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Morphine Tolerance and Reward but not Expression of Morphine Dependence are Inhibited by the Selective Glutamate Carboxypeptidase II (GCP II, NAALADase) Inhibitor, 2-PMPA

Piotr Popik*[,1], Ewa Kozela[1], Malgorzata Wróbel[1], Krystyna M Wozniak[2] and Barbara S Slusher[2]

[1]Institute of Pharmacology, Polish Academy of Sciences, Kraków, Poland; [2]Guilford Pharmaceuticals Inc., Baltimore, USA

Inhibition of glutamate carboxypeptidase II (GCP II; NAALADase) produces a variety of effects on glutamatergic neurotransmission. The aim of this study was to investigate effects of GCP II inhibition with the selective inhibitor, 2-PMPA, on: (a) development of tolerance to the antinociceptive effects, (b) withdrawal, and (c) conditioned reward produced by morphine in C57/Bl mice. The degree of tolerance was assessed using the tail-flick test before and after 6 days of twice daily (b.i.d.) administration of 2-PMPA and 10 mg/kg of morphine. Opioid withdrawal was measured 3 days after twice daily morphine (30 or 10 mg/kg) administration, followed by naloxone challenge. Conditioned morphine reward was investigated using conditioned place preference with a single morphine dose (10 mg/kg). High doses of 2-PMPA inhibited the development of morphine tolerance (resembling the effect of 7.5 mg/kg of the NMDA receptor antagonist, memantine) while not affecting the severity of withdrawal. A high dose of 2-PMPA (100 mg/kg) also significantly potentiated morphine withdrawal, but inhibited both acquisition and expression of morphine-induced conditioned place preference. Memantine inhibited the intensity of morphine withdrawal as well as acquisition and expression of morphine-induced conditioned place preference. In addition, 2-PMPA did not affect learning or memory retrieval in a simple two-trial test, nor did it produce withdrawal symptoms in morphine-dependent, placebo-challenged mice. Results suggest involvement of GCP II (NAALADase) in phenomena related to opioid addiction. *Neuropsychopharmacology* (2003) **28,** 457–467. doi:10.1038/sj.npp.1300048

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INTRODUCTION

Over the last decade, research has provided compelling evidence that glutamate receptors are crucially involved in the phenomena related to opioid tolerance, dependence, and reward (Bisaga and Popik, 2000). Glutamate, a major excitatory neurotransmitter in the brain, stimulates both ionotropic and metabotropic glutamate receptors (Monaghan *et al*, 1989; Conn and Pin, 1997). Antagonists of the ionotropic *N*-methyl-D-aspartate (NMDA) receptor complex, including memantine, the low affinity, and highly voltage-dependent clinically available NMDA receptor antagonist, inhibit the development of morphine tolerance (Trujillo and Akil, 1991; Marek *et al*, 1991; Popik *et al*, 2000a) and reverse pre-existing tolerance so that opiate-tolerant animals treated with NMDA receptor antagonists become sensitive to doses of morphine that previously did not evoke antinociception (Tiseo and Inturrisi, 1993; Popik *et al*, 2000a). Antagonists of this receptor complex also attenuate the development (Trujillo and Akil, 1991), expression (Cappendijk *et al*, 1993), and maintenance (Popik and Skolnick, 1996) of the continuing morphine dependence. The inhibitory effect on the expression of morphine dependence has also been demonstrated for memantine (Popik and Skolnick, 1996) (Bisaga and Popik, 2000).

The current work demonstrates similar effects of compounds that inhibit the function of metabotropic receptors. Thus, an agonist of group II metabotropic receptors for glutamate (mGluRII), (+)-2-aminobicyclo [3,1,0] hexane-2,6-dicarboxylic acid (LY354740), has been shown to inhibit the development of morphine tolerance (Popik *et al*, 2000b) as well as the expression of morphine dependence (Klodzinska *et al*, 1999; Vandergriff and Rasmussen, 1999).

In addition to the effects on opioid tolerance and dependence, NMDA receptor antagonists inhibit conditioned reward produced by morphine, a phenomenon thought to be related to drug addiction (Koob and LeMoal,

*Correspondence: Dr P Popik, Institute of Pharmacology, Polish Academy of Sciences, 12 Smetna Street, 31-343 Kraków, Poland, Tel: +48 12 6623375, Fax: +48 12 6374500, E-mail: nfpopik@cyf-kr.edu.pl
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2001). Conditioned reward can be studied using the conditioned place preference test wherein compounds may affect its acquisition, expression, or both. While the effects on acquisition provide heuristic insight into the role of a given neurotransmitter pathway in the development of conditioned reward, the inhibitory effects on its expression can be regarded as having potential therapeutic impact in the treatment of drug addiction. NMDA receptor antagonists, including memantine, have been shown to inhibit the acquisition of conditioned place preference produced by opiates (Bespalov *et al*, 1994; Tzschentke and Schmidt, 1995; Popik *et al*, 1998; Kotlinska and Biala, 1999; Popik and Danysz, 1997) as well as its expression (Bespalov *et al*, 1994; Tzschentke and Schmidt, 1997; Popik and Danysz, 1997; Popik *et al*, 1998).

Another, perhaps, more 'physiological' way of attenuating glutamate neurotransmission could potentially be achieved by inhibiting the metabolism of *N*-acetyl-aspartyl-glutamate (NAAG) (Slusher *et al*, 1999), an endogenous dipeptide, present in the brain in millimolar (0.5–2.7 mM) concentrations (Pouwels and Frahm, 1997) that has been immunohistochemically localized to neurons, particularly those known to be glutamatergic (Williamson and Neale, 1988; Tsai *et al*, 1990, 1993). NAAG has been hypothesized to be involved in neuronal communication as a neurotransmitter, neuromodulator, and precursor of glutamate (Blakely and Coyle, 1988). NAAG is released from neurons after depolarization by a calcium-dependent process upon synaptic stimulation (Tsai *et al*, 1990; Neale *et al*, 2000), suggesting its neurotransmitter-like properties.

Importantly, NAAG is hydrolyzed by the neuropeptide glutamate carboxypeptidase (GCP II; EC 3.4.17.21) [*N*-acetylated-α-linked-acidic dipeptidase (NAALADase)] to liberate *N*-acetyl-aspartate (NAA) and glutamate (Stauch *et al*, 1989), and the activity of this enzyme can be inhibited by the recently developed specific inhibitors (Jackson and Slusher, 2001). NAAG itself has been shown to act as a low-potency agonist at NMDA receptors (Koenig *et al*, 1994; Sekiguchi *et al*, 1992; Westbrook *et al*, 1986), and thus, according to these data, an inhibition of its metabolism might result in stimulation of NMDA receptors. However, in many other systems it has been shown to antagonize the effects of NMDA receptor activation (Burlina *et al*, 1994 ; Grunze *et al*, 1996 ; Puttfarcken *et al*, 1993), and therefore, according to these findings, an inhibition of its metabolism would actually inhibit NMDA receptors. Thus, as noted by Yamamoto *et al* (2001a) NAAG acts as an NMDA receptor antagonist at low concentrations but as a low-potency NMDA receptor agonist at high concentrations, and can be regarded as a mixed agonist/antagonist at the NMDA receptor depending on its concentration (Bruno *et al*, 1998; Thomas *et al*, 2000).

In addition, an increase in NAAG concentration may decrease glutamatergic tone mediated by presynaptic mGluRII receptors (mGluR3), because another line of evidence indicates that NAAG is an agonist at mGluRII receptors (Wroblewska *et al*, 1993; Wroblewska *et al*, 1997) with $EC_{50} \sim 26\,\mu M$ (Tortella *et al*, 2000). Lastly, the inhibition of metabolism of NAAG to glutamate could directly produce a reduction of extracellular concentration of glutamate, and via this action may then attenuate the stimulation of both ionotropic and metabotropic receptors for glutamate.

The pharmacological effects of inhibition of GCP II activity have not been investigated until recently, when specific and potent inhibitors of this enzyme were developed. Among them is 2-phosphonomethyl pentanedioic acid (2-PMPA) (Jackson *et al*, 1996) that potently inhibits GCP II activity with an inhibition constant (K_i) of 0.3 nM. 2-PMPA is selective for GCP II with no apparent affinity for over 100 different receptors, ion channels, transporters, and enzymes including several glutamatergic sites such as NMDA, AMPA, metabotropic glutamate receptors, and glutamate transporters (Slusher *et al*, 1999).

As a result of its potency and apparent specificity for GCP II, we used 2-PMPA as a prototype compound to explore the role of GCP II inhibition in the development of morphine tolerance, the expression of morphine dependence, and the acquisition and expression of conditioned reward produced by morphine.

METHODS

Subjects

Male C57/BL mice (IMP, Lodz, Poland), 22–24 g of body weight, were group-housed in the standard laboratory cages and kept in a temperature-controlled colony room ($21 \pm 2^{\circ}C$) with a 12-h light/dark cycle (light on: 07:00, off: 19:00). Commercial food and tap water were available *ad libitum*. Each experimental group consisted of 7–28 mice per treatment. All mice were used only once.

Apparatus for Experiments 1 and 2

A standardized tail-flick analgesia meter (Columbus, Ohio, USA, model 33), adjusted to a sensitivity of '10' with radiant heat source and connected to an automatic timer was used to assess antinociceptive responses. The intensity of the heat stimulus was adjusted so that the baseline tail-flick latency was ~3 s. A maximum latency of 10 s (ie cutoff) was used to minimize damage to the tail. The tail withdrawal latency was measured from the start of heat stimulus until the mouse exhibited a flick of the tail. Each response assessment consisted of two separate measurements taken at different portions of the tail (spaced by 1.5–2 cm) and separated by 15 s. The mean of these responses was used for subsequent comparisons.

Morphine antinociceptive potency was investigated with the use of cumulative dose–response curves that allowed for minimization of the animal number used (Paronis and Holtzman, 1991). After adaptation and baseline trials, each mouse was injected s.c. with a low dose of morphine (1 mg/kg). After 30 min, the mouse was retested and injected with the next dose of morphine that was increased by quarter of a log unit. Thus, because the initial dose of morphine was 1.0 mg/kg, the next dose was 1.78 mg/kg, for a cumulative dose of 2.8 mg/kg. This procedure continued until either the mouse did not move its tail within the cutoff time or until there was a plateauing of the dose–response curve, so that the latency did not increase from one dose to the next. Each

analgesic responder was not subjected to further tail-flick assessments but was injected with the subsequent dose of morphine, so that every animal received the same total dose of morphine during a given test.

Apparatus for Experiment 4

The place-preference apparatus consisted of three rectangular arms ($30 \times 15 \times 20$ cm) spaced at 120° from each other, which were all accessible from a triangular (central) platform (Stinus *et al*, 1990). The apparatus was made of Metaplex and the three arms differed in distinctive visual, tactile, and olfactory cues. Thus, the white arm had a black floor with small holes in it and was marked with peppermint odor, the one black arm with white rough floor was marked with anise odor and, the other black arm with plain black floor had no odor. These distinct cues served as conditioned stimuli (CSs). The use of tactile, texture floor cues allowed mice to be in direct contact with a CS to experience its conditioned effect during preference testing (Vezina and Stewart, 1987 (cf. Cunningham *et al*, 1992)). The guillotine doors, colored according to the respective wall colors, were inserted during conditioning sessions and removed during the pre-test and post-test. The ceiling of the three arms was made of transparent Plexiglass. During testing, mouse location was monitored through a closed circuit TV camera positioned directly above the apparatus. The testing room had dim indirect lighting, comprising two 15 W bulbs positioned about 1 m above the apparatus. A loudspeaker, also positioned above the apparatus, delivered white noise. The apparatus was kept free of urine and faeces; the floors were repeatedly washed and dried.

Apparatus for Experiment 5

The elevated plus maze (Lister, 1987), made of black painted plywood and consisting of two open arms (5×30 cm) and two enclosed arms ($5 \times 30 \times 15$ cm), was used to study the effect of 2-PMPA on learning and memory retrieval in mice. The arms extended from a central platform (5×5 cm). The apparatus was elevated to a height of 50 cm above the floor. The open arms were illuminated with two bright lamps. Testing was carried out in an experimental room supplied with a white noise by an experimenter blind to the treatment conditions.

Experimental Design

Effects on morphine tolerance (Experiment 1) and acute effects in the tail-flick test (Experiment 2) Experiment 1 was carried out to investigate the effect of 2-PMPA on the development of morphine tolerance. On day 1 (test 1), the first measurement of morphine antinociceptive potency was performed, followed by 6 days of b.i.d morphine injections (10 mg/kg, s.c., 7:30 and 17:30) (Elliott *et al*, 1994; Popik *et al*, 2000b). Pretreatment with 2-PMPA (30, 50, or 100 mg/kg, i.p.) or memantine (7.5 mg/kg, s.c., a 'positive control') was given at 30 min prior to each morphine dose on days 2–7. On day 8 (test 2), the second measurement of morphine antinociceptive potency was carried out. The degree of morphine tolerance was assessed by comparing the morphine antinociceptive potencies (cumulative dose–response curves) obtained in tests 1 and 2.

Experiment 2 was designed to determine whether 2-PMPA might itself produce antinociceptive effects and/or affect the antinociceptive effects of morphine. Morphine (1.5 or 3 mg/kg, s.c.) was administered 30 min after injection of 100 mg/kg of 2-PMPA or placebo, administered i.p. The 3 mg/kg dose of morphine corresponds to the antinociceptive ED_{50} dose in these test conditions (data not shown).

Effects on morphine withdrawal (Experiment 3) One week after their arrival to the laboratory, mice started receiving twice daily (b.i.d) s.c. injections of morphine, 30 mg/kg, (7:30 and 17:30) for 3 days as described previously (Popik and Skolnick, 1996). An additional dose of morphine was administered on the morning (07:00 h) of the test day (day 4). One and a half hours later, mice received various doses of 2-PMPA, memantine (7.5 mg/kg, used here as a 'positive control'), or saline (placebo). Then, 30 min later (ie 2 h after the last dose of morphine), mice were challenged with 4 mg/kg of naloxone (or placebo) (all injections via i.p. route). Immediately afterwards, subjects were placed singularly into glass beakers (21 cm of diameter, 41 cm of height). The typical response of morphine-dependent mouse to naloxone is intense jumping, and thus the number of jumps to the height of 7 cm or more was counted for 10 min by an experimenter blind to the treatment conditions.

Since in Experiments 1 and 4 morphine was used at a dose of 10 mg/kg, the possibility that 100 mg/kg of 2-PMPA might differently affect morphine withdrawal produced by 10 mg/kg of morphine was investigated.

Effects on morphine-induced conditioned place preference (CPP) (Experiment 4) The experiment was carried out according to an unbiased procedure and consisted of five phases (days): (1) adaptation, (2) pre-test, (3) conditioning with morphine, (4) conditioning with placebo, and (5) post-test. During the adaptation period, mice were carried into the testing room, weighed and handled by the experimenter by moving animals from one standard home cage to another in the proximity of the apparatus. This adaptation phase was intended to reduce the novelty and stress associated with handling, injections, and exposure to the apparatus. During the pre-test, mice were placed individually on the central triangular platform of the apparatus with free access to all three arms for 20 min. The time spent in each arm and the number of arm entries were recorded. For all mice, the two arms registering the most similar preferences were identified and one was then paired with morphine and the other with placebo. After assigning the arms, there were no significant differences between time spent in the 'to-be' morphine-paired and the placebo-paired arms during the preconditioning phase. This is an important step in the experimental procedure that avoids any preference bias before conditioning (Manzanedo *et al*, 2001).

During conditionings, mice randomly assigned to treatment groups were pretreated with placebo, 2-PMPA (100 mg/kg) or memantine (7.5 mg/kg, a 'positive control'), and injected with morphine (10 mg/kg) or placebo (or, to investigate its putative aversive effects, with 100 mg/kg of 2-PMPA), 20 min before being confined to the respective compartment. The post-test was carried out similarly to the pre-test, with the mice being placed individually on the

central triangular platform of the apparatus with free access to all arms for 20 min following treatment with placebo, 2-PMPA (100 mg/kg), or memantine (7.5 mg/kg). The time spent in each arm and the number of arm entries were recorded. The third arm was visited only during the pre-test and post-test (Mucha and Iversen, 1984).

Effects of 2-PMPA on learning and retrieval of memory (Experiment 5) Although the elevated plus maze has been used mainly in research on anxiety, it also allows investigation of the cognitive effects of drugs. The procedure used was similar to that described by Itoh *et al* (1991). On the first test, mice were individually placed at the end of one open arm facing away from the central platform. The latency of each mouse to find and enter with its four paws one of the enclosed arms was measured (transfer latency 1 [TL1]). Mice were allowed to explore freely the apparatus for the following 10 s. After 24 h, the second test was carried out. As in the first test, mice were individually placed at the end of one open arm facing away from the central platform and the latency of each mouse to enter one of the enclosed arms was measured again (TL 2). After each mouse, the apparatus was cleaned and dried.

To investigate the effects on *learning*, mice were pretreated with placebo, 50 and 100 mg/kg of 2-PMPA, or 0.1 mg/kg of MK-801 (a positive control), 20 min before the first test. To evaluate the effects on *memory retrieval*, mice were treated with 2-PMPA (50 and 100 mg/kg) 20 min before the second test. This closely mimicked the drug treatment of the place preference procedure (see above, Experiment 4). To keep the number and sequence of injections equal among various groups, control mice were treated with placebo instead of active compounds.

Drugs

Morphine HCl (Polfa, Krakow), naloxone HCl, MK-801 [(+)-5-methyl-10,11-dihydroxy-5H-dibenzo(a,d)cyclohepten-5,10-imine maleate] (Sigma-Aldrich St. Louis, MO, USA), and memantine HCl (generous gift of Professor Wojciech Danysz, MERZ & CO, Germany) were dissolved in sterile physiological saline that served as placebo. 2-phosphonomethyl pentanedioic acid (2-PMPA) was synthesized by Guilford Pharmaceuticals as described previously (Jackson *et al*, 1996) and was dissolved in sterile distilled water and the pH was adjusted to 6 ± 0.25 with 1N NaOH. 2-PMPA was stored at -20°C. All drugs were made fresh the day before experiment and stored at 4°C in the refrigerator. The dose of morphine is expressed as the base, the doses of all other compounds as their respective salts. Doses of morphine, naloxone and memantine were used based on our previous observations (Popik and Skolnick, 1996; Popik *et al*, 2000b). All compounds were administered in the volume of 10 ml/kg.

Data Presentation and Statistics

For the morphine tolerance study (Experiment 1), latencies (in s) of the tail-flick responses were converted to maximum possible effects (MPEs) (Paronis and Holtzman, 1991), according to the formula: $100 \times$ [(postinjectory latency−baseline latency)/(cut-off latency−baseline latency)]. MPE values were used to construct morphine cumulative dose–response curves by nonlinear regression; these curves were used to calculate antinociceptive ED_{50} values using GraphPad Prism ver. 3.00 (GraphPad Software, CA, USA) software. The ED_{50} values obtained on tests 1 and 2 were compared among groups, as were the fold shifts (determined by dividing individual test 2 ED_{50} values by the test 1 ED_{50} values) with one-way ANOVAs and *post hoc* Newman–Keul's test.

Effects of 2-PMPA with or without morphine on tail-flick responses (Experiment 2) were compared with the use of area under curve (AUC) assessments on MPE values, which were calculated using trapezoid rule ($\Delta X^*(Y1+Y2)/2$) on a series of measurements from 0 to 120 min. These data were subjected to one-way ANOVA and *post hoc* Newman–Keul's test.

Data from the morphine dependence study (Experiment 3) are expressed as mean $\pm$ SEM number of jumps per 10 min. Statistical analyses involved one way between subjects ANOVA followed by Newman–Keul's test and Student's *t*-test.

To assess the effects of 2-PMPA and memantine on acquisition and expression of morphine-induced conditioned place preference (Experiment 4), statistical analysis involved one-way ANOVA followed by Dunnett's test. The magnitude of CPP was assessed as the % preference (pre-test = 100%, post-test = *X*%, (Popik and Danysz, 1997)).

The effects of 2-PMPA and MK-801 on acquisition and retrieval of memory (Experiment 5) were assessed with one-way ANOVAs and *post hoc* Dunnett's test. The degree of shortening entrance latency is presented for each group by calculating the % difference (TL1 = 100%, TL2 = *X*%).

Ethics

All experiments were carried out according to the National Institutes of Health Guide for Care and Use of Laboratory Animals (publication No. 85-23, revised 1985) and were approved by the Institute of Pharmacology Animal Care and Use Bioethics Commission.

RESULTS

Effects of 2-PMPA on Development of Morphine Tolerance (Experiment 1)

There were no differences in antinociceptive morphine ED_{50} values on test 1 among groups (Table 1). Treatment with 10 mg/kg b.i.d. of morphine produced a 6.44-fold increase in the ED_{50} values as determined on test 2. In contrast, pretreatment with memantine, 50 or 100 (but not 30) mg/kg of 2-PMPA given prior to each dose of morphine attenuated the development of morphine tolerance. The effects of 2-PMPA were related to the dose. This was evidenced by a significant decrease in both test 2 ED_{50} values (statistically significant for the dose 100 mg/kg) and antinociceptive morphine fold shifts of 2-PMPA for the doses of 100 and 50 mg/kg, as compared with the control group that received placebo+morphine (Table 1). Similarly, memantine (7.5 mg/kg) produced an inhibition of morphine tolerance.

Table 1 Effects of 2-PMPA and Memantine on the Development of Tolerance to Morphine

Treatment/dose mg/kg (*N*)	Test 1 ED_{50}	Test 2 ED_{50}	Fold shift
Placebo+morphine (8)	1.49 ± 0.26	8.85 ± 2.22	6.44 ± 1.17
Placebo+placebo (8)	2.23 ± 0.42	3.28 ± 0.47*	1.70 ± 0.29*
2-PMPA 30+morphine (9)	2.00 ± 0.43	9.47 ± 2.13	5.20 ± 1.26
2-PMPA 50+morphine (9)	1.87 ± 0.34	5.41 ± 1.11	3.20 ± 0.66*
2-PMPA 100+morphine (10)	1.59 ± 0.30	3.49 ± 0.83*	2.70 ± 0.57*
Memantine 7.5+morphine (8)	1.51 ± 0.29	3.52 ± 0.88*	2.60 ± 0.49*
ANOVA: F(5,46) =	0.71; ns	3.891; $P<0.01$	4.555; $P<0.01$

Presented are mean ED_{50} values with ± SEM determined during test 1 (pre-morphine) and test 2 (post-morphine) as well as the resulting fold shifts. Asterisks indicate a statistically significant difference compared to the placebo+morphine group that received saline and morphine during the development of morphine tolerance (*$P<0.05$, Newman–Keul's test).

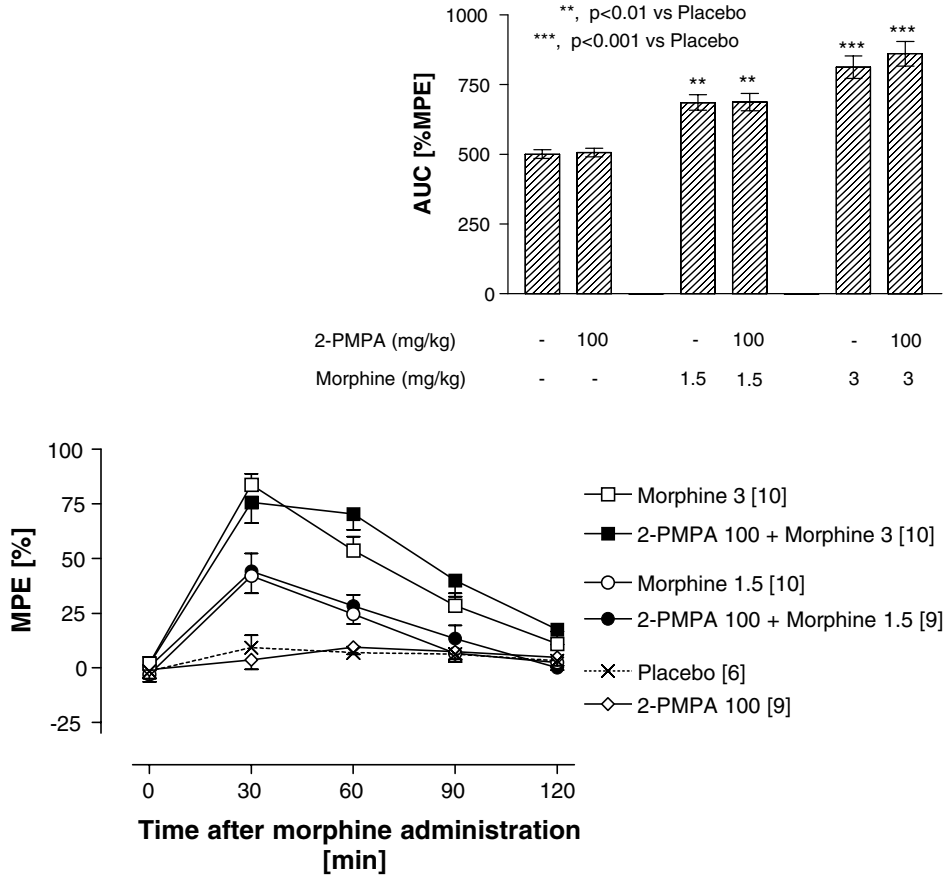

Figure 1 Effects of 2-PMPA on tail-flick responses and on morphine antinociception. Presented are the time courses of tail-flick responses of mice treated with combination of 2-PMPA and morphine. The *N* is given in brackets. *Inset*: Presented are mean ± SEM AUC values calculated on the same data. One way ANOVA $F(5,48) = 19.28$, $P<0.0001$, and *post hoc* Newman–Keul's test revealed that the treatment with placebo+morphine 1.5 mg/kg and with 100 mg/kg 2-PMPA+morphine 1.5 mg/kg differed significantly (**$P<0.01$) from placebo+placebo treatment. Similarly, treatment with placebo+morphine 3 mg/kg and that with 100 mg/kg 2-PMPA+morphine 3 mg/kg differed significantly (***$P<0.001$) from placebo+placebo treatment. Effects of 100 mg/kg of 2-PMPA+placebo treatment did not differ from placebo+placebo treatment. Effects of placebo+respective doses of morphine did not differ from the effects of 2-PMPA+respective doses of morphine.

Effects of 2-PMPA on the Tail-Flick Response and Antinociceptive Effects of Morphine (Experiment 2)

Analysis of AUC revealed that treatment with placebo +1.5 and 3 mg/kg of morphine produced significantly longer tail-flick responses compared to placebo+placebo treatment. In contrast, 100 mg/kg of 2-PMPA+placebo treatment did not affect tail-flick responses as compared to placebo+placebo treatment. Moreover, this dose of 2-PMPA did not affect antinociceptive effects of 1.5 or 3 mg/kg of morphine (Figure 1).

Effects of 2-PMPA and Memantine on Morphine Withdrawal Syndrome (Experiment 3)

Morphine-dependent, naloxone-challenged mice demonstrated robust morphine withdrawal signs (jumps) that were inhibited by 7.5 mg/kg of memantine. 2-PMPA pretreatment at doses 5–50 mg/kg did not affect their severity, however, pretreatment with 100 mg/kg of 2-PMPA potentiated the severity of morphine withdrawal (Table 2). Mice pretreated with 30 mg/kg of morphine and challenged with 2-PMPA (50 or 100 mg/kg) but no naloxone did not exhibit jumps.

Mice treated with 10 mg/kg of morphine, pretreated with placebo, and challenged with naloxone demonstrated severity of withdrawal similar to that produced by 30 mg/kg of morphine. Pretreatment with 100 mg/kg of 2-PMPA did not affect the number of jumps produced by 10 mg/kg morphine treatment (Table 2, bottom).

Effects on Morphine-Induced Conditioned Place Preference (Experiment 4)

One-way ANOVA performed on pretest values demonstrated differences among groups ($P<0.05$) and Dunnett's test detected that the pretest scores of mice treated with 2-PMPA before the pre-test differed (were higher than that) from morphine-only treated mice (Table 3). This suggests that for mice treated with 2-PMPA before the pre-test, it might be more difficult to demonstrate a decrease in morphine-induced CPP.

With regard to the effect of conditioning, control mice that received two injections of placebo during conditioning did not change their preference to the respective arm, because the mean ± SEM difference between pre-test and post-test time (δ) was 4.6 ± 16.6 s and the resulting preference was 104.2%. Morphine produced robust preference towards the morphine-associated arm, since the δ value for this group was 135.9 ± 17.2 s and the resulting preference was 157.8%. 2-PMPA inhibited place preference produced by morphine, because for the groups treated with this compound during acquisition and expression phases, the δ values were 64 ± 13.1 and 51 ± 19.2 s, respectively (for the resulting preferences (%), see Table 3). Similar inhibition was observed for the groups treated with memantine during acquisition and expression phases, since the respective δ values were 35.7 ± 22.7 and 68.4 ± 28.4 s. 2-PMPA given instead of morphine produced neither preference nor aversion toward the arm associated with its administration ($\delta = 36.2 \pm 23$ s).

Table 2 Effects of 2-PMPA and Memantine on Expression of Morphine Dependence

Subchronic treatment/treatment/challenge/(*N*)	Jumps/10 min
Morphine 30/placebo/naloxone (28)	21.4 ± 1.96
Morphine 30/2-PMPA 5/naloxone (9)	23.0 ± 3.47
Morphine 30/2-PMPA 10/naloxone (11)	18.2 ± 2.49
Morphine 30/2-PMPA 50/naloxone (10)	30.0 ± 6.03
Morphine 30/2-PMPA 100/naloxone (10)	39.5 ± 3.91***
Morphine 30/memantine 7.5/naloxone (27)	6.81 ± 0.90***
Morphine 30/2-PMPA 50/placebo (10)	0.0 ± 0.00***
Morphine 30/2-PMPA 100/placebo (10)	0.0 ± 0.00***
ANOVA: F(7,107) =	25.56; $P<0.001$
Morphine 10/placebo/naloxone (8)	24.8 ± 2.77
Morphine 10/2-PMPA 100/naloxone (10)	33.4 ± 3.87[a]

Presented are mean ± SEM number of jumps/10 min observation period of morphine-dependent mice pretreated with placebo, memantine or 2-PMPA 30 min before naloxone challenge. Symbols: ***$P<0.001$: statistically significant from placebo group, Newman–Keul's test:
[a]$t(16) = 1.729$, $P>0.05$, Student's *t*-test *vs* 'morphine 10/placebo/naloxone'.

Effects of 2-PMPA on Learning and Memory (Experiment 5)

One-way ANOVAs performed on difference (%) (but not TL1 values), demonstrated a significant difference among groups (Table 4). Although all treatments resulted in shorter TL2 than TL1, only the decrease of TL2 of mice treated with MK-801 was significantly less than that of Placebo.

DISCUSSION

Effects of GCP II Inhibition on Morphine Tolerance

Considering the impact of inhibition of NAAG metabolism on glutamatergic systems, the attenuation of development of morphine tolerance by 2-PMPA was not unexpected. As early as 1991, Marek *et al* (1991) and Trujillo and Akil

Table 3 Effects of 2-PMPA and Memantine on Acquisition and Expression of Morphine-induced Conditioned Place Preference

Treatment/dose/phase	*N*	Pretest	Posttest	Preference (%)
Morphine	15	247 ± 11.1	383 ± 18.8	157.8 ± 8.1
Placebo	15	268 ± 10.7	273 ± 11.9	104.2 ± 6.8***
2-PMPA 100 on acquisition	7	299 ± 10.8	363 ± 13.7	121.9 ± 4.6*
2-PMPA 100 on expression	8	303 ± 16.8*	354 ± 22.5	117.8 ± 6.2**
Memantine 7.5 on acquisition	8	275 ± 14.1	311 ± 33.6	111.3 ± 7.9**
Memantine 7.5 on expression	10	255 ± 13.2	323 ± 33.9	126.0 ± 11.3*
2-PMPA 100 itself	10	259 ± 17.5	295 ± 29.6	113.8 ± 10.1**
ANOVA F(6,66) =		2.274; $P<0.05$		5.55; $P<0.001$

Presented are mean ± SEM times spent in morphine-associated arm of the apparatus before (pre-test) and after (post-test) conditioning as well as the mean difference between these times for each group (preference (%)) expressed as an average relative % change between pretest (100%) and post-test for each mouse tested. Mice were conditioned to the effects of morphine (10 mg/kg) and treated with 2-PMPA (100 mg/kg) or memantine (7.5 mg/kg) during conditionings or before the post-test to investigate the effects on acquisition and expression, respectively. Symbols: *$P<0.05$; **$P<0.01$; ***$P<0.001$ *vs* morphine group (Dunnett's test).

Table 4 Effects of 2-PMPA on Acquisition and Retrieval of Memory

Treatment/dose/phase	*N*	TL 1 (s)	TL 2 (s)	Difference (%)
Placebo	20	50.75 ± 4.82	13.70 ± 1.71	29.3 ± 3.85
2-PMPA 50 acquisition	7	52.29 ± 8.83	11.71 ± 3.34	32.3 ± 12.79
2-PMPA 100 acquisition	7	50.00 ± 12.93	14.00 ± 4.95	27.5 ± 5.83
2-PMPA 50 retrieval	7	57.43 ± 10.66	13.43 ± 5.42	24.5 ± 8.78
2-PMPA 100 retrieval	7	59.71 ± 11.17	15.29 ± 3.23	40.9 ± 14.90
MK-801 0.1 acquisition	12	50.33 ± 7.58	39.66 ± 9.43	101.9 ± 35.85*
ANOVA F(5,54) =		0.204 $P>0.05$		2.76; $P<0.05$

Presented are mean ± SEM latencies (s) to enter the enclosed arm of the apparatus during the first (transfer latency 1 [TL 1]) and second (transfer latency 2 [TL 2]) tests, as well as the mean difference between these times for each group (Difference (%)) expressed as an average relative % decrease between TL 1 (100%) and TL 2 for each mouse tested. Statistical analyses involved one-way ANOVAs performed on TL 1 and difference (%). Significant difference compared to "Placebo" treatment is indicated as *$P<0.05$; Dunnett's test. Except for MK-801 treatment, other treatments did not produce significant effects compared to Placebo control.

(1991) demonstrated the inhibitory effects of kynurenic acid and MK-801 on the development of tolerance to the antinociceptive effects of morphine. Numerous subsequent studies revealed that coadministration of either competitive or noncompetitive NMDA receptor antagonists attenuate and/or reverse the development of tolerance to the antinociceptive effects of morphine. In the mouse, such effects have been shown for a number of NMDA receptor antagonists, including memantine (Lutfy *et al*, 1993; Elliott *et al*, 1994; Bilsky *et al*, 1996; Belozertseva and Bespalov, 1998; Gonzalez *et al*, 1997; Popik *et al*, 2000a). Similarly, the stimulation of presynaptic mGluRII receptors with (+)-2-aminobicyclo [3,1,0] hexane-2,6-dicarboxylic acid (LY354740) was shown to inhibit the development of morphine tolerance (Popik *et al*, 2000b). In the light of these findings, the inhibitory effect of 2-PMPA on morphine tolerance may be explained by its effects at NMDA, mGluRII, or both receptors.

Moreover, the results of Experiment 2 indicate that 2-PMPA, at the dose effectively inhibiting development of morphine tolerance, produced no antinociceptive effect in the tail-flick test, which is in agreement with a recent report of Yamamoto *et al* that showed lack of an antinociceptive effect of 2-PMPA in another acute pain (hot plate) test (Yamamoto *et al*, 2001b).

Effects of GCP II Inhibition on Morphine Dependence/Withdrawal

In the light of studies demonstrating that inhibition of GCP II activity inhibits glutamatergic neurotransmission, the results of Experiment 3 are unexpected and somewhat counterintuitive. This is because the expression of opioid withdrawal has been frequently shown to be inhibited by NMDA receptor antagonists and compounds that inhibit mGluR-mediated neurotransmission. Thus, attenuation of the intensity of morphine withdrawal has been reported after acute pretreatment with high- and low-affinity NMDA receptor uncompetitive antagonists, competitive antagonists, glycine, and polyamine site antagonists, as well as NMDAR1 subunit antisense oligonucleotides (Belozertseva *et al*, 2000; Bristow *et al*, 1997; Zhu and Ho, 1998; Layer *et al*, 1996; Popik and Skolnick, 1996; Farzin, 1999; Popik and Danysz, 1997; Brent and Chahl, 1993; Gonzalez *et al*, 1997). These studies have assessed various classical somatic and autonomic signs of withdrawal, as well as biochemical markers (Bristow *et al*, 1997; Rasmussen, 1995). Ability of NMDA receptor antagonists to block the expression of opioid withdrawal is consistent with the frequently reported increase in glutamate release in opioid-withdrawn animals (Sepulveda *et al*, 1998) and facilitation of the withdrawal signs by glutamate receptor agonist administration (Tokuyama *et al*, 1996). Similarly, stimulation of mGluRII receptors with (+)-2-aminobicyclo [3,1,0] hexane-2,6-dicarboxylic acid (LY354740) was shown to inhibit the expression of morphine dependence (Klodzinska *et al*, 1999; Vandergriff and Rasmussen, 1999).

The results of Experiment 3, indicating no effect of 2-PMPA on morphine withdrawal or even its intensification with the use of a relatively high dose, suggest that the inhibition of activity of GCP II produces a unique effect on the consequences of repeated exposure to morphine (inhibition of tolerance but not dependence). As mentioned earlier, Yamamoto *et al* (2001a) suggested that NAAG acts as an NMDA receptor antagonist at low concentrations, but as a low potency NMDA receptor agonist at high concentrations. If this hypothesis were correct, it could be speculated that administration of a high dose (100 mg/kg) of 2-PMPA leads to high concentrations of NAAG that acts as an agonist, rather than an antagonist at NMDA receptors, and thus increases the severity of morphine withdrawal. In support, it should be noted that during opioid withdrawal there is a massive release of glutamate (Rasmussen, 1995; Jhamandas *et al*, 1996; Sepulveda *et al*, 1998). The effects of massive glutamate release apparently may not be prevented by NAAG-induced mGluRII stimulation and/or a decrease in glutamate release. Such stimulation of glutamate receptors could increase the severity of morphine withdrawal as was reported for glutamate receptor agonists (Tokuyama *et al*, 1996). Interestingly, above a certain threshold (5–10 mg/kg), the dose of morphine used to produce dependence appears to have little effect on the intensity of opioid withdrawal (Popik *et al*, 1998), which Experiment 3 seems to confirm. However, in mice in which dependence was produced with 10 mg/kg of morphine, 100 mg/kg of 2-PMPA did not intensify opioid withdrawal, which suggests that this effect is indeed related to the intensity of neurochemical events associated with the withdrawal state. Martin *et al* (1997a, b) reported that glutamate release is attenuated by agonists selective

for μ-opioid receptors and mGluRII receptors located presynaptically, and that this inhibitory effect is further potentiated in morphine-tolerant animals (Martin *et al*, 1999). It remains to be assessed if the differential effects of a GCP II inhibitor on morphine tolerance and dependence may be explained solely by the concentration of glutamate. Interestingly, a similar set of effects was recently reported for another indirect inhibitor of glutamate availability, the glutamate transporter activator, (R)-(-)5-methyl-1-nicotinoyl-2-pyrazoline (MS-153) (Nakagawa *et al*, 2001). As in our report, these authors found inhibitory effects of MS-153 on the development of morphine tolerance, but no effect on morphine antinociceptive actions or expression of morphine dependence.

Alternatively, the results of Experiments 1 and 3 may suggest that morphine tolerance and dependence can be separated, at least at the level of glutamatergic neurotransmission. This has been earlier shown in behavioral (Rahman *et al*, 1994; Paronis and Woods, 1997a, b; Gold *et al*, 1994) and neurochemical (Lang and Schulz, 1989; Gudehithlu and Bhargava, 1996; Aley and Levine, 1997) studies and experiments with the use of genetically modified mice (Kest *et al*, 2001; Bohn *et al*, 2000).

Effects of GCP II Inhibition on Morphine-Induced Conditioned Reward and Learning and Memory

In our study, only one conditioning session with morphine in the compartment distinguished by visual, tactile, and olfactory cues was sufficient to induce significant preference to this compartment, as has been reported by other investigators (Mucha *et al*, 1982; Bardo and Neisewander, 1986). It has been demonstrated that use of multiple stimuli as cues in the CPP procedure produces stronger conditioning, as opposed to the procedure based on single modality stimulus (Mucha *et al*, 1982; Barr *et al*, 1985). This method of establishing CPP using only one drug pairing corresponds better to the literature on humans indicating that the initial drug experience is an important factor contributing to later drug use (Haertzen *et al*, 1983). Moreover, it eliminates other possible confounding factors like tolerance to the rewarding effect of morphine (Shippenberg *et al*, 1988).

2-PMPA, at the one, selected dose that significantly affected morphine tolerance and withdrawal inhibited both acquisition and expression of morphine-induced conditioned place preference. This effect resembles the effect of memantine (and other NMDA receptor antagonists, see the Introduction section). Slusher *et al*, have recently demonstrated that 2-PMPA as well as another GCP II inhibitor, GPI 5693, inhibited both the acquisition and the expression of cocaine-induced CPP in rats (Slusher *et al*, 2001).

There are various possible interpretations to the effects of compounds on place conditioning. For example, it has been suggested that if a compound by itself produces conditioned aversion, it may attenuate CPP not because of a specific effect on conditioned reward but because subjects may associate an aversive state with the rewarded arm of the apparatus (Carr *et al*, 1989). In this regard, data from Experiment 4 demonstrate that an effective dose of 2-PMPA did not produce any aversive or rewarding effect by itself when given 20 min before the conditioning, which agrees with observations in rats (Slusher *et al*, 2001) indicating no effect of 2-PMPA on place conditioning. The dose of memantine used in the present experiment (7.5 mg/kg), that inhibited conditioned morphine reward was also shown not to produce conditioned place preference or aversion (Popik and Danysz, 1997). Moreover, since it is known that the effects of NMDA receptor antagonists on morphine-induced CPP are not because of the state-dependent learning (Tzschentke, 1998), it may be speculated that the effects of 2-PMPA were also probably not because of the state-dependent learning phenomenon.

Another potentially confounding factor could be a direct effect on locomotor activity. Our CPP procedure involved recording the number of visits to arms (a rough measurement of locomotor activity). We found (data not shown) that 2-PMPA did not change the locomotion of mice, which agrees with similar observations in rats (Shippenberg *et al*, 2000). The unaffected locomotion following 2-PMPA administration is an important issue in interpreting its effects on expression of morphine-induced CPP as it assures that 2-PMPA-treated mice did not simply enter one of the compartments and remain there because their locomotor function was impaired.

Cognitive effects of a compound that modulates conditioned reward should also be carefully considered. For instance, if a compound produced learning or memory impairment, its inhibitory effects on conditioned drug reward would be considered unspecific. This issue has frequently been raised for the effects of NMDA receptor antagonists (Bisaga and Popik, 2000). To assess the effects of 2-PMPA on acquisition and/or retrieval of memory, we used the elevated plus maze model of spatial learning in mice (Itoh *et al*, 1991). This paradigm is sensitive to the amnesic effects of post-training administration of scopolamine or electroconvulsive shock (Itoh *et al*, 1990), pretraining effects of scopolamine, MK-801 and diazepam (Itoh *et al*, 1991), and pretest effects of MK-801 and scopolamine (Hlinak and Krejci, 2000). Results of Experiment 5 demonstrating amnesic effects of MK-801 confirm earlier observations (Itoh *et al*, 1991); however, we found that in contrast to MK-801, 2-PMPA (50 and 100 mg/kg) affected neither learning nor retrieval of memory. The lack of effects on learning and memory agrees with earlier observations (Slusher *et al*, 1999) and suggests that the effect of 2-PMPA on acquisition and expression of conditioned morphine reward were not because of its effects on remembering or retrieval of information.

In conclusion, the present study demonstrates that inhibition of activity of GCP II by 2-PMPA inhibits the development of tolerance to the antinociceptive effects of morphine without affecting the nociceptive response *per se* or interfering with morphine antinociception. Moreover, inhibition of activity of GCP II by 2-PMPA leaves the expression of morphine dependence unaffected (or potentiates it when used at the highest dose). In addition, 2-PMPA inhibited both the acquisition as well as expression of morphine-induced conditioned place preference. In the light of the recent data demonstrating that inhibition of GCP II results in reduction of alcohol intake in rats (Mckinzie *et al*, 2000) and attenuation of cocaine sensitization (Shippenberg *et al*, 2000), our findings indicate the

therapeutic potential of GCP II inhibition in the management of chronic pain and opioid addiction.

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