GCP II (NAALADase) Inhibition Suppresses Mossy Fiber-CA3 Synaptic Neurotransmission by a Presynaptic Mechanism

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INTRODUCTION

N-acetylaspartylglutamate (NAAG) is a neuropeptide ubiquitously distributed in millimolar concentrations in the mammalian brain (Coyle 1997; Neale et al. 2000). In the extracellular space, NAAG is enzymatically hydrolyzed into glutamate and N-acetylaspartate by glutamate carboxypeptidase II [GCP II; also known as N-acetylated alpha-linked acidic dipeptidase (NAALADase)] (Riveros and Orrego 1984; Stauch et al. 1989). This enzyme is expressed mainly in glial cells, where it is membrane bound (Cassidy and Neale 1993). Additional peptidases having a high affinity to NAAG were recently identified (Bacich et al. 2002). NAAG was originally considered to be an intermediate metabolite in the biosynthesis of glutamate (Coyle 1997; Curatolo et al. 1967). However, more recent data support a role for NAAG as a classical neurotransmitter (Coyle 1997; Neale et al. 2000). NAAG is concentrated in synaptic vesicles (Renno et al. 1997; Williamson and Neale 1988) and is co-localized with several neurotransmitters including GABA, glutamate, acetylcholine, and dopamine and is released in a calcium-dependent manner from synaptic terminals (Gafurov et al. 2001; Moffett et al. 1994; Tsai et al. 1993; Urazaev et al. 2001; Zollinger et al. 1988). NAAG binds to N-methyl-D-aspartate (NMDA) receptors (IC50: NAAG, 8.8 M, vs. glutamate, 0.4 M) but not to AMPA/kainate receptors (Valivullah et al. 1994). NAAG is a low-affinity agonist of the NMDA receptor (EC50, NAAG, 666 M, vs. NMDA, 29 M) at olfactory bulb neurons (Trombley and Westbrook 1990).

In contrast to its weak effects at NMDA receptors, NAAG binds to group II metabotropic glutamate receptors (mGluRs) with high affinity (IC50 < 5 M) (Schaffhauser et al. 1998; Shave et al. 2001). In transfected cell lines, NAAG activates the group II mGluR, mGluR3 (EC50: 65 M for NAAG vs. 58 M for trans-ACPD) (Wroblewska et al. 1997). Because activation of group II mGluRs results in presynaptic inhibition of transmitter release (Kamiya et al. 1996) NAAG may act as a potent presynaptic inhibitor. In support of this hypothesis are findings that exogenous NAAG reduces climbing fiber-mediated excitatory postsynaptic potentials (EPSPs) in Purkinje cells (Sekiguchi et al. 1989). In cortical cultures, exogenous NAAG selectively and potently inhibits GABA release via activation of mGluR3 receptors (EC50 = 28.6 M) (Zhao et al. 2001).

The recent introduction of potent GCP II inhibitors (Jackson et al. 1996; Rong et al. 2002; Slusher et al. 1999; Vornov et al. 1999) provides a means to increase endogenous NAAG and study the subsequent effects on synaptic transmission. We took advantage of the GCP II inhibitor 2-(3-mercaptopropyl)pentanedioic acid (2-MPPA) (Majer et al. 2003) to test the hypothesis that NAAG acts to presynaptically inhibit glutamate release at hippocampal mossy fiber-CA3 pyramidal cell synapses. The CA3 region was selected because it expresses GABAergic transmission, including GABA, glutamate, acetylcholine, and dopamine and is released in a calcium-dependent manner from synaptic terminals (Gafurov et al. 2001; Moffett et al. 1994; Tsai et al. 1993; Urazaev et al. 2001; Zollinger et al. 1988). NAAG binds to N-methyl-D-aspartate (NMDA) receptors (IC50: NAAG, 8.8 M, vs. glutamate, 0.4 M) but not to AMPA/kainate receptors (Valivullah et al. 1994). NAAG is a low-affinity agonist of the NMDA receptor (EC50, NAAG, 666 M, vs. NMDA, 29 M) at olfactory bulb neurons (Trombley and Westbrook 1990).

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Hippocampal slice preparations

Animal protocols used in this study complied with all pertinent institutional and federal regulations. Wistar male rats (21–30 day old) were anesthetized with ketamine (50 mg/kg) and decapitated. Brains were rapidly removed and placed in cold (4°C) sucrose-artificial cerebrospinal fluid (ACSF) containing (in mM) 75 sucrose, 3 KCl, 2 CaCl₂, 2 MgSO₄, 0.15 BES (N,N-bis(2-hydroxyethyl)2-aminoethanesulfonic acid), NaHCO₃, and 25 glucose, aerated with 95% O₂-5% CO₂ (pH 7.4; 305 mosM). Transverse hippocampal slices (400 μm) were cut with a Vibroslicer (OTIS-4000, EMS, Fort Washington, PA). Slices were allowed to recover in standard ACSF (see following text) at 34°C for ≥20 min and then transferred to room temperature before being placed in the recording chamber. In the heated recording chamber (32°C) slices were continuously perfused (at 1.5 ml/min) with ACSF containing (in mM) 125 NaCl, 3 KCl, 2 CaCl₂, 2 MgSO₄, 0.15 BES (N,N-bis(2-hydroxyethyl)2-aminoethanesulfonic acid), NaHCO₃, and 25 glucose, aerated with 95% O₂-5% CO₂, pH 7.4.

Electrophysiology

Recordings were made from visually identified pyramidal neurons in the CA3 region of the hippocampus. All cells were initially identified on the basis of somata shapes using differential interface contrast optics. Recording pipettes pulled from borosilicate glass had a resistance of 3–5 MΩ. To record miniature excitatory post synaptic currents (mEPSCs), patch electrodes were filled with a solution comprising (in mM) 120 K-glucuronate, 10 KCl, 2.5 Mg-ATP, 10 HEPES, 0.25 Na₂-GTP, 0.1 BAPTA, and biocytin 0.1% (pH 7.2; 287 mosM). Evoked EPSCs were recorded with pipettes containing a floride-based solution that intracellularly blocks GABAₐ-mediated inhibitory currents (Nelson et al. 1994). This solution contained (in mM) 135 KF, 10 KCl, 2 MgCl₂, 0.4 HEPES, 0.5 CaCl₂, and 0.1 BAPTA (pH 7.2) (Brager et al. 2002).

In experiments involving analysis of evoked EPSCs, 5 mM N-(2,6-dimethylphenylcarbamoyl-methyl) triethylammonium bromide (QX-314) was added to the intracellular solution to prevent Na⁺-dependent action potentials. Miniature mEPSCs were recorded in the presence of tetrodotoxin (TTX, 0.5 μM).

Intrinsic membrane properties were recorded in current-clamp mode using Axoclamp-2B amplifier. Currents and EPSC data were recorded with either an Axopatch1-D or Axoclamp-2B. Data were digitized at 10 kHz with a DIGIDATA 1300 A/D board (Axon Instruments). Pyramidal neurons were voltage clamped at −70 mV. Series and access resistance were monitored continually, and cells were discarded if access resistance changed by >20%.

Mossy fiber stimulation

To evoke mossy fiber synaptic responses, stimuli were delivered using Teflon-coated tungsten bipolar electrodes (10- to 20-μm tips) positioned in the hilar region of the dentate gyrus at the border of stratum lucidum (Jonas et al. 1993). Constant current pulses (100–150 μs) were delivered through a constant-current isolation unit (Isolflex, AMPI, Jerusalem). EPSCs were elicited by electrical stimulation of the mossy fiber pathway at a frequency of 0.05–0.033 Hz. In some experiments, stimulation frequency was increased ≥20 Hz to explore saturation of responses. Stimulus strengths were adjusted to evoke reproducible response amplitudes (~70% of the maximal amplitude). Stimulation intensities ranged from 20 to 200 μA. Paired pulse protocols (50-ms interpulse interval) were used to study short-term plasticity.

Chemicals

GCP II inhibitor 2-MPPA was obtained from Guilford Pharmaceuticals. TTX, gabazine, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and -2-aminophosphonopentanoic acid (AP-5) were obtained from Sigma (St. Louis, MO). 2S-2-amino-2-(1S,2S-2-carboxycyclopropyl-1-yl)-3-(xanth-9-yl) 1-propanoic acid (LY341495), 2S,3S,4S-CCG- (2S,1'S,2'S)-2-(carboxycyclopropyl)glycine (L-CCG-I), and 2S,2'R,3'R-2'(2'-3',4'-dicarboxycyclopropyl)glycine (DCG-IV) were obtained from Tocris Cookson, (Ellisville, MO). Other agents include biocytin (Molecular Probes, Eugene, OR) and QX-134 (Alomone labs, Jerusalem, Israel).

Analyses

Responses were analyzed off-line with MiniAnalysis software (Synaptosoft, Decatur, GA) and pCLAMP8 (Axon Instruments). Paired-pulse ratio (PPR) was calculated as the meanEPSC 1 / meanEPSC 2 (Kim and Alger 2001), where EPSC 1 is the amplitude of first evoked current and EPSC 2, the amplitude of the second synaptic current. Dose-response relationships for 2-MPPA were analyzed by fitting the data to the function f(x) = A2 + [(A1 − A2)/1 + (x/EC50)ⁿ], where x is the 2-MPPA concentration, A1 is a constant representing asymptotic maximum inhibition as determined by the percent of the control data, A2 is the asymptotic minimum, p is the slope. Data are presented as means ± SE. Statistical comparisons were performed with unpaired or paired (for the same cell) Student’s t-test, the nonparametric Kolgomorov-Smirnov statistic (KS test), one-way ANOVA with post hoc comparisons by Tukey’s honest statistical difference; P < 0.05 was considered significant.

RESULTS

Inhibition of the enzyme GCP II produces an increase in endogenous NAAG concentrations (Jackson and Slusher 2001). This increase in NAAG may impact NMDA receptors at which NAAG acts as a low-affinity agonist and partial antagonist (see introduction). To focus on the hypothesized group II mGluR-mediated presynaptic actions of NAAG, we performed all experiments in the presence of the NMDA receptor antagonist AP5 (50 μM).

Intrinsic membrane properties are not affected by GCP II inhibition

We first evaluated the effects of GCP II inhibition on intrinsic membrane properties, by recording from CA3 pyramidal

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Records from CA3 pyramidal cells (n = 7) before (control) and after application of the glutamate carboxypeptidase II (GCP II) inhibitor, 2- (3-mercaptopropyl)-pentanedioic acid (2-MPPA). Values are expressed as means ± SE. Vrn, resting membrane potential; Rmn, input resistance; τm, membrane time constant; AP, action potential; ADP, afterdepolarization; fAHP, fast afterhyperpolarizing potential. Statistical comparisons performed by unpaired Student’s t-test, P as indicated.
neurons in current-clamp (action potential properties) or voltage-clamp mode (whole cell currents). As summarized in Table 1, application of 2-MPPA (0.1–20 μM) had no significant effects on passive membrane properties, including resting membrane potential, membrane time constant, or input resistance (n = 10 cells). Similarly, 2-MPPA did not affect action potential properties, including firing threshold and amplitude, the base, or the amplitudes of afterdepolarizing and fast afterhyperpolarizing potentials (Table 1). In addition, 2-MPPA had no significant effects on responses to intra-somatic injections of hyperpolarizing and depolarizing currents (Fig. 1a).

To study the effects of the 2-MPPA on whole cell currents we recorded, in voltage-clamp mode, from 25 CA3 pyramidal neurons in the presence of TTX (0.5 μM; Fig. 1B). 2-MPPA (0.1–20 μM) had no effect on fast, rapidly inactivating inward currents (including IA current; Fig. 1B1), on hyperpolarization-activated anomalous rectifying currents (including IKir and IH; Fig. 1B, 2 and 3), or on sustained outward currents (reflecting Ca2+ and K+ conductances; Fig. 1B3).

These findings, and the current-clamp data in the preceding text, indicate that application of 2-MPPA and the presumed consequent increases in NAAG concentrations have no discernible effects on intrinsic membrane properties or whole cell conductances of CA3 neurons.

GCP II inhibition depressed mossy fiber-CA3 synaptic transmission

Next we examined the effects of the GCP II inhibitor 2-MPPA on stimulus-evoked AMPA/kainate-mediated EPSCs at mossy fiber-CA3 synapses. Stimulation in the hilar region of the dentate gyrus may evoke in CA3 pyramidal neurons EPSCs mediated by mossy fiber inputs or by other inputs, including those mediated by antidromically activated axon collaterals of

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**FIG. 1.** The glutamate carboxypeptidase II [GCP II, N-acetylated alpha-linked acidic dipeptidase (NAALADase)] inhibitor 2-(3-mercaptopropyl)pentanedioic acid (2-MPPA, 10 μM) does not affect intrinsic membrane properties of CA3 pyramidal neurons. A: voltage recordings of responses to current pulses (400-ms duration; −120–140 pA) before (control) and 10 min after drug application (2-MPPA). B: whole cell currents recorded in voltage-clamp mode. Numbered triangles depict time points analyzed in the I-V plots below. 2-MPPA has no effect on transient outward currents (B1), inwardly rectifying currents (B2 and B3), or sustained currents (B3).
CA3 cells, or polysynaptic inputs from neighboring CA3 neurons (for review, see Henze et al. 2000). We therefore included in our analyses only evoked EPSCs that satisfied criteria previously established as diagnostic of mossy-fiber-evoked EPSCs (Claiborne et al. 1993). These criteria include responses having a single component with a rapid rise time (<2.5 ms at 10–90%) and a constant, short latency (<3.5 ms) (Jonas et al. 1993; Xiang et al. 1994). In addition, mossy-fiber-evoked EPSCs are characterized by large paired-pulse facilitation (PPF), (Kobayashi et al. 1999; Salin et al. 1996; Xiang et al. 1994). We therefore accepted for analyses only responses that exhibited a PPF ≥130%, in response to a pair of stimuli delivered at 50-ms interstimulus interval. Finally, mossy-fiber-evoked EPSCs can be diagnosed by their sensitivity to agonists delivered at 50-ms interval. After drug washout (data not shown). Changes in EPSC amplitudes, but this effect was partially reversed within 20 min of the remaining two cells, 2-MPPA significantly reduced EPSC amplitudes, measured 45 min after drug application—were reduced to 62.6 ± 8.6% of control amplitudes (ANOVA, P < 0.001; Fig. 2, B and C). In the remaining two cells, 2-MPPA significantly reduced EPSC amplitudes, but this effect was partially reversed within 20 min after drug washout (data not shown). Changes in EPSC amplitudes were not accompanied by a significant change in input resistance (Fig. 2A2). Moreover, 2-MPPA-induced depression of EPSCs was accompanied by a twofold increase in the coefficient of variation (CV) of the responses (CVcontrol = 0.072 ± 0.01 vs. CV2-MPPA = 0.15 ± 0.01, n = 8, paired t-test, P < 0.001) consistent with a presynaptic mechanism of action. In contrast to its effect on EPSC amplitudes, 2-MPPA had no effect on EPSC rise or decay kinetics. This is shown in Fig. 2A3, depicting scaled and superimposed EPSCs recorded during the control, drug application, and washout periods. The 10–90% rise time of evoked EPSCs during the control periods (2.07 ± 0.4 ms) was not significantly different (P > 0.05, paired t-test, n = 10) from that recorded 10 min after 2-MPPA application (1.95 ± 0.5 ms). Similarly, the 10–90% decay time constants before (15.2 ± 3.2 ms) and during drug application (14.9 ± 2.4 ms) were indistinguishable (P > 0.05).

We studied dose-response relationships by analyzing the effects of varying the concentrations of 2-MPPA on inhibition of evoked EPSC. Bath application of 1 μM 2-MPPA produced a significant reduction (86.0 ± 4.2%, P < 0.001, ANOVA) of EPSC amplitudes measured 45 min after application. Higher 2-MPPA concentrations (20 and 50 μM) also induced a significant depression of synaptic responses (67.7 ± 2.7 and 64.8 ± 1.0%). However, the effect at 20 and 50 μM was not significantly different that depression produced by 10 μM 2-MPPA, suggesting that 10 μM is a saturating dose to inhibit synaptic responses at mossy fiber-CA3 synapses. The relation between dose and EPSC inhibition was well fit with a sigmoidal function (Fig. 2A4, r^2 = 0.99, see METHODS). From this sigmoidal function we estimated IC50 to be 1.33 μM.

GCP II inhibition increases PPF

The mechanisms by which 2-MPPA affects mossy fiber synaptic transmission may involve a decrease in presynaptic glutamate release, an alteration in postsynaptic responsiveness or a combination of both effects. Short-term plasticity is one approach to distinguish between these mechanisms. In response to a pair of stimuli, mossy fiber-CA3 synapses display short-term plasticity, in which the response to a second stimulus is facilitated (Xiang et al. 1994). This PPF is induced by presynaptic mechanisms (Zucker and Regehr 2002). We therefore reasoned that if GCP II inhibition-mediated increases in NAAG act presynaptically, then GCP II inhibition itself should affect PPF. In all cells examined (n = 10), pairs of stimuli (50-ms interval; 20 Hz) resulted in PPF ranging from 120 to 200% (145.8 ± 12.6%; Fig. 2A). Application of 2-MPPA (10 μM) resulted in a significant enhancement of PPF (Fig. 2A). Analysis of group data (n = 8; Fig. 2b) revealed a significant increase in PPF (paired t-test, P < 0.001, Fig. 2C), measured 45 min after drug application (183.7 ± 16.5%). Because an enhancement in PPF at these excitatory synapses depends on initial release probability (Dobrunz and Stevens 1997), these findings suggest that 2-MPPA increases [NAAG] which acts presynaptically at mossy fiber synapses.

GCP II inhibition in high [Ca2+]

As a further test for NAAG’s presynaptic locus of action, we tested the effects of GCP II inhibition under conditions of increased release probability, by increasing the ratio of extra-cellular [Ca2+]0/[Mg2+]0 from 2:1.3 to 3:1. This results in increased [Ca2+]0 in presynaptic terminals (Heidelberger et al. 1994) and a subsequent enhanced transmitter release probability (Thomson 2000; Tong and Jahr 1994). As a result, and as previously documented (Canepari and Cherubini 1998), evoked EPSCs exhibited larger amplitudes (by 35 ± 3% relative to control ACSF, n = 5). The enhanced release probability results in rapid vesicle depletion, such that the PPF recorded in normal ACSF was reverted to paired pulse depression (PPD, 5 of 5 cells; Fig. 3A1). We reasoned that if NAAG acts presynaptically to suppress glutamate release, GCP II inhibition should attenuate the effects of high [Ca2+]0 by suppressing responses to the first stimulus in a train and by suppressing the reduction in PPF. Indeed, 2-MPPA significantly reduced the amplitude of the first EPSC in the train to 69 ± 5.5% of predrug levels (t-test, P < 0.01; Fig. 3A2) and prevented the conversion of PPF to PPD in four of five cells (t-test, P < 0.01, Fig. 3B).

Taken together, these findings indicate that GCP II inhibition, likely resulting in increases in NAAG, exerts a potent modulation of presynaptic glutamate release mechanisms at mossy fiber synapses.

GCP II inhibition acts via group II mGluRs

The suppression of presynaptic glutamate release by NAAG is likely due to activation of presynaptic, group II mGluRs, which are expressed by mossy fiber terminals (Petraila et al. 1996; Tamaru et al. 2001). NAAG is a selective agonist at these receptors, mainly of the mGluR3 subtype (Wroblewska et al. 1997; Zhao et al. 2001). To test this hypothesis, we assessed the ability of a group II mGluR antagonist,
LY341495, to competitively prevent the effect of GCP II inhibition. LY341495 is a selective and potent group II mGluR antagonist at nanomolar concentrations (20–100 nM, Kingston et al. 1998). As shown in Fig. 4A, LY341495 (100 nM) had no significant effects on normalized EPSC amplitudes (102.8 ± 5.0%) or on PPF ratios (101.4 ± 8.2%). This is consistent with previous reports that LY341495 has little effect on basal activation of mGluRs (Schoepp 2001). However, LY341495 completely suppressed the presynaptic effects of 2-MPPA (10 μM; Fig. 4, A and B). Neither the amplitudes of mossy fiber evoked EPSCs (97.1 ± 5.2%) (Fig. 4, A and B) nor PPF ratios (101.9 ± 8.3%) were significantly different from controls (t-test, P = 0.12). These effects were reversible on washout of LY341495 when 2-MPPA produced the previously described suppression of EPSC amplitudes and enhancement of PPF ratios (Fig. 4, A and B). Analyses of group data (n = 10) reveal that both the amplitude suppression (74.8 ± 14.2%, 1-way ANOVA, F = 47.2, P < 0.001) and the enhancement of
PPF ratios (115.3 \pm 9.4\%, \ F = 70.3, \ P < 0.001) were statistically significant (Fig. 4, B–D). These findings indicate that GCP II inhibition depresses mossy fiber synapses by a mechanism involving activation of group II mGluRs.

**2-MPPA reduces mEPSC frequency but not their amplitude**

Processes that affect the frequency but not the amplitude of spontaneous, TTX-insensitive mEPSCs involve presynaptic, but not postsynaptic mechanisms (Scanziani et al. 1995). Therefore a finding that 2-MPPA results in a reduction in mEPSC frequencies, but has no effect on their amplitudes, would support a presynaptic action of NAAG. We tested this postulate in experiments performed in whole cell voltage-clamp mode (–70 mV) in the presence of TTX (0.5 \mu M), AP5 (50 \mu M), and gabazine (10 \mu M). As previously reported (Jonas et al. 1993), the 10–90% rise time of mEPSC recorded from CA3 pyramidal neurons varied considerably (0.3–12.0 ms). To focus our analysis on spontaneous events mediated by mossy fiber synapses, we restricted our analysis to mEPSCs having a rapid rise time \leq 3 ms (see METHODS). Application of non-NMDA antagonist CNQX (10–20 \mu M) completely suppressed all mEPSCs, indicating that they were mediated by AMPA/kainate receptors (data not shown).

Figure 5A depicts a representative sample of mEPSCs recorded in control ACSF (top) and 10 min after application of 2-MPPA (10 \mu M; bottom). To test for potential effects of 2-MPPA on mEPSC kinetics, we averaged, scaled, and superimposed mEPSCs recorded from a single neuron before and during drug application (Fig. 5A2). In this example, and from analyses of group data (\(n = 10\) neurons), we conclude that 2-MPPA had no significant effects on mEPSC kinetics, including the 10–90% rise time (ACSF = 2.85 \pm 0.07 ms; 2-MPPA = 2.69 \pm 0.09 ms, \(t\)-test, \(P = 0.15\)) and decay time constant (13.1 \pm 1.7 vs. 13.5 \pm 1.4 ms, \(t\)-test, \(P = 0.31\)). This finding confirms that 2-MPPA had no discernible effect on postsynaptic properties. The effects of 2-MPPA on the amplitudes and frequencies of mEPSCs recorded from a single CA3 pyramidal neuron are depicted in Fig. 5A. The amplitude histogram (Fig. 5A1) and the cumulative distribution of amplitudes (Fig. 5A3) revealed no significant changes in mEPSC amplitudes after 2-MPPA application (K-S test \(P = 0.09\)). By contrast, 2-MPPA resulted in a rightward shift in the cumulative distribution of interevent intervals, demonstrating a significant decrease in mEPSC frequencies (Fig. 5A4, K-S test, \(P = 0.008\)). Analyses of group data (\(n = 10\) neurons) are consistent with these findings, demonstrating that 2-MPPA significantly decreased mEPSC frequencies (Fig. 5C, K-S test, \(P < 0.001\)) or mean frequency (Fig. 5D, mean = 72.7 \pm 14.5\% relative to control, \(P < 0.05\)), while having no significant effects on their mean amplitudes (91.9 \pm 12.3\% relative to control; \(P > 0.05\); Fig. 5D) or their distribution (K-S test, \(P > 0.05\), Fig. 5, B and D).

These findings further strengthen our conclusion that inhibition of GCP II, and subsequent increases in NAAG, acts presynaptically to decrease the probability of glutamate release from the mossy fiber nerve synapses.

**2-MPPA-induced changes in mEPSC are mediated by group II mGluRs**

We next asked whether the 2-MPPA-mediated presynaptic suppression of mEPSCs—like the effects on evoked EPSCs described in the preceding text—are mediated via activation of group II mGluRs. Bath application of the mGluR antagonist LY351495 (100 \mu M) had no significant effect on the amplitude or frequency of mEPSCs (Fig. 6, A, B, and D). However, LY341495 completely prevented 2-MPPA-induced reduction in mEPSC frequencies (\(n = 6\); Fig. 6, A, C, and D). This effect was reversible, as after LY341495 washout, 2-MPPA produced a significant reduction in mEPSC frequencies but had no effect on their amplitudes (\(P > 0.05\); Fig. 6). Analysis of group data from these six CA3 pyramidal cells demonstrated that 2-MPPA produced a significant rightward shift in the cumulative distribution of interevent intervals (Fig. 6C, K-S test, \(P < 0.001\)) and produced a significant reduction in mean mEPSC frequency (65.4 \pm 4.6\% relative to control, ANOVA \(P < 0.01\); Fig. 6D). These findings support the conclusion that GCP II inhibition increases NAAG, which acts presynaptically by activating group II mGluRs, to suppress glutamate release from mossy fiber synapses.

**DISCUSSION**

Our aim was to test the hypothesis that endogenous NAAG acts presynaptically, via activation of mGluRs, to presynaptically suppress glutamate release. For this purpose, we took advantage of 2-MPPA, an inhibitor of GCP II, NAAG’s catalytic enzyme. 2-MPPA was recently demonstrated to potently inhibit NAALADase activity with an IC_{50} of 90 \text{nM} and a Ki of 30 \text{nM} (Majer et al. 2003). Further, 2-MPPA was recently evaluated for its selectivity for GCP II over >100 other potentially relevant proteins, such as metalloproteases and glutamate receptors (Majer et al. 2003; Wozniak and Slusher, unpublished observations). Even at a 100-fold higher concentration than its IC_{50} for GCP II (10 \mu M), 2-MPPA did not exhibit significant activity against any of the proteins examined. Fur-
further, preliminary studies demonstrate that a different GCP II inhibitor 2-(phosphonomethyl)pentanedioic acid (2-PMPA) (Witkin et al. 2002) also produces presynaptic suppression of glutamate release in the CA1 region of the hippocampus (Gar-rido Sanabria and Keller, unpublished observations), further supporting our conclusion that GCP II inhibition results in a specific increase in NAAG concentration. Thus the high affinity of 2-MPPA for GCP II, and its selective action on this enzyme, suggest that the effects we report are indeed due to an increase in endogenous NAAG concentrations and allow us to

FIG. 4. GCP II inhibition presynaptically suppresses transmitter release by acting on type II mGluRs. A1: representative EPSCs demonstrate that preincubation with the mGluR antagonist 2S-2-amino-2-(1S,2S,2-carboxycyclopropyl-1-yl)-3-(xanth-9-yl) propanoic acid (LY341495, 100 nM) reversibly suppressed the effects of 2-MPPA. Roman numerals refer to time points in A2. Time courses of EPSC amplitudes and paired-pulse ratio (PPR) are depicted in A2, and superimposed responses, scaled to the 1st EPSC, are shown in A3. B: time course of group data for normalized mean ± SE EPSC amplitudes and PPR (n = 10 cells). Mean ± SE (10 cells) normalized EPSC amplitudes (B) and PPR (C; ANOVA, *: P < 0.001).
assay the roles of endogenous NAAG in regulating synaptic transmission.

GCP II inhibition significantly suppressed the amplitudes of mossy-fiber-evoked EPSCs and resulted in a significant enhancement of PPF, a presynaptically mediated phenomenon of short-term plasticity. Further supporting a presynaptic mechanism is the finding that 2-MPPA suppressed the conversion of PPF to PPD in high [Ca\(^{2+}\)]. In addition, GCP II inhibition suppressed the frequency but not the amplitude of mEPSCs, consistent with a presynaptic action. In agreement with our findings, NAAG inhibits KCl-mediated GABA release from cortical neurons by acting via presynaptic mGluR3 receptors (Zhao et al. 2001). We found no evidence for postsynaptic effects of GCP II inhibition: there were no discernible effects on the intrinsic membrane properties of CA3 pyramidal neurons or on the decay kinetics of EPSCs evoked by trains of stimuli. In support of the postulate that NAAG acts via activation of group II mGluRs are findings that antagonists of these receptors suppressed the effects of 2-MPPA.

The hydrophilic structure of 2-MPPA predicts a rapid washout of the drug. However, in most experiments the effects of 2-MPPA did not reverse, even after 45 min of washout. It is possible that this reflects an irreversible inhibition of NAAG cleavage by GCP II, and a requirement for de novo NAAG synthesis, which may be suppressed in the slice. Additionally, it would be possible that 2-MPPA may act as a slow off rate inhibitor. Another possibility is that NAAG binding to presynaptic mGluRs activates a cascade of intracellular cascades that produce long-term, irreversible effects. Support for this comes from reports that other mGluR II agonists also produce irreversible suppression of synaptic transmission at mossy fiber-CA3 terminals (Kamiya et al. 1996; Manzoni et al. 1995).

**NAAG and GCP II expression in hippocampus**

The finding that GCP II inhibition strongly depresses synaptic transmission at mossy fiber-CA3 synapses is in agree-
ment with neuroanatomical findings on the expression of NAAG and GCP II in this region. Mossy fiber axon terminals express high levels of GCP II (Slusher et al. 1992). Biochemical studies detect high concentrations of NAAG (300 μM) and GCP II in the hippocampus (Fuhrman et al. 1994). The immunocytochemistry results of Passani et al. (1997b) argue that NAAG is localized in dendritic structures in the molecular layer of the dentate gyrus and in granule cells, suggesting that NAAG may be localized in mossy fibers. These results are contradicted by findings from another study using a high-affinity NAAG antibody which does not cross-react with N-acetyl-aspartate (Moffett and Namboodiri 1995). The latter study concluded that NAAG is localized in large nonpyramidal neurons in the stratum pyramidale of CA1 and CA3. A selective localization of NAAG in dentate and CA3 interneurons is also supported by the immunocytochemistry study of Anderson et al. (1986).

These anatomical findings, and our present finding, suggest that NAAG is released after activation of mossy fiber axons. NAAG may be released from a subpopulation of NAAG-containing neurons (putative interneurons) postsynaptic to these axons. Based on the distribution patterns of NAAG (see preceding text), it is unlikely that NAAG is directly released from mossy fiber terminals. After its release, NAAG activates presynaptic group II mGluR receptors (mGluR2 and mGluR3), which are expressed by mossy fiber axon terminals and by granule cell bodies (Blumcke et al. 1996; Shigemoto et al. 1997; Tamaru et al. 2001). Indeed, recent studies using [3H]-NAAG revealed strong labeling of NAAG binding sites in the hippocampus that was inhibited by DCG-IV, an agonist of group II mGluRs (Shave et al. 2001). NAAG is a potent mGluR3 agonist (Ghose et al. 1997; Lea et al. 2001; Wroblewska et al. 1997; Zhao et al. 2001) and also binds to mGluR2 with a lower affinity (Schaffhauser et al. 1998).

In addition to GCP II, a novel membrane-bound NAAG peptidase activity was recently identified in the brain, spinal cord, and kidney of GCP II knockout mice (Bacich et al. 2002). The affinity of 2-MPPA to these peptidases has not yet been determined. One possibility is that 2-MPPA inhibits all peptidases, including the novel variants. Alternatively, 2-MPPA may spare the novel peptidases. As a result, our findings may underestimate the effects of NAAG on presynaptic inhibition of transmitter release.

NAAG as a modulator of synaptic transmission

Our findings are consistent with those reported in previous studies, in which the effects of exogenous NAAG were studied.
Exogenous NAAG reduces the release of [H]norepinephrine in rat hippocampal slices (Puttfarken et al. 1993) and depresses climbing fiber-Purkinje cell excitatory synaptic transmission in cerebellar slices (Sekiguchi et al. 1989). NAAG—acting via mGlUR3—reduces GABA release from cortical neurons (Zhao et al. 2001).

In contrast, other studies report a postsynaptic action of exogenous NAAG. In the hippocampal medial perforant pathway, exogenous NAAG (50–200 μM) suppressed LTP by acting on postsynaptic mGluR3 (Lea et al. 2001). That study also reported that NAAG had no effect on PPD, arguing against a presynaptic mechanism (see also Huang et al. 1999). These postsynaptic actions of NAAG are related to the high density of group II mGluRs on granule cells postsynaptic to medial perforant path-axon terminals (Petraila et al. 1996; Shigemoto et al. 1997). By contrast, the CA3 pyramidal neurons we studied do not express these receptors (Tamaru et al. 2001), a finding that may explain the absence of postsynaptic NAAG effects in our study.

In our study, we suppressed the potential postsynaptic effects of NAAG on NMDA receptors (Koenig et al. 1994; Pai and Ravindranath 1991; Sekiguchi et al. 1992; Valivullah et al. 1994; Westbrook et al. 1986). We did this for technical reasons and because NAAG’s affinity for NMDA receptors is 23-fold lower than that of NMDA (Trombley and Westbrook 1990), suggesting that NAAG’s actions at NMDA receptors may not be physiologically relevant. However, endogenously released NAAG may have a physiological effect as a precursor for glutamate, which has a high affinity for NMDA receptors. Indeed, Thomas et al. (2000) found that GCP II inhibition prevents NMDA-dependent neurotoxicity by suppressing NAAG cleavage to glutamate.

Functional implications

The CA3 region contains a highly interconnected network of pyramidal neurons, rendering it susceptible to recurrent excitation and pathological hyper-excitability (Bains et al. 1999; Christian and Dudek 1988). Presynaptic NAAergic modulation of synaptic efficacies may be essential for preventing pathological processes associated with excessive glutamate release. Thus pharmacological tools that enhance NAAG activity, such as the GCP II inhibitor 2-MPPA we used in this study, may be an effective therapeutic tool to treat disorders such as epilepsy and neurotoxicity. Indeed, GCP II inhibitors have been successfully used to inhibit the expression and development of cocaine-induced seizures (Witkin et al. 2002). Furthermore, GCP II inhibition exerts a neuroprotective action in animal models of degeneration such as ischemic-hypoxic damage and long-term diabetic neuropathy (Cai et al. 2002; Jackson and Slusher 2001; Lu et al. 2000; Zhang et al. 2002).

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