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

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Abstract

Glycyl-glutamine (Gly-Gln; β-endorphin30-31) is an endogenous dipeptide synthesized from β-endorphin1-31. Previous investigations have shown that Gly-Gln inhibits the cardiovascular and respiratory depression caused by morphine and β-endorphin1-31 but it does not interfere with opioid analgesia. In this study, we tested whether Gly-Gln administration would influence morphine conditioned place preference, tolerance, dependence or withdrawal. For place preference experiments, rats were conditioned with morphine sulfate (2.5 mg/kg ip) or saline on alternate days for 6 days and tested on day 7. Glycyl-glutamine (1-100 nmol icv) pretreatment inhibited acquisition of a conditioned place preference to morphine significantly. Glycyl-glutamine (100 nmol icv) 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 did not produce place preference or aversion when given alone to morphine naïve animals. To induce antinociceptive tolerance, rats were treated with morphine (10 mg/kg ip) twice daily for 7 days and morphine antinociception was evaluated with the tail flick test. Glycyl-glutamine (100 nmol icv) 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 ip) 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 conditioned place preference, tolerance, dependence and withdrawal without compromising morphine analgesia.
Introduction

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 non-opioid phenotype (Raffin-Sanson et al., 2003). Alternatively, non-opioid 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 physiologic functions (Parish, 1983; Smith and Funder, 1988). Early studies showed that Gly-Gln is an inhibitory peptide that reduces the firing frequencies of brainstem neurons following 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$_{1-27}$, 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\textsubscript{1-31} 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\textsubscript{1-27} 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\textsubscript{1-27} and Gly-Gln, capable of modulating the effects of β-endorphin\textsubscript{1-31}, albeit through different receptor mechanisms.

In this study, we tested whether Gly-Gln inhibits morphine 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 naïve 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.
Methods

Animals and Surgical Procedures: Male Sprague-Dawley rats (250-300 g; Charles River 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% O₂, 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 (icv) 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 one week prior to experimentation. The animal protocols were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

For icv 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. Glycylglutamine was dissolved in 5 µl 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 sec 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 (Nores et al., 1999; Slusher et al., 2001; Tzschtentke, 1998). The two conditioning chambers were identical in size (45 x 45 x 30 cm) but were distinguishable both
visually and tactualy. 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 x 25 x 30 cm) painted a neutral gray color, 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 ± 16 sec) as the striped chamber (251 ± 25 sec; n = 8).

The conditioned place preference procedure consisted of three components. Habituation: The animals were transported to the testing room and handled daily for 3 consecutive days to habituate them to the testing room and human handling. Conditioning: Conditioning sessions were performed once daily for six days. Half 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 ip; Elkins-Skin, 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 ip) on days 2, 4 and 6 and were confined in the alternate chamber for 30 min. Control animals received saline ip before all 6 conditioning sessions; one of the two chambers was designated as the ‘primary’ conditioning chamber. Testing: Test 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 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 Chemical Co., St. Louis, MO) or saline (5 µl) icv followed, 2 min later, by morphine sulfate (2.5 mg/kg) or saline ip 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 icv 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 animal’s normal diet was restricted to 5 pellets/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 icv 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 5, all animals were treated with saline icv 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 ip) twice daily between 07:00 and 08:00 a.m. and 18:00 and 19:00 p.m. 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 icv 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 percent maximal possible effect (%MPE) by using the formula: %MPE = (post drug 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 ip injection, rather than subcutaneous pellet implantation, to more closely parallel the presumed time course of Gly-Gly following icv injection. Accordingly, rats were treated with Gly-Gln (100 nmol) or saline icv followed, 2 min later, by morphine sulfate (10 mg/kg; ip) 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 ip; Sigma Chemical Co, St. Louis, MO). 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 (Gellert and Holtzman, 1978). 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 icv and withdrawal was precipitated 5 min later with naloxone hydrochloride (1 mg/kg; sc). 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 ip) 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 six 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 ip) also produced a significant, albeit less robust, place preference response (165 ± 66 sec; 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 pre-treating rats with Gly-Gln (100 nmol) or saline icv 2 min before they were given morphine sulfate (2.5 mg/kg) or saline ip 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 pre-treated with Gly-Gln (100 nmol icv) 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). Importantly, rats treated with Gly-Gln icv followed by saline ip, in lieu of morphine, did not show a preference for either chamber during place preference testing (Fig. 1). Glycyl-glutamine (100 nmol icv) 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 sec; saline + Gly-Gln = -127 ± 75 sec; morphine + saline = 315 ± 85 sec; morphine + Gly-Gln = -29 ± 38 sec; 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) icv 2 min before they received morphine (2.5 mg/kg) ip 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 nmol and 100 nmol icv [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 icv injection. This is an important consideration, of course, because glycine, itself, is a neurotransmitter. To test this, rats were pretreated with glycine and glutamine (100 nmol of each amino acid) icv 2 min before ip 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 ip instead of morphine (Table 1). The inactive 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 non-specific peptide effects are likely to explain Gly-Gln’s ability to inhibit acquisition of a morphine conditioned place preference.

**State Dependence:** An additional caveat, is the possibility that Gly-Gln’s inhibitory activity is attributable to state-dependent learning (Tzschenkte and Schmidt, 1997; Tzschenkte, 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 icv) + morphine (2.5 mg/kg ip) for six days as done previously then treated them again with Gly-Gln (100 nmol icv) + morphine (2.5 mg/kg ip) 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). Glycyl-glutamine’s ability 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 non-selectively interferes with all rewards, both drug and non-drug. 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 during conditioning. On days 1, 3 and 5, rats were treated with Gly-Gln (100 nmol) or saline icv 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 icv 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, however; 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, 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 ip for six days without concurrent Gly-Gln treatment and were given a single dose of Gly-Gln (100 nmol) or saline icv 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 prior to testing. Together, these experiments 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 ip) twice daily for seven 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 four days, tail flick latencies had declined to $39.0 \pm 5.2\%$ MPE (Fig. 6A). To test whether Gly-Gln influences the development of morphine tolerance, rats were pretreated with Gly-Gln (100 nmol) or saline icv twice daily 2 min before they were given morphine (10 mg/kg) or saline ip. Twice daily Gly-Gln administration to saline treated control animals did not affect tail flick latencies (Fig. 6A), consistent with earlier 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).

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 ip) twice daily for 4 days then, beginning on the morning of the fifth day, half the animals were given Gly-Gln (100 nmol) and half were given saline icv 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. Interestingly, 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 ip) for four days and Gly-Gln (100 nmol) or saline was injected icv 2 min before each morphine injection. Somatic withdrawal symptoms were scored at 5 min intervals for 20 min after naloxone (4 mg/kg ip) 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 8 individual withdrawal symptoms measured only two, sniffing and rearing, were reduced significantly, however.

**Withdrawal:** To study withdrawal, morphine dependence was induced by implanting a 75 mg sustained release morphine pellet subcutaneously. Control animals received a placebo pellet. Approximately 72 h thereafter, rats were treated with Gly-Gln (100 nmol) or saline icv and, 5 min later, they were injected with naloxone (1 mg/kg sc). 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 following 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 following 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 the 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 (Owen et al. 2000; Unal et al. 1994, 1997) and certain behavioral effects (Hirsch and O’Donohue, 1986) produced by morphine and β-endorphin_{1-31}. Glycyl-glutamine’s effect on place 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 earlier studies, we also found that [³H]Gly-Gln was not hydrolyzed to a significant extent in vivo, after icv 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 (unpublished data). Glycyl-D-glutamine also failed to prevent morphine-induced place preference conditioning and antinociceptive tolerance and so non-specific peptide interactions do not readily explain Gly-Gln’s inhibitory activity.

Nevertheless, Gly-Gln’s ability 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 non-specific effects would influence food conditioning as well. Control experiments also ruled out the possibility that Gly-Gln’s inhibitory activity is attributable to state dependent learning. Nevertheless, it is important to emphasize that Gly-Gln’s pharmacological effects have not been thoroughly investigated and alternative explanations for its efficacy remain viable.

It is interesting to note that Gly-Gln’s inability 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 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 (Scoubis et al., 2005).

As one might predict, β-endorphin1-27 also inhibits acquisition of a conditioned place preference to β-endorphin1-31 and other opioids (Bals-Kubik et al., 1988) and inhibits the rise in extracellular dopamine evoked by β-endorphin1-31 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 co-release β-endorphin1-27 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-endorphin1-31 to β-endorphin1-27 and β-endorphin1-26 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-endorphin1-27 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 delayed 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 earlier 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 Gly-Gln’s
ability 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 (Parish et al., 1983; Zakarian and Smyth, 1982) 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 al., 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 hours 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-endorphin1-31 or morphine analgesia (Owen et al., 2000) and because it fails to displace [3H]naloxone binding to rat brain membranes even at inordinately high concentrations (Unal et al., 1994). NovaScreen analysis (NIMH/NovaScreen Drug Discovery & Development Program) failed to detect any significant displacement by Gly-Gln of a wide variety of radioligands for opioid, glutamate, glycine, GABA or other receptors (unpublished data). Preliminary radioligand binding experiments showed that [3H]Gly-Gln binds to brain membrane preparations in a saturable and stereospecific manner, albeit with a relatively low affinity (Kd = 44 nM), and is not displaced by ligands for opioid receptors. It remains to be seen whether this [3H]Gly-Gln binding site represents a physiologically relevant receptor, however.

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 glycineB 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; Ossipov et al., 2004; Trujillo, 2000). To initially assess the possibility that Gly-Gln inhibits morphine withdrawal by blocking glycineB receptors, we tested whether D-serine, a glycineB receptor agonist (Witkin et al., 1997), would prevent Gly-Gln from suppressing morphine withdrawal symptoms. D-Serine (1 µmol icv) 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 naïve animals and, importantly, without interfering with morphine analgesia (Owen et al., 2000; Unal et al., 1997). Perhaps the most intriguing aspect of these findings, is the demonstration that a dipeptide synthesized from β-endorphin1-31, 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.
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Footnotes

This study was supported by DA018029 from the National Institute on Drug Abuse.
Legends for Figures

**Figure 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) icv followed, 2 min later, by morphine sulfate (2.5 mg/kg) or saline ip 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.

**Figure 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 icv followed, 2 min later, by morphine (2.5 mg/kg ip) 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.

**Figure 3:** The effect of Gly-Gln on the acquisition of morphine conditioned place preference is not state-dependent. Rats were treated with saline icv + morphine (2.5 mg/kg) ip or Gly-Gln (100 nmol) icv + morphine ip 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 ip 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 icv) + morphine (2.5 mg/kg ip), 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).

**Figure 4:** Glycyl-glutamine does not inhibit acquisition of a conditioned place preference to palatable food. Groups of 5 rats were treated with Gly-Gln (100 nmol) or saline icv 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.

**Figure 5:** Glycyl-glutamine inhibits expression of a conditioned place preference to morphine. Rats were treated with morphine sulfate (2.5 mg/kg) or saline ip 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 ip and confined to the alternate chamber for 30 min. On day 7, they were treated with Gly-Gln (100 nmol) or saline icv 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.
**Figure 6:** Glycyl-glutamine pretreatment delays the development of morphine tolerance. Panel A: Rats were treated with Gly-Gln (100 nmol) or saline icv followed, 2 min later, by morphine (10 mg/kg) or saline ip twice daily for 7 days. Panel B: Rats received Gly-D-Gln (100 nmol) or saline icv followed by morphine (10 mg/kg) ip 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.

**Figure 7:** Glycyl-glutamine reverses morphine tolerance. Two groups of 6 rats were treated with saline icv followed by morphine sulfate (10 mg/kg ip) twice daily for 4 days. On days 5 - 8 one group was pretreated with Gly-Gln (100 nmol icv; 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.

**Figure 8:** Gly-Gln 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 icv followed, 5 min later, by naloxone (1 mg/kg) sc 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 bars; n = 7) or Gly-Gln (hatched bars; 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.

**Figure 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 icv followed, 5 min later, by naloxone (1 mg/kg) sc and wet dog shakes (upper panel) and teeth chattering (lower panel) 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.

**Figure 10:** Gly-Gln 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 icv followed, 5 min later, by naloxone (1 mg/kg) sc. 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.
Table 1. Pretreatment with glycine and glutamine or glycyl-D-glutamine does not inhibit acquisition of a place preference to morphine.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Preference (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline/Saline (5)</td>
<td>26 ± 19</td>
</tr>
<tr>
<td>Saline/Morphine (6)</td>
<td>316 ± 74***</td>
</tr>
<tr>
<td>Gly + Gln/Saline (5)</td>
<td>52 ± 35</td>
</tr>
<tr>
<td>Gly + Gln/Morphine (6)</td>
<td>332 ± 25***</td>
</tr>
<tr>
<td>Gly-D-Gln/Saline (3)</td>
<td>30 ± 12</td>
</tr>
<tr>
<td>Gly-D-Gln/Morphine (5)</td>
<td>309 ± 39***</td>
</tr>
</tbody>
</table>

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) icv followed, 2 min later, by saline or morphine sulfate (2.5 mg/kg) ip and were confined in a conditioning chamber for 30 min; days 2, 4 and 6 they were treated with saline ip 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.

*** $P<0.001$ compared to the corresponding saline treated controls.
Figure 2
Figure 3

![Graph showing the time (sec) for different conditioned and tested substances.]

- Conditioned: Saline Morphine, Gly-Gln Morphine, Gly-Gln Morphine, Gly-Gln Morphine, Gly-Gln Morphine
- Tested: Gly-Gln Morphine, Gly-Gln, Morphine
Figure 4

The figure shows a bar chart comparing the time (in seconds) taken by two groups of animals under two conditions: Saline and Gly-Gln. The chart is divided into two sections, one for each condition.

- **Saline**:
  - Food paired: Approximately 350 seconds
  - No-food paired: Approximately 45 seconds
  - Statistical significance: **p < 0.01**

- **Gly-Gln**:
  - Food paired: Approximately 400 seconds
  - No-food paired: Approximately 35 seconds
  - Statistical significance: **p < 0.01**

The chart indicates that the Gly-Gln condition resulted in a significantly increased time for the food paired group compared to the saline condition, while the no-food paired group showed a slight decrease in time.
Figure 5

![Bar chart showing time (sec) for different treatment groups: Saline + Saline (5), Saline + Gly-Gln (5), Morphine + Saline (10), Morphine + Gly-Gln (8). The bar for Morphine + Saline is significantly higher than the other groups.](chart.png)
Figure 8

- Wet Dog Shakes
- Weight Loss
- Teeth Chattering
- Diarrhea
- Mouth Movements
- Ptosis
- Defecation
- Penile Erection
- Withdrawal Score
Figure 10