug.

Drugs were tested for possible effects on presynaptic release of glutamate using a paired-pulse paradigm. Two subthreshold stimuli (10 μs) were given to the MPP at interpulse intervals of 20, 30, and 80 ms. The initial slope of the second EPSP was normalized to the slope of the first EPSP of a pair (as shown in Bramham et al. 1997).

“Blind” whole cell patch-clamp recording was used to measure AMPA and NMDA receptor-mediated current in granule cells of the acute hippocampal slice (Blanton et al. 1989). Patch pipettes (4–8 MΩ) were pulled in two stages (Model p-80/PC Flaming-Bronn Micropipette Puller; Sutter Instruments) and filled with a solution of: (in mM) 140 CsF, 1 CaCl₂, 2 MgCl₂, 10 EGTA, 10 HEPES, 2 TEA, 5 Na₂-phosphocreatine, 5 QX-314, 0.1 leupeptin, and 4 Na₂-ATP; pH was adjusted to 7.35 with CsOH. Osmolality was adjusted to 290 mOsmol/1,000 g with sucrose. During patch experiments, 20 μM bicuculline and 50 nM CGP-55845 were added to the ACSF. The whole cell configuration was established in voltage-clamp mode approximately 10 min after establishing a seal in the cell-attached configuration (Hamill et al. 1981). Current measurements were made at holding potentials of −80, −30, and +20 mV (250-ms voltage steps from −80 to −30 and +20 mV; Axopatch 1D, Digidata 1200 series interface, pClamp 8.0). The late EPSC component (NMDA receptor current) was separated from the early EPSC component.
(AMPA receptor current) (Hestrin et al. 1990) by taking current measurements approximately 40 ms after stimulus. Resting membrane potential was also measured at the time of whole cell formation. Cells with resting potentials near −80 mV were accepted for data analysis. All other conditions of our preparations were the same as those described during extracellular recording.

**Cell cultures**

Primary cultures of rat cerebellar granule cells were prepared from 8-day-old Sprague-Dawley pups (Taconic, Germantown, NY) as described previously (Gallo et al. 1982). Cells were plated on the poly-L-lysine-coated dishes at the density 1.25 × 106 cells/ml and cultured in basal Eagle’s medium supplemented with 10% fetal bovine serum (heat inactivated), 2 mM glutamine, 50 mg/ml gentamicin, and 25 mM KCl. To prevent proliferation of the nonneuronal cells, cytosine arabinoside was added (10 μM) to the culture 24 h after plating. Cells kept in vitro for 6–8 days were used.

**Transfected cell lines**

Mammalian cell lines expressing metabotropic glutamate receptor mRNAs were prepared as described previously (Wroblewska et al. 1997). Briefly, mGluR2 and mGluR3 cDNAs (kindly provided by Dr. S. Nakanishi) were inserted into an EcoRI site of the mammalian expression vector pcDNA3 (Invitrogen) containing a neomycin-resistant gene. Mammalian cells (Chinese hamster ovary cells, CHO, and baby hamster kidney cells, BHK) were transfected with these constructs using the calcium phosphate method as described by Chen and Okayama (1987). The BHK cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) with l-glutamine (2 mM), sodium pyruvate, 10% fetal bovine serum (FBS, Gibco) and penicillin/streptomycin (pen/strep, Biofluids). The CHO cells were maintained in the medium described previously (Tanabe et al. 1992). Gentamycin (G-418, Gibco) was used to select stable neomycin-resistant cell lines expressing mGluR mRNAs. Positive clones were identified by reverse transcription PCR reaction with specific primers and the response to forskolin stimulated cAMP formation as described previously (Wroblewska et al. 1997).

**Assays**

**IP3.** Cells were prepared and assayed for inositol trisphosphate (IP3) using methods previously described (Wroblewska et al. 1993).

**cAMP.** Rat cerebellar granule cells, BHK-mGluR3 or CHO-mGluR2 stably transfected cell lines, were inoculated on the 24-well plates and grown for 6–8 days in the growing medium. Cells were preincubated for 10 min in medium containing 1 μM MK801, 10 μM CNQX, and after 500 μM 3-isobutyl-1-methylxanthine (IBMX) for granule cells or PBS and IBMX for cell lines. Cells were then incubated for 7 min with either forskolin (10 μM) alone, forskolin + agonist, or forskolin + agonist + β-NAAG (100 μM) as described previously (Wroblewska et al. 1993, 1997). The measurements of cAMP were performed with Amerlex cAMP 125I kit (Amersham). Curve fitting of the data was performed using GraphPad Prism 2.0 (GraphPad Software).

**Data analysis and statistics**

**LTP-EXTRACELLULAR RECORDINGS.** Responses were amplified, filtered (d.c. – 3 kHz), digitized at 20 kHz (DAS-20 interface, Keithley

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**FIG. 1.** N-Acetylaspartylglutamate (NAAG) blocks long-term potentiation (LTP). A: schematic representation of the rat hippocampal slice. Extracellular recording electrodes placed in the granule cell layer and in the mid-molecular layer (medial perforant path, MPP) of the dentate gyrus record characteristic potentials during MPP stimulation. In this figure, a subthreshold excitatory postsynaptic potential (EPSP) recorded in both the MPP and granule cell layer is presented. Higher stimulation intensity elicited a population spike (not shown). B: LTP induced by high-frequency stimulation (HFS, 100 Hz, 2 s) at 0 min lasted more than 2 h (artificial cerebrospinal fluid, ACSF; n = 5; P < 0.05; paired t-test). Perfusion with 200 μM NAAG 20 min prior to HFS blocked LTP (NAAG; n = 4; P > 0.05; paired t-test). B: top inset (ACSF): shows a representative baseline EPSP prior to HFS, overlayed with the EPSP after HFS. Bottom inset (NAAG): a representative baseline EPSP just prior to HFS, overlayed with an EPSP 60 min after HFS. C: perfusion of 50 μM NAAG 20 min prior to HFS blocked maintenance of LTP (NAAG; n = 3; P > 0.05; paired t-test). The initial negative slope of the EPSP recorded in the MPP is plotted as percent of baseline EPSP slope. Each data point represents the mean ± SE. The horizontal bar above the abscissa is the bath perfusion time for NAAG.
Metabyte, Taunton, MA), and stored for analysis using the Labman data-acquisition analysis program (a gift of Dr. T. Teyler, NeuroScientific Laboratories, Rootstown, OH). Measures of synaptic efficacy were made using the initial slope of the EPSP. Values were normalized to percentages of the mean baseline value. Data are expressed as the means ± SE. Statistical analyses of drug effects in hippocampal slices were carried out in Statview (Abacus Concepts, Berkeley, CA) using a two-tailed paired t-test or ANOVA plus a post hoc Bonferroni/Dunn test for multiple comparisons.

WHOLE CELL PATCH-CLAMP RECORDINGS. Responses were amplified, digitized at 20 kHz, and stored for analysis (Axopatch 1D, Digidata 1200 series interface, pClamp 8.0). Measurements of NMDA current were made when AMPA current subsided (approximately 40 ms).

BIOCHEMISTRY. Statistical analyses of drug effects in cultured cells were carried out in Sigma Plot using the Student's t-test. Data were fit to a sigmoidal log dose-response curve using Prism 2.0 (GraphPad Software). A probability of 0.05 was selected as the level of statistical significance for all data.

RESULTS

NAAG blocks LTP

High-frequency stimulation (HFS; 100 Hz, 2 s) of the MPP increased subthreshold EPSP slopes recorded in the MPP (Fig. 1B; see top trace in inset). Long-term potentiation (LTP; approximately 120% baseline) was significant 120 min after HFS (Fig. 1B; n = 5; P < 0.05; paired t-test). To test the prediction that NAAG will block LTP in the dentate gyrus, we bath perfused hippocampal slices with 200 μM NAAG for 20 min prior to giving HFS (100 Hz, 2 s). NAAG perfusion had no detectable effect on EPSP slope or amplitude during the acquisition of baseline (see solid squares, bottom trace in Fig. 1B; n = 4; P > 0.05; ANOVA). NAAG (200 μM) blocked the increase in slope and amplitude normally seen in control LTP (n = 4; P > 0.05; paired t-test; Fig. 1B; see bottom trace in inset).

To test the effects of a lower concentration of NAAG on LTP, the tissue was perfused with 50 μM NAAG for 20 min prior to giving HFS (100 Hz, 2 s). As predicted, 50 μM NAAG prevented the maintenance phase of LTP (n = 3; P > 0.05; paired t-test; Fig. 1C). The ability to obtain posttetanic potentiation at a lower concentration of NAAG, but not with 200 μM NAAG, suggested that the higher concentration of NAAG was in some way affecting presynaptic release of transmitter. This was supported by the findings of Macek et al. (1996), who report that group II mGluR autoreceptors decrease EPSPs at the MPP-gc synapse. In contrast, Huang et al. (1997) reported that NAAG acts at a postsynaptic site. In light of these previous findings, further analysis of the effects of NAAG needed to be performed.

NAAG has no effect on NMDA or AMPA receptor current

To confirm that NAAG did not affect NMDA or AMPA receptor currents in granule cells in the acute hippocampal slice, we used blind whole cell patch-clamp recordings of MPP excitatory postsynaptic currents (EPSCs). Neither 50 nor 200 μM NAAG had any effect on the NMDA receptor-mediated slow EPSC or on the AMPA receptor-mediated fast EPSC at any potential (n = 3; Fig. 2). To verify that NAAG itself does not induce an inward current, potentially mediated through the ionotropic glutamate receptors, currents were compared at potentials between −80, −30, and +20 mV before eliciting an EPSC in the absence and presence of 50 and 200 μM NAAG. Neither 50 nor 200 μM NAAG induced any current at any potential (ANOVA; n = 3; P > 0.05; data not shown). We conclude from these experiments that the ability of 200 μM NAAG to inhibit the posttetanic potentiation seen with 50 μM NAAG is not caused by NAAG acting as an agonist or antagonist at either NMDA or AMPA receptors.

FIG. 2. NAAG has no effect on N-methyl-D-aspartate (NMDA) receptor-mediated current in dentate gyrus granule cells. Neither 50 nor 200 μM NAAG significantly altered NMDA receptor activation in dentate gyrus granule cells of intact 400-μm-thick hippocampal slices. Excitatory postsynaptic currents (EPSCs) were elicited at potentials of −80, −30, or +20 mV (n = 3). Measurements were taken approximately 40 ms after stimulation (see vertical bar in inset) to isolate NMDA receptor current from AMPA receptor current. Wash values were taken after both 50 and 200 μM NAAG treatments. Inset: representative EPSCs at −80, −30, and +20 mV. Data are from EPSCs in the absence of and 20 min after exposure to NAAG.
Ethyl glutamate and βNAAG rescue LTP from NAAG

To further characterize the NAAG-induced block of LTP, we tested the group II mGluR specific antagonist ethyl glutamate for its ability to antagonize the effects of NAAG. Ethyl glutamate prevented the blockade of LTP by NAAG (Fig. 3A; n = 3; P < 0.05; paired t-test). This supports the hypothesis that NAAG is acting through a group II mGluR, either mGluR2 or mGluR3. Although we previously demonstrated that the synthetic structural isomer of NAAG, β-NAAG, failed to act as an mGluR3 agonist (Wroblewska et al. 1993, 1997), it was not examined for antagonist activity at the mGluRs. When tested in our LTP model, β-NAAG mimicked the effects of ethyl glutamate and prevented the blockade of LTP by NAAG (Fig. 3B; n = 4; P < 0.05; paired t-test). These data suggest that β-NAAG is a group II mGluR antagonist and that it rescues LTP at either the group II mGluR2 or mGluR3 subtype. Because we have previously shown that NAAG is a specific agonist at the mGluR3 receptor, but not the mGluR2 receptor (Wroblewska et al. 1997), we hypothesized that β-NAAG was acting as an antagonist at the group II mGluR3 subtype.

While β-NAAG is an inhibitor (Kᵢ = 1 μM) of extracellular peptidase activity against NAAG (GCP II), this peptidase activity is also inhibited by those concentrations of PO₄³⁻ and SO₄²⁻ (1.25 mM KH₂PO₄, 1.3 mM MgSO₄) that are found in the ACSF used for these experiments (IC₅₀ for GCP II = 100 μM and 1 mM for PO₄³⁻ and SO₄²⁻) (Robinson et al. 1987).

β-NAAG has no effect on EPSPs

To characterize the response of the hippocampal slice to β-NAAG, 100 μM β-NAAG was bath applied for 60 min without administering HFS. Our results showed that β-NAAG had no effect on MPP evoked EPSPs (Fig. 3C).

β-NAAG and NAAG have no effect on presynaptic transmitter release

There is evidence for both a presynaptic (Neale et al. 2000) and postsynaptic (Huang et al. 1999) site of action for NAAG presumably through the group II mGluR3 subtype. We utilized a typical paired-pulse paradigm (McNaughton 1982) to test the hypothesis that NAAG and β-NAAG act at presynaptic mGluR3 to affect transmitter release (Neale et al. 2000) and that this mechanism may underlie the peptide’s action on LTP.

**FIG. 3.** Ethyl glutamate and β-NAAG rescue LTP from NAAG. A: 100 μM ethyl glutamate administered for 20 min prior to, during, and for 20 min after 50 μM NAAG perfusion rescued LTP in the medial perforant path (MPP) of the dentate gyrus. The EPSP was significantly increased from baseline 120 min post-HFS (100 Hz; 2 s; n = 3; P < 0.05; paired t-test). Top and bottom horizontal bars along the abscissa represent the infusion time for NAAG and ethyl glutamate, respectively. B: 100 μM β-NAAG administered 20 min prior to, during, and after 200 μM NAAG perfusion, rescued LTP in the medial perforant path of the dentate gyrus. LTP was significantly increased from baseline 120 min post-HFS (100 Hz; 2 s; n = 4; P < 0.05; paired t-test). Top and bottom horizontal bars along the abscissa represent the infusion time for NAAG and β-NAAG, respectively. Inset: taken just prior to and 60 and 120 min after HFS, traces show an increase in EPSP slope after HFS. C: 100 μM β-NAAG perfusion for 60 min, without giving HFS, had no significant effect on EPSP slope measured just prior to and 50 and 100 min after perfusion was begun (n = 16; P > 0.05; ANOVA). The horizontal bar above the abscissa indicates bath infusion of β-NAAG. D: neither 50 μM NAAG (n = 3) nor 100 μM β-NAAG (n = 16) affected paired-pulse depression measured at 20- and 80-ms interpulse intervals (P > 0.05; paired t-test). The slope of the 2nd EPSP is plotted as percent of maximum slope of the 1st EPSP of each pair. Each data point represents the mean ± SE.
Two subthreshold stimuli (10 μs) were given to the MPP with interpulse intervals (IPIs) of 20 and 80 ms. Neither exposure to 50 μM NAAG (n = 3; P > 0.05; paired t-test) nor exposure to 100 μM β-NAAG (n = 5; P > 0.05; paired t-test) affected paired-pulse depression of granule cell EPSP slopes recorded during paired-pulse paradigms (Fig. 3D). If necessary, stimulus intensity was adjusted prior to the paired-pulse paradigms to ensure that the EPSP caused by the first 10-μs stimulus of each pair matched that measured during baseline recordings. While it remains possible that NAAAG is acting at presynaptic GluR3 to suppress LTP following high-frequency stimulation, the peptide’s failure to influence paired-pulse depression is not supportive of this view.

β-NAAG affects group II but not group I or III mGluRs

The ability of β-NAAG to rescue LTP from blockade by NAAG, together with our previous data demonstrating that NAAG is a selective mGluR3 agonist (Wroblewska et al. 1997), suggests that β-NAAG may be an mGluR3 antagonist. To test this hypothesis, we examined the activity of β-NAAG on cells expressing group I (mGluR1 and 5), group II (mGluR2 and 3), and group III receptors.

Glutamate stimulates IP3 formation in CHO cells stably expressing mGluR1 or mGluR5 (Wroblewska et al. 1997). Dose-response studies using up to 100 μM glutamate on mGluR1α expressing cells and up to 30 μM glutamate on mGluR5 expressing cells gave IC50 values of 15 and 5 μM, respectively. Parallel glutamate dose-response assays in the presence of 100 μM β-NAAG were not significantly different.


\[ \text{\textbeta-NAAG RESCUES LTP FROM BLOCK BY NAAG VIA mGluR3} \]

\[ \beta\text{-NAAG rescues LTP from block by NAAG via mGluR3} \]

\[ \beta\text{-NAAG has no effect on the mGluR2 receptor subtype. } \]

\[ \beta\text{-NAAG has no significant effect on DCG-IV (A) or trans-ACPD (B)-mediated decreases in forskolin-stimulated cyclic AMP formation in the CHO-mGluR2 cell line ( } n = 5; \ P > 0.05; \ \text{Student’s } t\text{-test). The cells were preincubated for 10 min in the medium (phosphate-buffered saline, pH 7.4) and incubated for 7 min with forskolin (10 } \mu \text{M) and increasing concentrations of DCG-IV (0.1–30 } \mu \text{M, A) or trans-ACPD (0.1–300 } \mu \text{M, B) without ( ) and with ( ) } \beta\text{-NAAG (100 } \mu \text{M). The data (means } \pm \text{ SE, in triplicate) are expressed as a percentage of the cAMP levels in the presence of 10 } \mu \text{M forskolin. Data were fit to a log dose-response curve using Prism 2.0 (GraphPad Software).} \]

\[ \beta\text{-NAAG antagonizes mGluR3, but not mGluR2, receptors} \]

\[ \text{We have shown previously that NAAG decreases forskolin-stimulated cAMP levels in cerebellar granule cells in culture (Wroblewska et al. 1993). Moreover, using cell lines expressing single subtypes of metabotropic glutamate receptors, we have shown that NAAG selectively activates the group II metabotropic glutamate receptor subtype mGluR3 (Wro-} \]

\[ \text{from those obtained in the absence of this peptide. When these cell lines were stimulated with glutamate at these IC}_{50} \text{ values (Fig. 4), 1–300 } \mu \text{M } \beta\text{-NAAG was found to be without a significant effect. We conclude that } \beta\text{-NAAG does not act as a group I mGluR agonist or antagonist.} \]

\[ \text{We tested the effects of } \beta\text{-NAAG on both group II and group III mGluRs in cerebellar granule cells in culture (Fig. 5A). We used glutamate (groups II and III), trans-ACPD (group II), or L-AP4 (group III) to activate mGluRs in these cells. We have previously shown that group II and III mGluR activation in cerebellar granule cells results in substantial decreases in forskolin-induced cAMP levels (Wroblewska et al. 1993). We found that } \beta\text{-NAAG (100 } \mu \text{M) blocked the ability of glutamate and trans-ACPD to decrease cAMP levels induced by forskolin. In contrast, } \beta\text{-NAAG failed to block the ability of L-AP4 to decrease cAMP via the group III mGluRs (Fig. 5A).} \]

\[ \text{To verify the efficacy of } \beta\text{-NAAG at group II mGluRs, increasing concentrations of } \beta\text{-NAAG (10–100 } \mu \text{M) were applied to cerebellar granule cells that were treated with forsk-} \]

\[ \text{kolin (10 } \mu \text{M) and DCG-IV (3 } \mu \text{M), an mGluR2 and mGluR3 agonist (Fig. 5B). Both 30 and 100 } \mu \text{M } \beta\text{-NAAG were found to block the action of DCG-IV in these neurons (Fig. 5B; } n = 6; \ P < 0.05; \ \text{Student’s } t\text{-test). We conclude from these results that } \beta\text{-NAAG is an antagonist at the group II mGluRs but not at the group I or group III mGluRs.} \]

\[ \text{FIG. 6. } \beta\text{-NAAG has no effect on the mGluR2 receptor subtype. } \beta\text{-NAAG has no significant effect on DCG-IV (A) or trans-ACPD (B)-mediated decreases in forskolin-stimulated cyclic AMP formation in the CHO-mGluR2 cell line ( } n = 5; \ P > 0.05; \ \text{Student’s } t\text{-test). The cells were preincubated for 10 min in the medium (phosphate-buffered saline, pH 7.4) and incubated for 7 min with forskolin (10 } \mu \text{M) and increasing concentrations of DCG-IV (0.1–30 } \mu \text{M, A) or trans-ACPD (0.1–300 } \mu \text{M, B) without ( ) and with ( ) } \beta\text{-NAAG (100 } \mu \text{M). The data (means } \pm \text{ SE, in triplicate) are expressed as a percentage of the cAMP levels in the presence of 10 } \mu \text{M forskolin. Data were fit to a log dose-response curve using Prism 2.0 (GraphPad Software).} \]

\[ \text{FIG. 7. } \beta\text{-NAAG blocks NAAG inhibition of forskolin-stimulated cAMP formation via mGluR3. A: rat cerebellar granule cells, which express native group I–III mGluR subtypes and respond to 10 } \mu \text{M forskolin, were utilized to test the effects of increasing concentrations of NAAG (1–300 } \mu \text{M) on cAMP formation in the absence or presence of 100 } \mu \text{M } \beta\text{-NAAG. NAAG decreased cAMP levels in a concentration-dependent manner ( ). } \beta\text{-NAAG (100 } \mu \text{M) in the presence of NAAG blocked this decrease in cAMP levels ( ) and with ( ) } \beta\text{-NAAG (100 } \mu \text{M) on cAMP formation in the baby hamster kidney (BHK)-mGluR3 cell line ( } n = 5; \ P < 0.05; \ \text{Student’s } t\text{-test). The data (means } \pm \text{ SE, in triplicate) are expressed as a percentage of the cAMP levels in the presence of 10 } \mu \text{M forskolin. •, significant difference from NAAG-treated cells ( } P < 0.05). B: } \beta\text{-NAAG blocks the inhibitory effect of NAAG on forskolin-stimulated cAMP formation in the baby hamster kidney (BHK)-mGluR3 cell line ( } n = 5; \ P < 0.05; \ \text{Student’s } t\text{-test). The cells were preincubated for 10 min in the medium (phosphate-buffered saline, pH 7.4) and incubated for 7 min with forskolin (10 } \mu \text{M) and increasing concentrations of NAAG (1–300 } \mu \text{M) without ( ) or with ( ) } \beta\text{-NAAG (100 } \mu \text{M). The data (means } \pm \text{ SE, in triplicate) are expressed as a percentage of the cAMP levels in the presence of 10 } \mu \text{M forskolin. Curves were fit to a log dose-response curve using Prism 2.0 (GraphPad Software). •, significant difference from NAAG-treated cells ( } P < 0.05).} \]
lewksa et al. 1997). To determine if one or both of the two group II mGluR subtypes (mGluR2 or mGluR3) is antagonized by β-NAAG, we tested for specificity using similar methods.

When tested on CHO cells stably transfected with mGluR2, β-NAAG had no significant effect on either DCG-IV (Fig. 6A) or trans-ACPD (Fig. 6B)-mediated decreases in forskolin-stimulated cAMP. In the same mGluR2-expressing cell line, up to 300 μM NAAG failed to significantly affect forskolin-stimulated cAMP levels (102 ± 4.6% of forskolin stimulation; data not shown).

Cerebellar granule cells, which express group I–III mGluRs, were used to test the specificity of β-NAAG for the native mGluR3 receptor subtype. At 10 μM and higher, NAAG significantly inhibited the forskolin-stimulated increase in cAMP levels, while β-NAAG (100 μM) blocked the effect of all concentrations of NAAG (Fig. 7A).

To further confirm the ability of β-NAAG to block the effects of NAAG via the mGluR3 receptor, a BHK-mGluR3 cell line was stimulated with increasing concentrations of NAAG in the absence and presence of 100 μM β-NAAG (Fig. 7B). As little as 3 μM NAAG significantly inhibited the forskolin-stimulated cAMP levels, while β-NAAG blocked the effect of NAAG. Taken together these data provide the first evidence that β-NAAG is a selective mGluR3 antagonist.

DISCUSSION

The data presented in this paper demonstrate that NAAG blocks LTP of extracellularly recorded EPSPs at the MPP-gc synapse, 50 and 200 μM NAAG does not affect NMDA receptor current in granule cells of the acute hippocampal slice, β-NAAG and ethyl glutamate relieve the blockade of LTP by NAAG, β-NAAG alone does not affect MPP-gc EPSPs, NAAG and β-NAAG do not affect paired-pulse depression of the MPP, and β-NAAG is the first compound to be identified as a selective mGluR3 antagonist.

The peptide neurotransmitter, NAAG, is a selective agonist for mGluR3 in neurons (Wroblewska et al. 1993, 1997) and glia (Wroblewska et al. 1998). This receptor is coupled to a G protein that mediates a reduction in cytoplasmic levels of cAMP. We have reported the presence of NAAG in interneurons in the dentate gyrus as well as the presence of extracellular peptidase activity against NAAG in this tissue (Anderson et al. 1986; Bzdedge et al. 1997; Fuhrman et al. 1994; Moffett et al. 1993, 1995). Additionally, group II receptors (mGluR2/3) have been demonstrated in neurons and glia in the dentate gyrus (Petralia et al. 1996). We previously observed that LTP in the dentate gyrus requires norepinephrine, acting on a β-adrenergic receptor, which stimulates adenylyl cyclase and produces an increase in cAMP (Stanton and Sarvey 1985b). These results, coupled with the fact that the mGluR3 receptor inhibits adenylyl cyclase, suggests that the mGluR3 receptor may regulate the induction of LTP in this region of the hippocampus. Our results militate in favor of this hypothesis.

We found that, similar to the group II selective mGluR antagonist ethyl glutamate, the synthetic β-isomer of NAAG, β-NAAG, blocked the action of NAAG on LTP. The only previously reported action of β-NAAG was as a nonhydrolyzable inhibitor of nervous system GCP II activity (Serval et al. 1990). However, in the ACSF used to perfuse the in vitro hippocampal slice preparation in this study, GCP II was inhibited by the concentrations of phosphate and sulfate used in the perfusion medium (Robinson et al. 1987). Inhibition of GCP II should enhance rather than diminish the actions of NAAG. Because NAAG is a known agonist at the mGluR3 receptor and β-NAAG reversed the effects of NAAG, we speculated that β-NAAG may act as an mGluR3 receptor antagonist.

In testing the efficacy of β-NAAG as an mGluR antagonist, we found that it did not affect glutamate stimulation of group I mGluRs expressed in CHO cells. Similarly β-NAAG had no effect on the stimulation of cerebellar granule cell group III mGluRs by L-AP4. In contrast, β-NAAG antagonized the action of trans-ACPD on group II mGluRs (mGluR2 and mGluR3) and the action of NAAG on mGluR3 in cerebellar granule cells.

To confirm that β-NAAG discriminates mGluR3 from mGluR2, we examined its effect in cell lines transfected with either mGluR2 or mGluR3. Again the selectivity of NAAG was demonstrated as up to 300 μM NAAG failed to activate mGluR2 and 30 μM NAAG maximally activated mGluR3 expressed in transfected cells. With similar selectivity, β-NAAG antagonized the mGluR3 but not mGluR2 receptors in these cells. These data support the conclusion that β-NAAG is an mGluR3 selective antagonist and that induction of LTP in the MPP of the dentate gyrus can be regulated by mGluR3.

β-NAAG is the first subtype selective mGluR3 antagonist to be identified.

Beyond functioning as an mGluR3 agonist, NAAG clearly has been demonstrated to act as a low-potency agonist at NMDA receptors (Sekiguchi et al. 1992; Trombley and Westbrook 1990; Westbrook et al. 1986). In contrast, Sekiguchi et al. (1989) found that 5 μM NAAG decreased the depolarization induced by both NMDA and quisqualate in oocytes that had been injected with rat brain mRNA. These results led to speculation that the partial inhibition of NMDA-induced transmitter release that was observed following 200 μM NAAG application may be mediated by the peptide acting as an antagonist at this receptor (Puttfarcken et al. 1993). Binding studies suggest that the peptide’s affinity for rat brain NMDA receptors is 30-fold less than glutamate (Valivullah et al. 1994). Given NAAG’s low potency as an NMDA agonist in physiological studies, a potentially more parsimonious explanation of the findings of Sekiguchi et al. (1989), and Puttfarcken et al. (1993) is the possibility that NAAG may function as a partial agonist at this receptor. Additionally, it seems likely that NAAG interacts differentially with NMDA receptor subtypes expressed at various synapses (Benke et al. 1995; Hess et al. 1999; Monyer et al. 1994; Wenzel et al. 1995).

We have shown previously that MPP EPSP amplitude and area are decreased by NMDA receptor antagonists (Dahl et al. 1990). Neither effect was seen during NAAG perfusion in our study. Additionally, we find here that NAAG has no significant effect on NMDA receptor currents in the granule cells of the acute hippocampal slice. These data support our hypothesis that NAAG is affecting LTP via the mGluR3 receptor rather than acting as an agonist or partial agonist at the NMDA receptor.

The location of the mGluR3 receptors that NAAG activates to block LTP remains to be defined. An antibody that reacts with both mGluR2 and mGluR3 has been used to identify receptors on both pre- and postsynaptic membranes (Petralia et al. 1996; Shigemoto et al. 1997; Testa et al. 1994). The
activation of group II receptors decreases voltage-dependent calcium currents (Bischofberger and Schild 1996; Chavis et al. 1994; Sayer et al. 1992) and group II mGluR agonists have been shown to suppress synaptic release in several systems (Hayashi et al. 1993; Ishida et al. 1993; Poncer et al. 1995; Schaffhauser et al. 1998; Vignes et al. 1995). We recently found that NAAG acting via presynaptic mGluR3 receptors reduces depolarization-induced release of GABA from cortical neurons and that this action is blocked by ethyl glutamate and β-NAAG (Zhao et al. 2001). In the present study, however, neither NAAG (50 or 200 μM) alone nor β-NAAG (100 μM) alone affected the EPSPs evoked by single or paired-pulse stimulation of the MPP. Since the paired-pulse paradigms reflect the efficacy of presynaptic transmitter release, these data do not support a presynaptic action for NAAG or β-NAAG at the MPP-ge synapse. It remains possible, however, that NAAG acting via mGluR3 may render presynaptic targets insensitive to the potentiating effects of high-frequency stimulation.

Huang et al. (1999) reported that NAAG and DCG-IV induce long-lasting depression in the dentate gyrus following MPP stimulation. In their hippocampal slice preparation, NAAG had no detectable effect on paired-pulse depression but did cause a significant increase in MPP evoked EPSPs. In contrast, NAAG had no effect on either EPSPs or paired-pulse depression in our experiments. These contrasting findings may be related to differences in methodological approach that include submersed versus interface chambers, picrotoxin inhibited versus naturally inhibited slices, and age differences in the animals (40- to 80-g vs. 80- to 210-g rats).

The relative contributions of endogenous glutamate and NAAG to the activation of mGluR3 are unknown. While our discovery of β-NAAG’s antagonist properties may permit detection of endogenous ligand activation of the mGluR3 receptor, discrimination between the actions of endogenous glutamate and NAAG will be more complex. The development and application of inhibitors (Nan et al. 2000) of the extracellular peptidase activity that hydrolyzes NAAG will contribute to the nagging question of the function of N-acetylaspartylglutamate.

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