0022-3565/05/3152-949–958$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
JPET 315:949–958, 2005

Vol. 315, No. 2
91553/3056726
Printed in U.S.A.

Glycyl-Glutamine, an Endogenous β-Endorphin-Derived Peptide, Inhibits Morphine-Induced Conditioned Place Preference, Tolerance, Dependence, and Withdrawal

Sinan Cavun, Gökhan Göktalay, and William R. Millington

Department of Basic and Pharmaceutical Sciences, Albany College of Pharmacy, Union University, Albany, New York (S.C., G.G., W.R.M.); Ion Technologies, Inc., Winston-Salem, North Carolina (G.G.); and Department of Pharmacology and Clinical Pharmacology, Uludag University School of Medicine, Bursa, Turkey (S.C., G.G.)

Received June 23, 2005; accepted July 28, 2005

ABSTRACT

Glycyl-glutamine (Gly-Gln; β-endorphin$_{30-31}$) is an endogenous dipeptide synthesized from β-endorphin$_{1-31}$. Previous investigations have shown that Gly-Gln inhibits the cardiovascular and respiratory depression caused by morphine and β-endorphin$_{1-31}$, but it does not interfere with opioid analgesia. In this study, we tested whether Gly-Gln administration would influence morphine-induced conditioned place preference, tolerance, dependence, or withdrawal. For place preference experiments, rats were conditioned with morphine sulfate (2.5 mg/kg i.p.) or saline on alternate days for 6 days and tested on day 7. Glycyl-glutamine (1–100 nmol i.c.v.) pretreatment inhibited acquisition of a conditioned place preference to morphine significantly. Glycyl-glutamine (100 nmol i.c.v.) also blocked expression of a pre-established morphine place preference, but it did not interfere with acquisition of a conditioned place preference to palatable food, and it did not produce place preference or aversion when given alone to morphine-naive animals. To induce antinociceptive tolerance, rats were treated with morphine (10 mg/kg i.p.) twice daily for 7 days, and morphine antinociception was evaluated with the tail-flick test. Glycyl-glutamine (100 nmol i.c.v.) pretreatment delayed the onset of morphine tolerance significantly and partially reversed pre-established tolerance. Morphine dependence and withdrawal were assessed by measuring naloxone-precipitated withdrawal symptoms. Glycyl-glutamine inhibited the development of morphine dependence when given to rats twice daily immediately before they received morphine (10 mg/kg i.p.) and suppressed withdrawal symptoms of rats with subcutaneously implanted morphine pellets when administered 5 min before withdrawal was induced with naloxone. Glycyl-glutamine thus attenuates morphine-induced conditioned place preference, tolerance, dependence, and withdrawal without compromising morphine analgesia.

Soon after the discovery of β-endorphin$_{1-31}$ nearly 30 years ago, a series of structurally related β-endorphin peptides were isolated from mammalian brain and pituitary (Zakarian and Smyth, 1982). Subsequent work revealed that virtually every tissue that expresses the proopiomelanocortin (POMC) gene converts some or all the β-endorphin$_{1-31}$ it synthesizes to carboxy-terminal-truncated and amino-terminal-acetylated derivatives (Smith and Funder, 1988). For the most part, these β-endorphin$_{1-31}$-derived peptides are biologically inactive, although one, β-endorphin$_{1-27}$, displays opioid receptor antagonist activity and blocks β-endorphin$_{1-31}$-induced antinociception (Deakin et al., 1980; Nicolas and Li, 1985; Smith et al., 1992; Tseng, 2001). It is not really known why some neurons convert β-endorphin$_{1-31}$ to opioid-inactive and receptor antagonist derivatives, although POMC serves as the precursor for a number of other peptides, and so it may provide a mechanism for converting POMC neurons from an opioid to a nonopioid phenotype (Raffin-Sanson et al., 2003). Alternatively, nonopioid β-endorphin peptides may function as neurotransmitters with, as yet, unknown functions.

A dipeptide, glycyl-glutamine (Gly-Gln; β-endorphin$_{30-31}$), is also produced when β-endorphin$_{1-31}$ is converted to β-endorphin$_{1-27}$ (Parish et al., 1983). Glycyl-glutamine is the single most abundant β-endorphin-related peptide produced in some brain regions and most peripheral tissues that synthesize POMC, although relatively little is know about its physiological functions (Parish et al., 1983; Smith and Funder, 1988). Early studies showed that Gly-Gln is an inhibitory peptide that reduces the firing frequencies of brainstem neu-

This study was supported by Grant DA018029 from the National Institute on Drug Abuse.
Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.105.091553.

ABBREVIATIONS: POMC, proopiomelanocortin; Gly-Gln, glycyl-glutamine; Gly-D-Gln, glycyl-D-glutamine; %MPE, maximal possible effect; ANOVA, analysis of variance; NMDA, *N*-methyl-D-aspartate.

rons after iontophoretic application (Parish et al., 1983) and produces trophic (Koelle et al., 1988; Lotwick et al., 1990) and immune (McCain et al., 1987) effects in peripheral tissues. The simple observation that Gly-Gln is synthesized with β-endorphin$_{1\text{-}27}$, an opioid receptor antagonist, prompted us to investigate whether it might also influence the pharmacological effects of opioids. We found that Gly-Gln effectively inhibited the hypotension and respiratory depression caused by β-endorphin$_{1\text{-}31}$ or morphine, although it did not affect cardiovascular or respiratory function when given alone to otherwise untreated animals (Unal et al., 1994, 1997; Owen et al., 2000). By contrast, Gly-Gln had no effect at all on the antinociception evoked by opioids in the tail-flick and paw-lift assays, even at doses over 100-fold higher than required to inhibit morphine-induced respiratory depression (Owen et al., 2000). This latter finding implies that Gly-Gln is unlikely to act as an opioid receptor antagonist, as β-endorphin$_{1\text{-}27}$ does, a conclusion supported by evidence from radioreceptor binding experiments (Unal et al., 1994). These data suggest that some POMC neurons release two peptides, β-endorphin$_{1\text{-}27}$ and Gly-Gln, capable of modulating the effects of β-endorphin$_{1\text{-}31}$, albeit through different receptor mechanisms.

In this study, we tested whether Gly-Gln inhibits morphine-induced conditioned place preference, tolerance, dependence, or withdrawal. Conditioned place preference is widely used to study the incentive or rewarding properties of drugs and other stimuli (Tzschentke, 1998). Morphine and other opioids produce a robust conditioned place preference response after relatively short treatment periods (Reid et al., 1989; Tzschentke, 1998). Here, we report that Gly-Gln pretreatment prevents the acquisition and expression of a place preference response to morphine without causing place preference or place aversion in morphine-naive animals. Glycyl-glutamine administration also inhibited development of morphine tolerance and dependence and suppressed naloxone-precipitated withdrawal symptoms. Together with previous data, these findings support the idea that Gly-Gln inhibits the adverse effects of opioids without compromising opioid analgesia.

Materials and Methods

Animals and Surgical Procedures. Male Sprague-Dawley rats (250–300 g; Charles River Breeding Laboratories, Wilmington, MA) were housed in plastic cages in a climatically controlled animal room under a 12-h light/dark cycle with free access to food and water. Animals were allowed to acclimate to the animal care facility for 3 days before surgery. Each rat was anesthetized with 4% halothane and maintained with 1.5% halothane in 100% O_2, mounted in a stereotaxic frame, and a 23-gauge stainless steel guide cannula (Plastics One, Roanoke, VA) was implanted in the left lateral ventricle for intracerebroventricular (i.c.v.) drug injections. The tip of the guide cannula was positioned 1.3 mm lateral and 0.9 mm posterior to bregma and 4.0 mm below the skull surface, and the cannula was fixed to the skull with dental acrylic. Animals were allowed to recover from surgery for 1 week before experimentation. The animal protocols were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

For i.c.v. injections, a 28-gauge stainless steel injection cannula was connected to a 5-μl Hamilton syringe with polyethylene tubing and inserted through the guide cannula. Glycyl-glutamine was dissolved in 5 μl of 0.9% saline, pH 7.3, and injected at a constant rate over a 1-min period. The injection volume was monitored by observing the movement of an air bubble placed in the tubing, and the injection cannula was maintained in the guide cannula for 30 s after drug or saline administration was complete. At the end of each experiment, cannula placements were verified with India ink (5 μl). Only data from animals with correct cannula placement were used for statistical analysis.

Conditioned Place Preference. Place preference conditioning was conducted in a three-chambered apparatus (Tzschentke, 1998; Nores et al., 1999; Slusher et al., 2001). The two conditioning chambers were identical in size (45 $\times$ 45 $\times$ 30 cm) but were distinguishable both visually and tactually. The interior of one chamber was entirely white and the Plexiglas floor was covered with wood chips; the other chamber was painted with white and black vertical stripes and had no wood chips on the floor. The conditioning chambers were connected through sliding doors to a third compartment (45 $\times$ 25 $\times$ 30 cm) painted a neutral gray, with a Plexiglas floor without wood chips, which served as the entrance to the conditioning chambers during place preference testing. Rats did not display a preference for either chamber and, when allowed to freely explore the two chambers for 15 min, spent essentially the same amount of time in the white chamber (266 $\pm$ 16 s) as the striped chamber (251 $\pm$ 25 s; n = 8). The conditioned place preference procedure consisted of three components: habituation, conditioning, and testing.

Habituation. The animals were transported to the testing room and handled daily for three consecutive days to habituate them to the testing room and human handling.

Conditioning. Sessions were performed once daily for 6 days. Half of the animals were conditioned in the striped chamber and half in the white chamber, and they were randomly assigned to both the conditioning chambers and treatment groups. The rats were treated with morphine sulfate (2.5 mg/kg i.p.; Elkins-Sin, Cherry Hill, NJ) on days 1, 3, and 5 and were confined in one of the two conditioning chambers for 30 min. They were treated with saline (1 ml/kg i.p.) on days 2, 4, and 6 and were confined in the alternate chamber for 30 min. Control animals received saline i.p. before all six conditioning sessions; one of the two chambers was designated as the "primary" conditioning chamber.

Testing. Sessions were conducted on day 7 approximately 24 h after the final conditioning period. On the test day, each rat was placed in the entrance chamber and allowed to move freely between the two conditioning chambers for 15 min, and the amount of time it spent in each chamber was recorded. Data are reported as the amount of time spent in the morphine-paired chamber minus the time spent in the saline-paired chamber. For saline-treated controls, data represent the time spent in the primary conditioning chamber minus the time spent in the alternate saline-paired chamber.

To test the effect of Gly-Gln on acquisition of a morphine-induced conditioned place preference, rats were randomly assigned to one of four treatment groups: saline + saline, Gly-Gln + saline, saline + morphine, and Gly-Gln + morphine. On days 1, 3, and 5, the animals were given Gly-Gln (1–100 nmol; Sigma-Aldrich, St. Louis, MO) or saline (5 μl) i.c.v. followed 2 min later by morphine sulfate (2.5 mg/kg) or saline i.p. and were confined in the appropriate conditioning chamber for 30 min. On days 2, 4, and 6, all animals were given saline and were placed in the alternate chamber for 30 min. To test whether Gly-Gln influences the expression of a conditioned place preference to morphine, rats were conditioned with morphine or saline and given a single dose of Gly-Gln (100 nmol) or saline i.c.v. immediately before place preference testing.

Place preference conditioning for food was conducted as described above except that the conditioning substance was food rather than morphine. The normal diet of the animal was restricted to five pellets per day for 5 days before and throughout the duration of the conditioning and test sessions (Slusher et al., 2001). They were conditioned with the breakfast cereal Fruit Loops (30 pieces), which rats consume eagerly and which generates a place preference response reproducibly (Slusher et al., 2001). Rats were randomly assigned to one of four treatment groups: saline + food, Gly-Gln + food, saline +

no food, and Gly-Gln + no food. On days 1, 3, and 5, rats were treated with either Gly-Gln (100 nmol) or saline i.c.v. and were placed in a chamber containing food (designated as the food-paired chamber) or no food (designated as the no-food-paired chamber) for 30 min. On days 2, 4, and 6, all animals were treated with saline i.c.v. and were confined to the opposite chamber, which contained no food, for 30 min. On the test day, the animals were placed in the entrance chamber of the conditioning apparatus with no drug treatment or food presentation, they were allowed to move freely within the two conditioning chambers for 15 min, and the time spent in each chamber was recorded.

Tolerance. Rats were treated with morphine sulfate (10 mg/kg i.p.) twice daily between 7:00 and 8:00 AM and between 6:00 and 7:00 PM for 7 days, and nociceptive response latencies were measured with the tail-flick reflex test. Tail-flick latencies were determined before and 30 min after each morning morphine treatment. After measuring baseline tail-flick latencies, Gly-Gln (100 nmol) or saline was administered i.c.v. followed 2 min later by morphine. The cut-off time was set at 10 s to prevent tissue damage. Tail-flick latencies were converted to percentage of maximal possible effect (%MPE) by using the formula %MPE = (postdrug latency − baseline latency)/(cut-off latency − baseline latency).

Dependence. Morphine dependence was evaluated by measuring naloxone-precipitated withdrawal symptoms (Gellert and Holtzman, 1978). Dependence was produced by treating rats with morphine sulfate by i.p. injection, rather than subcutaneous pellet implantation, to more closely parallel the presumed time course of Gly-Gly after i.c.v. injection. Accordingly, rats were treated with Gly-Gln (100 nmol) or saline i.c.v. followed, 2 min later, by morphine sulfate (10 mg/kg i.p.) twice daily for 6 days. On day 7, they were treated with morphine and, 4 h later, withdrawal was precipitated with naloxone hydrochloride (4 mg/kg i.p.; Sigma-Aldrich). Animals were observed for 20 min and scored for a series of eight withdrawal symptoms (wet dog shakes, stretching, grooming, sniffing, rearing, teeth chattering, ptosis, and diarrhea) (Chen et al., 2003). At the end of the experiment, the individual scores were combined and divided by the number of withdrawal symptoms to derive a mean total withdrawal score. The observer was "blind" to drug administration.

Withdrawal. Morphine withdrawal was investigated by using a modification of the procedure used to study dependence. Rats were rendered dependent on morphine by using sustained release morphine pellets to avoid the necessity of daily injections and because morphine pellet implantation reportedly produces more intense withdrawal behavior than twice daily morphine injections (Cicero and Meyer, 1973). The behavioral scale was also modified to include withdrawal signs that could be evaluated more objectively and reproducibly. Rats were anesthetized with halothane and a 75-mg sustained release morphine pellet wrapped in nylon mesh (pore size 0.3 mm; Small Parts, Inc., Miami Lakes, FL) was implanted subcutaneously between the scapulas. Controls were implanted with placebo pellets. Seventy-two hours after pellet implantation, the animals were administered Gly-Gln (1–300 nmol) or saline i.c.v. and withdrawal was precipitated 5 min later with naloxone hydrochloride (1 mg/kg s.c.). Immediately after naloxone administration, each rat was placed in a transparent observation cage and withdrawal signs were scored at 5-min intervals for 40 min. Withdrawal signs were divided into two classes, graded signs (wet dog shakes and weight loss) and checked signs (teeth chattering, diarrhea, defecation, ptosis, penile erection, and mouth movements) using a modification of a scale originally described by Gellert and Holtzman (1978). Weight loss was measured by weighing the animals immediately before and 2 h after naloxone injection. A mean withdrawal score was calculated by adding the scores for individual withdrawal symptoms and dividing the total withdrawal score by the total number of signs. The observer was blind to treatment protocol.

Data Analysis. Data are expressed as the mean ± S.E.M. and were analyzed by analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. Paired data were analyzed by two-tailed Student's t test. The criterion for statistical significance was $P < 0.05$.

Results

Morphine-Induced Conditioned Place Preference. To produce a morphine-conditioned place preference, rats were treated with morphine sulfate (2.5 mg/kg i.p.) or saline on alternate days for 6 days and confined to a conditioning chamber for 30 min; control animals were conditioned with saline on all 6 days. Morphine conditioning produced a significant place preference. After 6 days of conditioning, morphine-treated rats spent significantly more time in the morphine-paired chamber than in the saline-paired chamber when allowed to freely explore the two chambers during testing on day 7 (Fig. 1). A lower morphine dose (1.25 mg/kg i.p.) also produced a significant, albeit less robust, place preference response (165 ± 66 s; $n = 5$). Control animals conditioned with saline on all 6 days did not display a preference for either chamber when tested on day 7 (Fig. 1). These data confirm that morphine produces a conditioned place preference response as shown in many previous investigations (Reid et al., 1989; Tzschentke, 1998).

The effect of Gly-Gln on the acquisition of a place preference to morphine was tested by pretreating rats with Gly-Gln (100 nmol) or saline i.c.v. 2 min before they were given morphine sulfate (2.5 mg/kg) or saline i.p. on days 1, 3, and 5 of place preference conditioning; all animals received saline on days 2, 4, and 6. Glycyl-glutamine pretreatment inhibited the acquisition of a morphine conditioned place preference completely [$F(3,18) = 15.4$; $P < 0.001$] (Fig. 1). Animals that were pretreated with Gly-Gln (100 nmol i.c.v.) before they received morphine failed to develop a significant place preference response and spent approximately the same amount of time in the two chambers during place preference testing on day 7 (Fig. 1). It is noteworthy that rats treated with

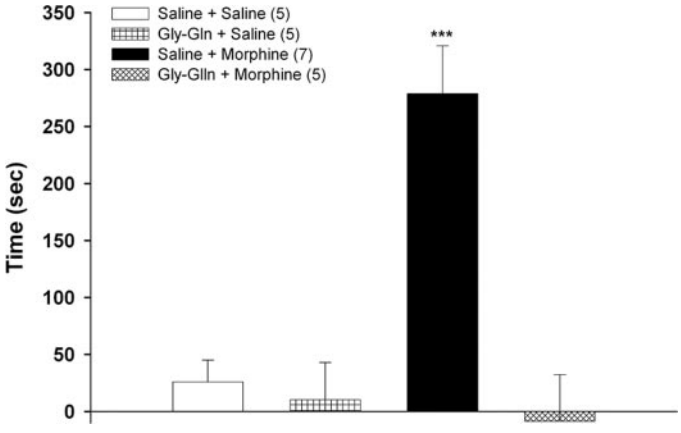

Fig. 1. Glycyl-glutamine pretreatment prevents acquisition of a conditioned place preference to morphine. Rats were conditioned with morphine or saline on alternate days for 6 days. On days 1, 3, and 5, they were treated with Gly-Gln (100 nmol) or saline (5 μl) i.c.v. followed 2 min later by morphine sulfate (2.5 mg/kg) or saline i.p. and were confined to a conditioning chamber for 30 min; on days 2, 4, and 6, all animals received saline and were confined to the alternate chamber for 30 min. The columns represent the difference in the amount of time (mean ± S.E.M.) animals spent in the two conditioning chambers during place preference testing on day 7. The numbers in parentheses indicate the number of animals in each group. Data were analyzed by ANOVA followed by the Student-Newman-Keuls test. ***, $P < 0.001$ differs from saline-treated controls.

Gly-Gln i.c.v. followed by saline i.p., in lieu of morphine, did not show a preference for either chamber during place preference testing (Fig. 1). Glycyl-glutamine (100 nmol i.c.v.) also inhibited acquisition of a morphine conditioned place preference using a biased procedure (Tzschentke, 1998) in which animals showed a clear preference for one of the two conditioning chambers [saline + saline, −137 ± 70 s; saline + Gly-Gln, −127 ± 75 s; morphine + saline, 315 ± 85 s; morphine + Gly-Gln, −29 ± 38 s; $n = 6–9$; [$F(1,26) = 7.7$; $P < 0.01$]]. Glycyl-glutamine thus inhibits acquisition of a place preference to morphine in both biased and unbiased procedures without inducing place preference or aversion when given alone to otherwise untreated animals.

Dose-Response and Control Experiments. In an earlier study, we found that Gly-Gln inhibited morphine-induced respiratory depression at doses considerably lower than the 100-nmol dose shown here to block acquisition of morphine place preference (Owen et al., 2000). To test whether Gly-Gln is effective in the same dose range, dose-response experiments were conducted with lower Gly-Gln doses. Rats were pretreated with Gly-Gln (1, 10, 30, or 100 nmol) i.c.v. 2 min before they received morphine (2.5 mg/kg) i.p. during place preference conditioning on days 1, 3, and 5; once again, all rats were conditioned with saline on days 2, 4, and 6. Figure 2 shows that Gly-Gln inhibited morphine place preference significantly at doses between 1 and 100 nmol i.c.v. [$F(4,23) = 9.2$; $P < 0.001$].

One important caveat to the conclusion that Gly-Gln inhibits morphine place preference is the possibility that it may be hydrolyzed to glycine and glutamine after i.c.v. injection. This is an important consideration because glycine itself is a neurotransmitter. To test this, rats were pretreated with glycine and glutamine (100 nmol of each amino acid) i.c.v. 2 min before i.p. morphine (2.5 mg/kg) or saline administration. Once again, morphine administration produced a significant place preference response (Table 1). Combined treatment with glycine and glutamine had no significant effect on the acquisition of a conditioned place preference to morphine and did not produce place preference or aversion when given to rats that received saline i.p. instead of morphine (Table 1). The Gly-Gln stereoisomer glycyl-D-glutamine (Unal et al., 1994) also failed to influence the acquisition of a morphine place preference and was inactive when given alone to saline-treated control animals (Table 1). These data indicate that neither Gly-Gln hydrolysis nor nonspecific peptide effects are likely to explain the ability of Gly-Gln to inhibit acquisition of a morphine-conditioned place preference.

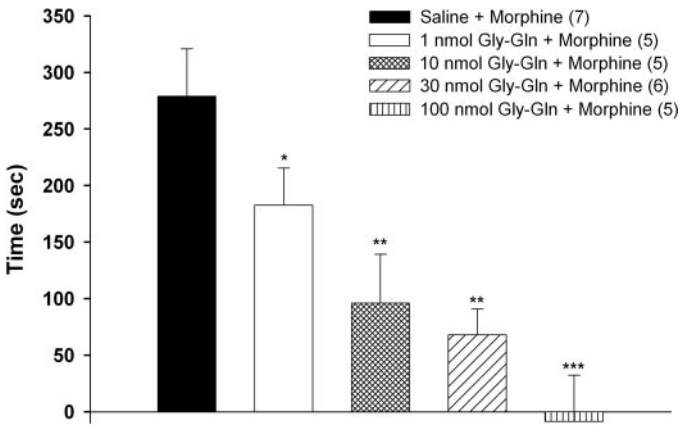

Fig. 2. Dose-response effect of Gly-Gln on the acquisition of a conditioned place preference to morphine. Rats were treated with the indicated dose of Gly-Gln or saline i.c.v. followed 2 min later by morphine (2.5 mg/kg i.p.) during place preference conditioning on days 1, 3, and 5 and were conditioned with saline on days 2, 4, and 6. The columns represent the amount of time spent in the morphine-paired chamber minus the time spent in the saline-paired chamber during place preference testing on day 7. Data were analyzed by ANOVA followed by Student-Newman-Keuls test. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ differs from saline + morphine-treated animals.

TABLE 1

Pretreatment with glycine and glutamine or glycyl-D-glutamine does not inhibit acquisition of a place preference to morphine

Rats were conditioned with morphine or saline on alternate days for 6 days. On days 1, 3, and 5 of place preference conditioning, they were treated with saline, glycine (Gly) and glutamine (Gln) (100 nmol each amino acid), or glycyl-D-glutamine (Gly-D-Gln; 100 nmol) i.c.v. followed 2 min later by saline or morphine sulfate (2.5 mg/kg) i.p. and were confined in a conditioning chamber for 30 min. On days 2, 4, and 6, they were treated with saline i.p. and confined to the alternate conditioning chamber for 30 min. The numbers in parentheses indicate the number of animals in each group. The data represent the difference in the amount of time rats spent in the two conditioning chambers during place preference testing and were analyzed by Student's t test.

Treatment	Preference
	s
Saline/saline (5)	26 ± 19
Saline/morphine (6)	316 ± 74***
Gly + Gln/saline (5)	52 ± 35
Gly + Gln/morphine (6)	332 ± 25***
Gly-D-Gln/saline (3)	30 ± 12
Gly-D-Gln/morphine (5)	309 ± 39***

*** $P < 0.001$ compared with the corresponding saline-treated controls.

State Dependence. An additional caveat is the possibility that the inhibitory activity of Gly-Gln is attributable to state-dependent learning (Tzschentke and Schmidt, 1997; Tzschentke, 1998). That is, rats may fail to display a place preference because they have learned to associate morphine treatment with a conditioning chamber only in the presence of internally perceived cues produced by Gly-Gln. If so, then rats conditioned with both Gly-Gln and morphine should once again display a place preference if they are given Gly-Gln and morphine immediately before testing. Accordingly, we conditioned rats with Gly-Gln (100 nmol i.c.v.) + morphine (2.5 mg/kg i.p.) for 6 days as done previously and then treated them again with Gly-Gln (100 nmol i.c.v.) + morphine (2.5 mg/kg i.p.) 5 min before testing on day 7. Figure 3 shows that treating rats with Gly-Gln + morphine prior to testing failed to produce a place preference response, however. Treatment with either Gly-Gln or morphine alone before place preference testing was also ineffective (Fig. 3). The ability of Gly-Gln to prevent acquisition of a morphine conditioned place preference is thus unlikely to be due to state-dependent learning.

Food-Induced Conditioned Place Preference. It is also possible that Gly-Gln-treated animals fail to display a place preference to morphine, not because Gly-Gln selectively inhibits morphine reward, but because it nonselectively interferes with all rewards, both drug and nondrug. To investigate this possibility, we tested whether Gly-Gln inhibits acquisition of a conditioned place preference to food. Rats were conditioned with a food they reportedly find highly palatable, the breakfast cereal Fruit Loops (Slusher et al., 2001). Food conditioning was conducted using the same protocol as used for morphine, except that Fruit Loops were placed in one chamber and no food in the alternate chamber

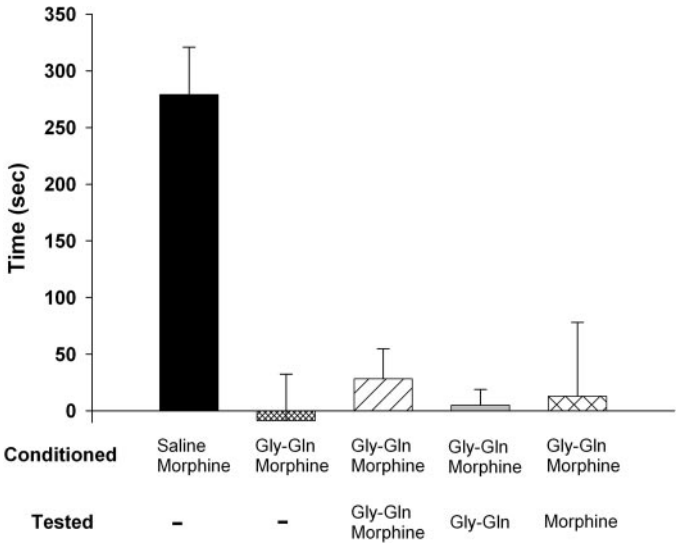

Fig. 3. Effect of Gly-Gln on the acquisition of morphine-induced conditioned place preference is not state-dependent. Rats were treated with saline i.c.v. + morphine (2.5 mg/kg) i.p. or Gly-Gln (100 nmol) i.c.v. + morphine i.p. on days 1, 3, and 5 of place preference conditioning and were confined to a conditioning chamber for 30 min; on days 2, 4, and 6 they were treated with saline i.p. and confined to the alternate conditioning chamber for 30 min. On day 7, they either received no pretreatment (indicated by a hyphen) or were treated with Gly-Gln (100 nmol i.c.v.) + morphine (2.5 mg/kg i.p.), Gly-Gln or morphine 5 min before place preference testing. The data represent the difference in the amount of time rats spent in the two conditioning chambers during place preference testing (n = 3–7).

during conditioning. On days 1, 3, and 5, rats were treated with Gly-Gln (100 nmol) or saline i.c.v. and were confined to a conditioning chamber containing either food or no food for 30 min. On days 2, 4, and 6, all rats were treated with saline i.c.v. and confined to the alternate chamber without food presentation.

On the test day, rats displayed a significant preference for the food-paired chamber [$F(1,13) = 45.0$; $P < 0.001$] (Fig. 4) as shown previously (Slusher et al., 2001). Pretreatment with Gly-Gln had no significant effect on acquisition of a food-induced place preference; Gly-Gln-treated rats spent approximately the same amount of time in the food-paired chamber as did saline-treated animals during place preference testing on day 7 (Fig. 4). Control animals given Gly-Gln with no food presentation during place preference conditioning spent approximately the same amount of time in the Gly-Gln- and saline-paired chambers during testing, again indicating that Gly-Gln treatment by itself did not produce either preference or aversion for either chamber (Fig. 4). Glycyl-glutamine thus inhibits morphine-induced, but not food-induced, place preference conditioning.

Expression of Morphine Place Preference. Next, we tested whether Gly-Gln inhibits expression of a pre-established conditioned place preference to morphine. Rats were conditioned with morphine (2.5 mg/kg) or saline i.p. for 6 days without concurrent Gly-Gln treatment and were given a single dose of Gly-Gln (100 nmol) or saline i.c.v. 5 min before place preference testing on day 7. Glycyl-glutamine inhibited expression of a pre-established place preference to morphine completely [$F(3,24) = 15.0$; $P < 0.001$] (Fig. 5). As shown for acquisition experiments, Gly-Gln did not produce either place preference or place aversion when given to saline conditioned animals before testing. Together, these experiments

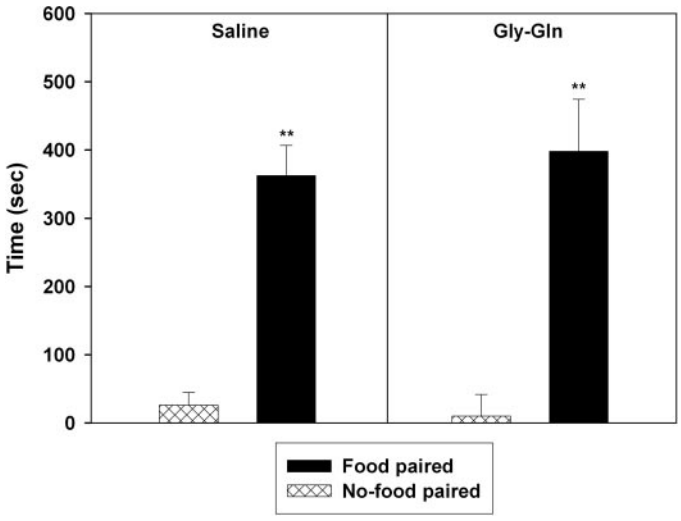

Fig. 4. Glycyl-glutamine does not inhibit acquisition of a conditioned place preference to palatable food. Groups of five rats were treated with Gly-Gln (100 nmol) or saline i.c.v. on days 1, 3, and 5 of place preference conditioning and were confined to a conditioning chamber containing either the breakfast cereal Fruit Loops or no food for 30 min; on days 2, 4, and 6 they were treated with saline and confined to the alternate chamber with no food presentation. The data represent the difference in the amount of time spent in the two conditioning chambers during place preference testing on day 7. ***, $P < 0.001$ differs from control animals conditioned with no food.

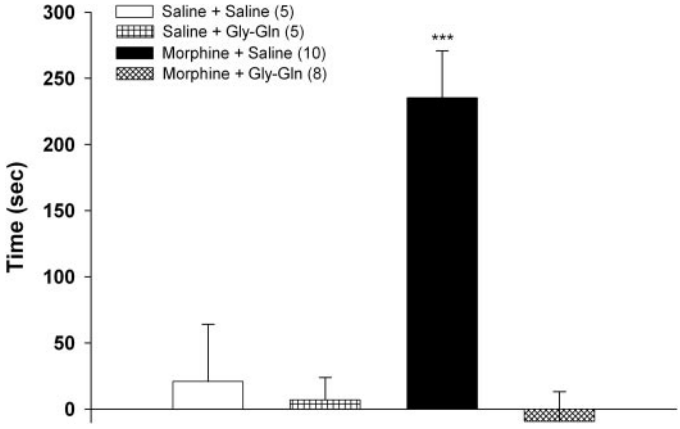

Fig. 5. Glycyl-glutamine inhibits expression of a conditioned place preference to morphine. Rats were treated with morphine sulfate (2.5 mg/kg) or saline i.p. on days 1, 3, and 5 of place preference conditioning and were confined to a conditioning chamber for 30 min; on days 2, 4, and 6 they were treated with saline i.p. and confined to the alternate chamber for 30 min. On day 7, they were treated with Gly-Gln (100 nmol) or saline i.c.v. 5 min before place preference testing. Data represent the difference in the amount of time spent in the two conditioning chambers during the test session. ***, $P < 0.001$ differs from saline-treated controls.

show that Gly-Gln inhibits both acquisition and expression of a conditioned place preference to morphine.

Tolerance. The finding that Gly-Gln inhibits morphine place preference raises the possibility that it may influence other effects of morphine. We subsequently tested whether Gly-Gln pretreatment inhibits the development of morphine tolerance. To induce tolerance, rats were treated with morphine sulfate (10 mg/kg i.p.) twice daily for 7 days, and tail-flick latencies were measured daily 30 min after the first morphine dose. Tolerance developed rapidly with this protocol. The antinociceptive response to morphine was reduced significantly by the second day of treatment ($P < 0.01$) and,

within 4 days, tail-flick latencies had declined to 39.0 ± 5.2 %MPE (Fig. 6A). To test whether Gly-Gln influences the development of morphine tolerance, rats were pretreated with Gly-Gln (100 nmol) or saline i.c.v. twice daily 2 min before they were given morphine (10 mg/kg) or saline i.p.. Twice daily Gly-Gln administration to saline-treated control animals did not affect tail-flick latencies (Fig. 6A), consistent with previous evidence that acute Gly-Gln administration had no effect on nociceptive response latencies or morphine antinociception (Owen et al., 2000). When given to morphine-treated animals, Gly-Gln delayed the development of tolerance to morphine significantly [$F(18,128) = 24.4$; $P < 0.001$] (Fig. 6A). Among Gly-Gln pretreated rats, the antinociception response to morphine was not reduced significantly until the fourth day of treatment ($P < 0.001$). Pretreatment with the Gly-Gln stereoisomer glycyl-D-glutamine was ineffective (Fig. 6B).

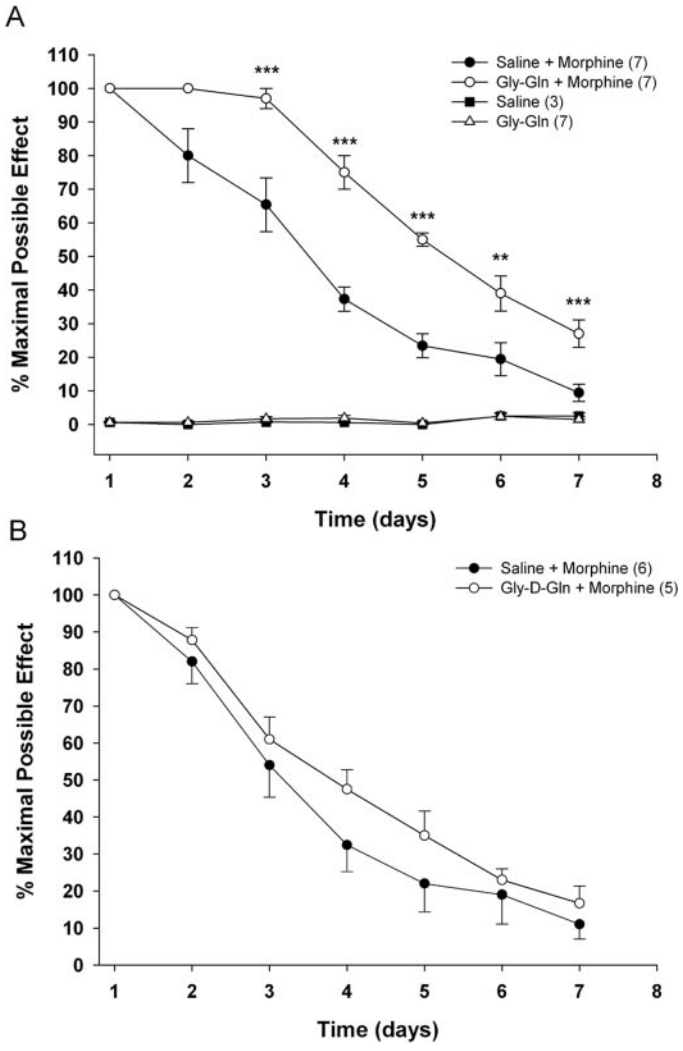

Fig. 6. Glycyl-glutamine pretreatment delays the development of morphine tolerance. A, rats were treated with Gly-Gln (100 nmol) or saline i.c.v. followed 2 min later by morphine (10 mg/kg) or saline i.p. twice daily for 7 days. B, rats received Gly-D-Gln (100 nmol) or saline i.c.v. followed by morphine (10 mg/kg) i.p. twice daily. Tail-flick latencies were measured 30 min after the first daily morphine administration. The data represent the mean % maximal possible effect ± S.E.M. and were analyzed by ANOVA followed by Student-Newman-Keuls test. **, $P < 0.01$ and ***, $P < 0.001$ differs from saline + morphine-treated animals at the corresponding time point.

Subsequently, we tested whether Gly-Gln would reverse pre-established antinociceptive tolerance in animals that continued to receive morphine. Rats were treated with morphine sulfate (10 mg/kg i.p.) twice daily for 4 days, and then beginning on the morning of the fifth day, one-half of the animals were given Gly-Gln (100 nmol) and one-half were given saline i.c.v. 2 min before each morphine treatment. Glycyl-glutamine administration prevented further development of tolerance and partially restored tail-flick latencies toward baseline values (Fig. 7). Statistical analysis revealed significant treatment [$F(1,10) = 7.9$; $P < 0.01$] and time effects [$F(7,78) = 15.4$; $P < 0.001$], although the interactive effect was not significant. It is interesting that tail-flick latencies were higher, although not significantly so, 30 min after the first Gly-Gln treatment on day 5. Glycyl-glutamine did not inhibit morphine tolerance completely, however, and tail-flick latencies remained below baseline values throughout the experiment. Glycyl-glutamine thus inhibits the development of antinociceptive tolerance and partially restores sensitivity to morphine in tolerant animals that continue to receive morphine.

Dependence. To investigate morphine dependence, we measured naloxone-precipitated withdrawal symptoms in animals treated chronically with morphine. Rats were treated twice daily with morphine sulfate (10 mg/kg i.p.) for 4 days, and Gly-Gln (100 nmol) or saline was injected i.c.v. 2 min before each morphine injection. Somatic withdrawal symptoms were scored at 5-min intervals for 20 min after naloxone (4 mg/kg i.p.) administration and averaged to determine the mean withdrawal score. Glycyl-glutamine pretreatment lowered the mean withdrawal score significantly from 9.34 ± 0.32 ($n = 11$) in saline-treated control animals to 6.00 ± 0.22 ($n = 10$) in Gly-Gln-treated animals ($P < 0.05$). Of the eight individual withdrawal symptoms measured, only two (sniffing and rearing) were reduced significantly.

Withdrawal. To study withdrawal, morphine dependence was induced by implanting a 75-mg sustained release mor-

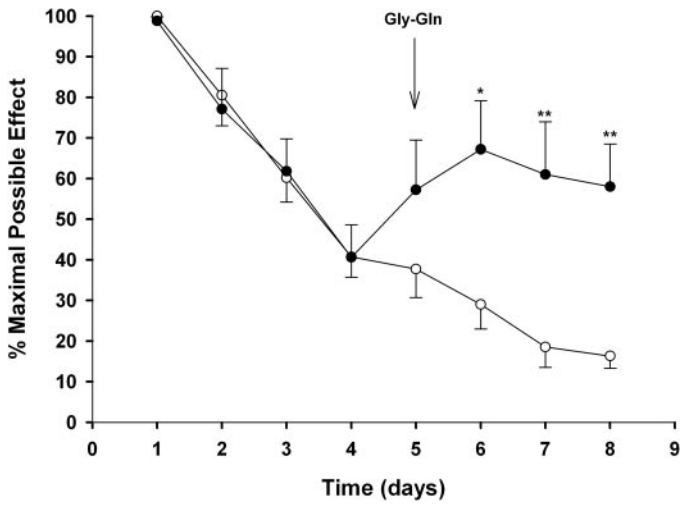

Fig. 7. Glycyl-glutamine reverses morphine tolerance. Two groups of six rats were treated with saline i.c.v. followed by morphine sulfate (10 mg/kg i.p.) twice daily for 4 days. On days 5 to 8, one group was pretreated with Gly-Gln (100 nmol i.c.v.; filled symbols) and the second group continued to receive saline (open symbols). Tail-flick latencies were measured 30 min after the first daily morphine treatment. Data were analyzed by ANOVA followed by Student-Newman-Keuls test. *, $P < 0.05$ and **, $P < 0.01$ differs from saline + morphine-treated animals at the corresponding time point.

phine pellet subcutaneously. Control animals received a placebo pellet. Approximately 72 h thereafter, rats were treated with Gly-Gln (100 nmol) or saline i.c.v. and, 5 min later, they were injected with naloxone (1 mg/kg s.c.). Withdrawal symptoms were scored at 5-min intervals for 40 min. Glycyl-glutamine pretreatment reduced the intensity of naloxone-precipitated withdrawal symptoms significantly [$F(8,99) = 6.1$; $P < 0.001$] (Fig. 8). Statistical analysis of individual withdrawal symptoms confirmed that Gly-Gln inhibited each of the eight withdrawal symptoms significantly and reduced the amount of weight animals lost during the 2-h period after naloxone administration (Fig. 8). Analysis of the time course of wet dog shakes and teeth chattering revealed that these two withdrawal signs occurred with highest frequency during the 15 min immediately after naloxone injection (Fig. 9). Glycyl-glutamine treatment reduced the incidence of wet dog shakes and teeth chattering throughout the 40-min test period (Fig. 9). Analysis of variance demonstrated a significant interactive effect of Gly-Gln on wet dog shakes [$F(8,99) = 3.2$; $P < 0.01$]; there were significant treatment [$F(1,11) = 15.8$; $P < 0.001$] and time [$F(8,99) = 5.6$; $P < 0.001$] effects on teeth chattering as well, although the interactive effect was not significant. The effect of Gly-Gln on naloxone-precipitated withdrawal was dose-dependent [$F(6,27) = 53.8$; $P < 0.001$] (Fig. 10), although somewhat higher Gly-Gln doses (30–300 nmol) were required to suppress morphine withdrawal symptoms than shown previously for place preference responding. Glycyl-glutamine had no obvious effect on withdrawal symptoms in placebo pellet-implanted control animals. These data indicate that, at an appropriate dose, Gly-Gln pretreatment suppresses naloxone-precipitated withdrawal symptoms almost entirely.

Discussion

This study shows that Gly-Gln prevents acquisition and expression of a morphine-induced conditioned place preference, a test of morphine's rewarding and/or incentive properties, and inhibits morphine tolerance, dependence, and withdrawal. These data extend earlier evidence that Gly-Gln attenuates the hypotension, respiratory depression (Unal et al., 1994, 1997; Owen et al., 2000), and certain behavioral effects (Hirsch and O'Donohue, 1986) produced by morphine and β-endorphin$_{1\text{-}31}$. The effect of glycyl-glutamine on place

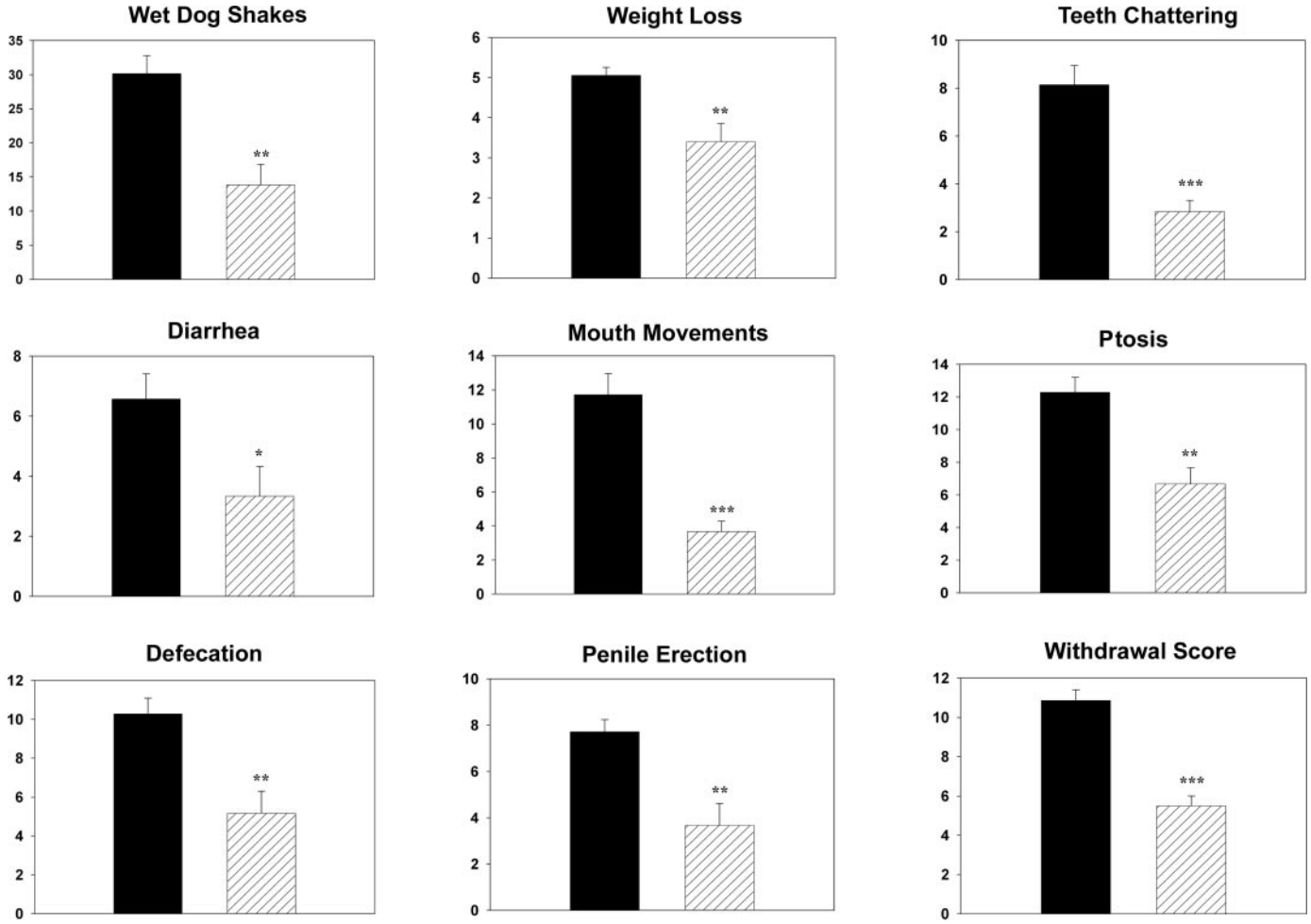

Fig. 8. Glycyl-glutamine inhibits naloxone-precipitated withdrawal symptoms in morphine-dependent animals. Rats were implanted with a 75-mg sustained release morphine pellet subcutaneously. Seventy-two hours later, they received a single injection of Gly-Gln (100 nmol) or saline i.c.v. followed, 5 min later, by naloxone (1 mg/kg) s.c., and withdrawal signs were measured at 5-min intervals for 40 min. Weight loss was determined by recording the animal's weight immediately before and 2 h after Gly-Gln or saline administration. The data illustrate withdrawal symptoms of rats treated with saline (filled columns; $n = 7$) or Gly-Gln (hatched columns; $n = 6$) and were analyzed by ANOVA followed by Student's t test. $*$, $P < 0.05$; $**$, $P < 0.01$; and $***$, $P < 0.001$ differs from saline-treated animals.

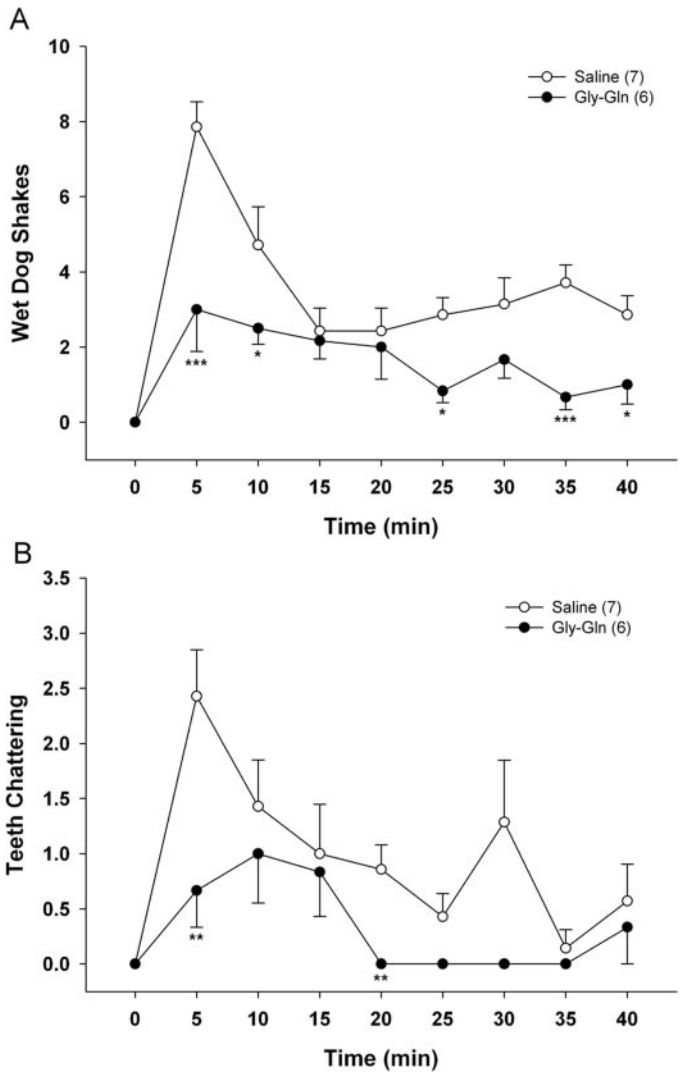

Fig. 9. Gly-Gln inhibits naloxone-precipitated morphine withdrawal: time course of the effect on wet dog shakes and teeth-chattering. Rats were implanted with a 75-mg morphine pellet subcutaneously, and 72 h later, they received either Gly-Gln (100 nmol) or saline i.c.v. followed 5 min later by naloxone (1 mg/kg) s.c., and wet dog shakes (top) and teeth-chattering (bottom) were measured at 5-min intervals for 40 min. Data were analyzed by ANOVA followed by the Student-Newman-Keuls test. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ differs from saline-treated control animals at the corresponding time point.

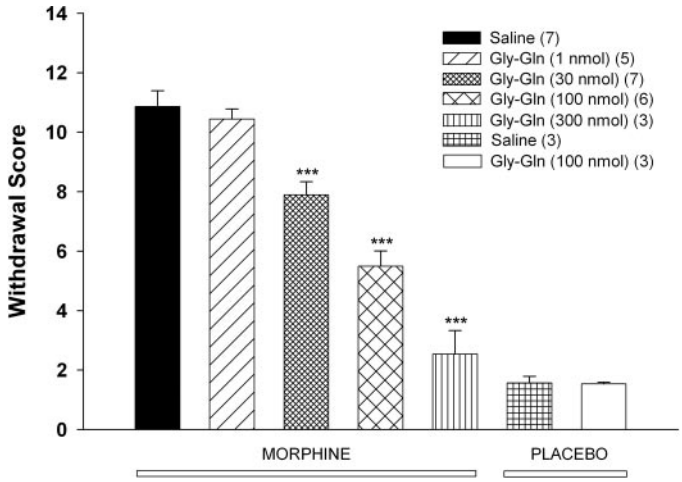

Fig. 10. Glycyl-glutamine inhibits naloxone-precipitated morphine withdrawal: dose response. Rats were implanted with a 75-mg sustained release morphine or placebo pellet subcutaneously. Seventy-two hours later, they were treated with the indicated dose of Gly-Gln or saline i.c.v. followed 5 min later by naloxone (1 mg/kg) s.c. Withdrawal symptoms were measured at 5-min intervals for 40 min, and a mean withdrawal score was calculated for each animal. Data were analyzed by ANOVA followed by the Student-Newman-Keuls test. ***, $P < 0.001$ differs from saline-treated morphine-dependent animals.

preference conditioning was not reproduced by glycine and glutamine and is thus unlikely to result from Gly-Gln hydrolysis and subsequent activation of glycine receptors. In previous studies, we also found that [^{3}H]Gly-Gln was not hydrolyzed to a significant extent in vivo after i.c.v. injection or in vitro during short-term incubation with rat brain membrane preparations, which further obviates concern that Gly-Gln acts indirectly through this mechanism (D. C. Pendergrass and W. R. Millington, unpublished data). Glycyl-D-glutamine also failed to prevent morphine-induced place preference conditioning and antinociceptive tolerance, and so nonspecific peptide interactions do not readily explain the inhibitory activity of Gly-Gln.

Nevertheless, the ability of Gly-Gln to prevent morphine place preference could result from an effect on sensory or behavioral mechanisms other than drug reward. Conceivably, Gly-Gln could interfere with place preference conditioning by producing dysphoria or otherwise causing animals to associate the morphine-conditioned chamber with an aversive experience. Conditioned place aversion is unlikely to explain Gly-Gln's efficacy, however, because control animals given Gly-Gln, but not morphine, did not display aversion for either conditioning chamber. Alternatively, Gly-Gln could interfere with learning and memory processes necessary for acquiring a place preference (White, 1996). It is not known whether Gly-Gln influences memory, but its inability to prevent acquisition of a place preference for palatable food argues against this explanation. Glycyl-glutamine's inhibitory activity could also result from an effect on locomotor activity, sensory information processing, or other cognitive or behavioral processes, but if so, it would seem logical to assume that such nonspecific effects would influence food conditioning as well. Control experiments also ruled out the possibility that the inhibitory activity of Gly-Gln is attributable to state-dependent learning. Nevertheless, it is important to emphasize that the pharmacological effects of Gly-Gln have not been thoroughly investigated and alternative explanations for its efficacy remain viable.

It is interesting to note that the inability of Gly-Gln to produce place aversion distinguishes it from naloxone and other opioid receptor antagonists. Naloxone produces conditioned place aversion, not only in morphine-dependent rats, but also in animals that have never received opioids (Mucha and Walker, 1987; Skoubis et al., 2005). This suggests that opioid neurons normally maintain a certain level of "hedonic homeostasis" that naloxone interrupts (Koob and Le Moal, 1997). Evidently, Gly-Gln does not share this property, perhaps because the basal hedonic state is mediated by neuronal enkephalin release, not β-endorphin (Skoubis et al., 2005).

As one might predict, β-endorphin$_{1\text{-}27}$ also inhibits acquisition of a conditioned place preference to β-endorphin$_{1\text{-}31}$ and other opioids (Bals-Kubik et al., 1988) and inhibits the rise in extracellular dopamine evoked by β-endorphin$_{1\text{-}31}$ in the nucleus accumbens (Spanagel et al., 1991). Based on

these observations, as well as the present data, it is tempting to speculate that some POMC neurons corelease β-endorphin$_{1\text{-}27}$ and Gly-Gln to counteract the potentially addictive effects of exogenous, or even endogenous, opioids. A report that chronic morphine administration enhances the conversion of β-endorphin$_{1\text{-}31}$ to β-endorphin$_{1\text{-}27}$ and β-endorphin$_{1\text{-}26}$ is consistent with this speculation (Bronstein et al., 1990). As tempting as this may be, however, there is really no direct evidence to support the idea that endogenous β-endorphin$_{1\text{-}27}$ or Gly-Gln influences opioid reward or other manifestations of opioid addiction.

We also found that Gly-Gln administration inhibits the development of morphine dependence and delays the onset of antinociceptive tolerance. The inhibition of morphine tolerance is unlikely to result from an antinociceptive effect of the dipeptide itself, because Gly-Gln did not prolong tail-flick latencies when administered chronically to otherwise untreated animals. This latter observation extends previous evidence that acute Gly-Gln administration has no effect on tail-flick or paw-lift latencies and does not block morphine antinociception, even at very high doses (Owen et al., 2000). Nevertheless, Gly-Gln may act differently in rats treated with morphine chronically than it does after acute opioid administration (Ossipov et al., 2004). In fact, when it was given to rats already tolerant to morphine, Gly-Gln increased tail-flick latencies rapidly, within 30 min after the initial Gly-Gln dose. This suggests that the ability of Gly-Gln to reverse morphine tolerance does not require long-term changes in gene expression or other subcellular processes associated with tolerance (Bailey and Connor, 2005).

The reason Gly-Gln is able to selectively inhibit morphine place preference, tolerance, dependence, and withdrawal without compromising morphine antinociception remains to be determined. Early studies of β-endorphin processing showed that β-endorphin is extensively converted to carboxy-terminal shortened peptides and Gly-Gln in the caudal medulla (Dores et al., 1986), brainstem (Zakarian and Smyth, 1982; Parish et al., 1983), and some forebrain regions (Zakarian and Smyth, 1982) to a considerably greater extent than it is in the in the midbrain periaqueductal gray (Berglund et al., 1989), an area that serves a pivotal role in pain control. Hence, it is conceivable that Gly-Gln does not influence morphine analgesia simply because it is not synthesized to a significant extent by POMC neurons that modulate pain perception.

Glycyl-glutamine completely suppressed morphine withdrawal symptoms, although the dose response was shifted to the right compared with the dose response for inhibition of morphine place preference. This could be interpreted to mean that Gly-Gln inhibits morphine place preference and withdrawal through two different mechanisms. However, it is also consistent with evidence that morphine withdrawal symptoms are somewhat resistant to pharmacological intervention. Considerably higher doses of the NMDA receptor antagonist dizocilpine are required to inhibit somatic signs of morphine withdrawal than are necessary to block morphine tolerance and dependence (González et., 1997), for example, and even naloxone must be given in higher doses to precipitate somatic withdrawal signs than to induce conditioned place aversion and other behavioral indices of opioid withdrawal (Schulteis et al., 1994). In preliminary experiments, Gly-Gln also suppressed some spontaneous withdrawal symptoms, including wet dog shakes, ptosis, and teeth chattering, 12 h after morphine pellet withdrawal, although other spontaneous withdrawal symptoms were not inhibited significantly.

These findings remain somewhat phenomenological without a clear understanding of Gly-Gln's mechanism of action. At this juncture, we do not know what Gly-Gln's mechanism of action is, only what it is not. Glycyl-glutamine is not an opioid receptor antagonist because it does not inhibit β-endorphin$_{1\text{-}31}$ or morphine analgesia (Owen et al., 2000) and because it fails to displace [^{3}H]naloxone binding to rat brain membranes, even at inordinately high concentrations (Unal et al., 1994). NovaScreen analysis (National Institute of Mental Health/NovaScreen Drug Discovery and Development Program, National Institutes of Health, Bethesda, MD) failed to detect any significant displacement by Gly-Gln of a variety of radioligands for opioid, glutamate, glycine, GABA, or other receptors. Preliminary radioligand binding experiments showed that [^{3}H]Gly-Gln binds to brain membrane preparations in a saturable and stereospecific manner, albeit with a relatively low affinity (K_d = 44 nM), and is not displaced by ligands for opioid receptors. It remains to be seen whether this [^{3}H]Gly-Gln binding site represents a physiologically relevant receptor.

Considering its sequence, it is conceivable that Gly-Gln acts as an antagonist for the strychnine-sensitive glycine$_A$ receptor or the NMDA-associated glycine$_B$ receptor, despite receptor binding data to the contrary. Although there is little evidence that glycine$_A$ receptors participate in morphine addiction, it is well understood that NMDA receptor antagonists, including drugs that block glycine$_B$ receptors, produce a pharmacological profile in tests of morphine place preference, tolerance, dependence, and withdrawal similar to what is shown here for Gly-Gln (Kolesnikov et al., 1994; Trujillo, 2000; Ossipov et al., 2004). To initially assess the possibility that Gly-Gln inhibits morphine withdrawal by blocking glycine$_B$ receptors, we tested whether D-serine, a glycine$_B$ receptor agonist (Witkin et al., 1997), would prevent Gly-Gln from suppressing morphine withdrawal symptoms. D-Serine (1 μmol i.c.v.) was ineffective, however; Gly-Gln produced approximately the same reduction in the incidence of wet dog shakes/40 min in D-serine-pretreated rats (D-serine/saline, 31 ± 7; D-serine/Gly-Gln, 13 ± 6; n = 3–4) as it did in saline-pretreated animals (saline/saline, 30 ± 3; saline/Gly-Gln, 14 ± 3; n = 6–7). This does not support the hypothesis that Gly-Gln blocks NMDA receptors and leaves questions about its mechanism of action unanswered.

In summary, these data show that Gly-Gln prevents acquisition and expression of morphine conditioned place preference and inhibits morphine tolerance, dependence, and withdrawal. These findings extend earlier evidence that Gly-Gln inhibits morphine hypotension and respiratory depression without influencing cardiovascular or respiratory function in opioid naive animals and, importantly, without interfering with morphine analgesia (Unal et al., 1997; Owen et al., 2000). Perhaps the most intriguing aspect of these findings is the demonstration that a dipeptide synthesized from β-endorphin$_{1\text{-}31}$, an endogenous opioid, is capable of suppressing multiple indices of opiate addiction. Moreover, they suggest that further investigation of Gly-Gln pharmacology could ultimately lead to new therapeutic strategies for selectively

preventing the adverse effects of opioids or, perhaps, for treating opiate dependence and withdrawal.

Acknowledgments

We thank Dr. Larry Reid (Department of Cognitive Science, Rensselaer Polytechnic Institute, Troy, NY) for stimulating discussions and invaluable advice during the initial phase of this study. We also acknowledge the National Institute on Drug Abuse Drug Supply Program for providing morphine pellets and thank Mark Levendusky, Janice Kidney, and Jeffrey Steele for technical assistance.

References

Bailey CP and Connor M (2005) Opioids: cellular mechanisms of tolerance and physical dependence. *Curr Opin Pharmacol* **5:**60–68.

Bals-Kubik R, Herz A, and Shippenberg TS (1988) β-Endorphin-(1-27) is a naturally occurring antagonist of the reinforcing effects of opioids. *Naunyn-Schmiedeberg's Arch Pharmacol* **338:**392–396.

Berglund LA, Millington WR, and Simpkins JW (1989) Gonadal steroid and chronic morphine treatment do not change the post-translational processing of β-endorphin in rat brain. *Life Sci* **44:**591–601.

Bronstein DM, Przewlocki R, and Akil H (1990) Effects of morphine treatment on pro-opiomelanocortin systems in rat brain. *Brain Res* **519:**102–111.

Chen JC, Tao PL, Li JY, Wong CH, and Huang EY (2003) Endomorphin-1 and -2 induce naloxone-precipitated withdrawal syndromes in rats. *Peptides* **24:**477–481.

Cicero TJ and Meyer ER (1973) Morphine pellet implantation in rats: quantitative assessment of tolerance and dependence. *J Pharmacol Exp Ther* **184:**404–408.

Deakin JF, Doströvsky JO, and Smyth DG (1980) Influence of N-terminal acetylation and C-terminal proteolysis on the analgesic activity of β-endorphin. *Biochem J* **189:**501–506.

Dores RM, Jain M, and Akil H (1986) Characterization of the forms of beta-endorphin and alpha-MSH in the caudal medulla of the rat and guinea pig. *Brain Res* **377:**251–260.

Gellert VF and Holtzman SG (1978) Development and maintenance of morphine tolerance and dependence in the rat by scheduled access to morphine drinking solutions. *J Pharmacol Exp Ther* **205:**536–546.

González P, Cabello P, Germany A, Norris B, and Contreras E (1997) Decrease of tolerance to and physical dependence on, morphine by glutamate receptor antagonists. *Eur J Pharmacol* **332:**257–262.

Hirsch MD and O'Donohue TL (1986) Structural modifications of pro-opiomelanocortin-derived peptides alter their behavioral effects markedly. *J Pharmacol Exp Ther* **237:**378–385.

Koelle GB, Massoulie J, Eugene D, and Melone MA (1988) Effects of glycyl-L-glutamine *in vitro* on the molecular forms of acetylcholinesterase in the preganglionically denervated superior cervical ganglion of the cat. *Proc Natl Acad Sci USA* **85:**1686–1690.

Kolesnikov YA, Maccechini ML, and Pasternak GW (1994) 1-Aminocyclopropane carboxylic acid (ACPC) prevents mu and delta opioid tolerance. *Life Sci* **55:**1393–1398.

Koob GF and Le Moal M (1997) Drug abuse: hedonic homeostatic dysregulation. *Science (Wash DC)* **278:**52–58.

Lotwick HS, Haynes LW, and Ham J (1990) Glycyl-L-glutamine stimulates the accumulation of A_{12} acetylcholinesterase but not of nicotine acetylcholine receptors in quail embryonic myotubes by a cyclic AMP-independent mechanism. *J Neurochem* **54:**1122–1129.

McCain HW, Bilotta J, and Lamster IB (1987) Endorphinergic modulation of immune function: potent action of the dipeptide glycyl-L-glutamine. *Life Sci* **41:**169–176.

Mucha RF and Walker MJK (1987) Aversive property of opioid receptor blockade in drug-naive mice. *Psychopharmacology* **93:**483–488.

Nicolas P and Li CH (1985) β-Endorphin-(1-27) is a naturally occurring antagonist to etorphine-induced analgesia. *Proc Natl Acad Sci USA* **82:**3178–3181.

Nores WL, Olson RD, Olson GA, Vaccarino AL, Bell RL, Zadina JE, and Kastin AJ (1999) Tyr-W-MIF-1-induced conditioned place preference. *Peptides* **20:**479–484.

Ossipov MH, Lai J, King T, Vanderah TW, Malan TP, Hruby VJ, and Porreca F (2004) Antinociceptive and nociceptive actions of opioids. *J Neurobiol* **61:**126–148.

Owen MD, Unal CB, Callahan MF, Triveda K, York C, and Millington WR (2000) Glycyl-glutamine inhibits the respiratory depression, but not the antinociception, produced by morphine. *Am J Physiol* **279:**R1944–R1948.

Parish DC, Smyth DG, Normanton JR, and Wolstencroft JH (1983) Glycyl glutamine, an inhibitory neuropeptide derived from β-endorphin. *Nature (Lond)* **306:**267–270.

Raffin-Sanson ML, de Keyzer Y, and Bertagna X (2003) Proopiomelanocortin, a polypeptide precursor with multiple functions: from physiology to pathological conditions. *Eur J Endocrinol* **149:**79–90.

Reid LD, Marglin SH, Mattie ME, and Hubbell CL (1989) Measuring morphine's capacity to establish a place preference. *Pharmacol Biochem Behav* **33:**765–775.

Schulteis G, Markou A, Gold LH, Stinus L, and Koob GF (1994) Relative sensitivity to naloxone of multiple indices of opiate withdrawal: a quantitative dose-response analysis. *J Pharmacol Exp Ther* **271:**1391–1398.

Skoubis PD, Lam HA, Shoblock J, Narayanan S, and Maidment NT (2005) Endogenous enkephalins, not endorphins, modulate basal hedonic state in mice. *Eur J Neurosci* **21:**1379–1384.

Slusher BS, Thomas A, Paul M, Schad CA, and Ashby CR (2001) Expression and acquisition of the conditioned place preference response to cocaine in rats is blocked by selective inhibitors of the enzyme N-acetylated-α-linked-acidic dipeptidase (NAALADase). *Synapse* **41:**22–28.

Smith AI and Funder JW (1988) Proopiomelanocortin processing in the pituitary, central nervous system and peripheral tissues. *Endocr Rev* **9:**159–179.

Smith DJ, Robertson B, Monroe PJ, Taylor DA, Leedham JA, and Cabral JDY (1992) Opioid receptors mediating antinociception from β-endorphin and morphine in the periaqueductal gray. *Neuropharmacology* **31:**1137–1150.

Spanagel R, Herz A, and Shippenberg TS (1991) Modulation of the mesolimbic dopaminergic system by β-endorphin-(1-27) as assessed by microdialysis. *Eur J Pharmacol* **200:**319–324.

Trujillo KA (2000) Are NMDA receptors involved in opiate-induced neural and behavioral plasticity? A review of preclinical studies. *Psychopharmacology* **151:**121–141.

Tseng LF (2001) Evidence for ϵ-opioid receptor-mediated β-endorphin-induced analgesia. *Trends Pharmacol Sci* **22:**623–630.

Tzschentke TM (1998) Measuring reward with the conditioned place preference paradigm: a comprehensive review of drug effects, recent progress and new issues. *Prog Neurobiol* **56:**613–672.

Tzschentke TM and Schmidt WJ (1997) Interactions of MK-801 and GYKI 52466 with morphine and amphetamine in place preference conditioning and behavioral sensitization. *Behav Brain Res* **84:**99–107.

Unal CB, Owen MD, and Millington WR (1994) Inhibition of β-endorphin-induced cardiorespiratory depression by glycyl-L-glutamine, a dipeptide derived from β-endorphin processing. *J Pharmacol Exp Ther* **271:**952–958.

Unal CB, Owen MD, and Millington WR (1997) Cyclo(Gly-Gln) inhibits the cardiorespiratory depression produced by β-endorphin and morphine. *Brain Res* **747:**52–59.

White NM (1996) Addictive drugs as reinforcers: multiple partial actions on memory systems. *Addiction* **91:**921–949.

Witkin JM, Steele TD, and Sharpe LG (1997) Effects of strychnine-insensitive glycine receptor ligands in rats discriminating dizocilpine or phencyclidine from saline. *J Pharmacol Exp Ther* **280:**46–52.

Zakarian S and Smyth DG (1982) Distribution of β-endorphin-related peptides in rat pituitary and brain. *Biochem J* **202:**561–571.

Address correspondence to: Dr. William R. Millington, Department of Basic and Pharmaceutical Sciences, Albany College of Pharmacy, Union University, 106 New Scotland Ave., Albany, NY 12208. E-mail: millingw@acp.edu