Available online at www.sciencedirect.com
SCIENCE DIRECT
European Journal of Pharmacology 530 (2006) 95–102
www.elsevier.com/locate/ejphar

Glycyl-glutamine inhibits nicotine conditioned place preference and withdrawal

Gökhan Göktalay [a,b,c], Sinan Cavun [a,c], Mark C. Levendusky [a], Jonathan R. Hamilton [a], William R. Millington [a,b,*]

[a] Department of Basic and Pharmaceutical Sciences, Albany College of Pharmacy, Union University, 106 New Scotland Ave, Albany, NY 12208, USA
[b] Ion Technologies, Inc., 131 Wing Haven Circle, Winston-Salem, North Carolina, USA
[c] Department of Pharmacology and Clinical Pharmacology, Uludag University School of Medicine, Bursa, Turkey

Received 25 August 2005; received in revised form 8 November 2005; accepted 14 November 2005
Available online 20 December 2005

Abstract

Glycyl-glutamine (Gly-Gln) is an inhibitory dipeptide synthesized from β-endorphin$_{1-31}$. Previously, we showed that Gly-Gln inhibits morphine conditioned place preference, tolerance, dependence and withdrawal. In this study, we tested whether Gly-Gln's inhibitory activity extends to other rewarding drugs, specifically nicotine. Rats were conditioned with nicotine (0.6 mg/kg, s.c.) for four days and tested on day five. Glycyl-glutamine (100 nmol i.c.v.) inhibited acquisition and expression of a nicotine place preference significantly. Cyclo(Gly-Gln) (100 nmol i.c.v. or 25 mg/kg i.p.), a cyclic Gly-Gln derivative, blocked expression of nicotine place preference but Gly-D-Gln (100 nmol i.c.v.) was ineffective. To study nicotine withdrawal, rats were treated with nicotine (9 mg/kg/day) for seven days and conditioned place aversion was induced with mecamylamine (1 mg/kg, s.c.). Glycyl-glutamine blocked acquisition of place aversion to mecamylamine but not U50,488, a kappa opioid receptor agonist. Glycyl-glutamine thus inhibits the rewarding effects of nicotine and attenuates withdrawal in nicotine dependent rats.

Keywords: Opioid; Nicotine; β-endorphin; Dipeptide; Pro-opiomelanocortin; Conditioned place preference

1. Introduction

Nicotine is indirectly responsible for the premature death of nearly 5 million people annually worldwide but our ability to treat nicotine addiction is remarkably limited considering the severity of the health risk it entails (Ezzati and Lopez, 2003). A wide variety of treatments for nicotine addiction have been evaluated but with limited success (Lerman et al., 2005). Evidence that some aspects of nicotine addiction are mediated by opioid neurons (Walters et al., 2005; Zarrindast et al., 2003) suggests that naloxone and other opioid receptor antagonists may effectively reduce nicotine craving, withdrawal and recidivism. Preclinical investigations have, in fact, shown that naloxone blocks the acquisition (Zarrindast et al., 2003) and expression (Walters et al., 2005) of a conditioned place preference to nicotine, a measure of the rewarding and/or incentive properties of drugs and other stimuli (Tzschentke, 1998), and precipitates withdrawal in nicotine dependent animals (Malin et al., 1993). Nicotine fails to produce a conditioned place preference in mu opioid receptor knock-out mice (Berrendero et al., 2002; Walters et al., 2005), again suggesting that mu receptor activation mediates nicotine reward. The results of clinical trials with opiate receptor antagonists have been disappointing, however. Naloxone and naltrexone reportedly suppress craving for, and satisfaction from, cigarette smoking and reduce the number of cigarettes consumed by smokers in some (Epstein and King, 2004; Krishnan-Sarin et al., 2003; Rukstalis et al., 2005), but by no means all, short-term studies (Nemeth-Coslett and Griffiths, 1986; Sutherland et al., 1995). Data from long-term clinical trials have generally been negative (Covey et al., 1999; David et al., 2001; Wong et al., 1999).

* Corresponding author. Department of Basic and Pharmaceutical Sciences, Albany College of Pharmacy, Union University, 106 New Scotland Avenue, Albany, NY 12208, USA. Tel.: +1 518 694 7242; fax: +1 518 694 7202.
E-mail address: millingw@acp.edu (W.R. Millington).

0014-2999/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2005.11.034

In this study, we tested the hypothesis that nicotine place preference and withdrawal can be inhibited with glycyl-glutamine (Gly-Gln). Glycyl-glutamine is an endogenous dipeptide synthesized through the post-translational processing of β-endorphin$_{1-31}$ (Loh, 1992; Parish et al., 1983). β-Endorphin$_{1-31}$ is extensively processed to Gly-Gln, as well as β-endorphin$_{1-26}$, β-endorphin$_{1-27}$ and their N-terminally acetylated analogs, in the brain, pituitary gland and peripheral tissues that express the pro-opiomelanocortin (POMC) gene (Loh, 1992; Raffin-Sanson et al., 2003; Smith and Funder, 1988). Glycyl-glutamine and other post-translationally derived β-endorphin peptides display little or no affinity for opioid receptors (Akil et al., 1981; Loh, 1992; Unal et al., 1994) with the exception of β-endorphin$_{1-27}$ which is a relatively potent opioid receptor antagonist that blocks the antinociceptive (Nicolas and Li, 1985) and reinforcing effects of opioids (Bals-Kubik et al., 1988). By eliminating the opioid agonist potency of β-endorphin$_{1-31}$, post-translational processing apparently converts POMC neurons to a non-opioid phenotype.

This concept is supported by reports that Gly-Gln inhibits some of the pharmacological effects produced by opioids. Glycyl-glutamine attenuates the hypotension and respiratory depression caused by central β-endorphin$_{1-31}$ or morphine administration (Unal et al., 1994, 1997; Owen et al., 2000) and inhibits the grooming response produced by β-endorphin$_{1-31}$ (Hirsch and O'Donohue, 1986). It does not inhibit morphine antinociception, however, even at inordinately high doses (Owen et al., 2000). When given alone to opiate naïve animals, Gly-Gln has no discernable effect on respiration, cardiovascular function or nociceptive response latencies (Owen et al., 2000). These findings are consistent with data showing that β-endorphin$_{1-31}$ is extensively processed to Gly-Gln and other non-opioid β-endorphin peptides in brainstem areas that regulate cardiovascular and respiratory function (Dores et al., 1986; Parish et al., 1983; Zakarian and Smyth, 1982) and extend earlier evidence that Gly-Gln functions as a brain neurotransmitter (Haynes, 1991; Parish et al., 1983).

Recently we extended these findings by testing whether Gly-Gln would counteract the addictive properties of morphine. We found that Gly-Gln blocked the acquisition and expression of a conditioned place preference to morphine without producing place preference or aversion when given to otherwise untreated animals (Cavun et al., 2005). Glycyl-glutamine did not interfere with the acquisition of a conditioned place preference to palatable food, suggesting that its inhibitory activity is not readily attributable to non-specific effects on memory, state-dependent learning or conditioned place aversion. Glycyl-glutamine also delayed the onset of morphine tolerance, inhibited the development of morphine dependence and suppressed somatic withdrawal symptoms in morphine dependent rats (Cavun et al., 2005).

Here, we report that Gly-Gln prevents the acquisition and expression of a conditioned place preference to nicotine and blocks mecamylamine-induced conditioned place aversion in nicotine dependent rats (Suzuki et al., 1996). These data indicate that Gly-Gln's ability to inhibit drug reward and withdrawal is not limited to opioids.

2. Materials and methods

2.1. Animals and surgery

Male Sprague–Dawley rats (250–300 g; Charles River Laboratories, Wilmington, MA) were housed in groups of three in a temperature controlled room with free access to food and water under a 12 h light/dark cycle. Each rat was anesthetized with 4% halothane and maintained with 1.5% halothane in 100% O_2 and a 23 gauge stainless steel guide canulla (Plastics One, Roanoke, VA) was implanted in the lateral ventricle 1.3 mm lateral and 0.9 mm posterior to bregma with the tip 4.0 mm below the skull surface. The cannula was fixed to the skull with dental acrylic. The 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 intracerebroventricular (i.c.v.) injections, a 28 gauge stainless steel injection cannula was connected to a 10 μl Hamilton syringe with polyethylene tubing and inserted through the guide cannula. Peptides were dissolved in 5 μl 0.9% saline (pH 7.3) and injected at a constant rate over a 1 min time period. The injection was monitored by observing the movement of an air bubble placed in the tubing. The injection cannula was left in the place for 30 s after the injection was completed. At the end of each experiment the canulla placement was confirmed by injecting 5 μl Chicago sky blue dye i.c.v. and inspecting the ventricles for presence of the dye.

2.2. Conditioned place preference

2.2.1. Apparatus

Place preference conditioning was conducted in a three-chambered apparatus (Cavun et al., 2005; Slusher et al., 2001; Tzschentke, 1998). The two conditioning chambers were identical in size (30 × 30 × 35 cm) but were distinguishable both visually and tactually. The interior of one chamber was painted white and the floor was covered with wood chips whereas the second 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 (30 × 12 × 35 cm) painted a neutral gray color which served as the entrance to the conditioning chambers during place preference testing. Untreated rats did not display a preference for either chamber and, when allowed to freely explore the place preference apparatus, spent approximately the same amount of time in each of the two conditioning chambers (Cavun et al., 2005).

2.2.2. Experimental procedure

The conditioned place preference procedure was conducted in three phases; habituation, conditioning and testing. During the habituation phase, the animals were handled and habituated

to the test room daily for three days. On the final day of habituation they were placed in the place preference apparatus and allowed to explore the chambers for 15 min and the amount of time each rat spent in the two chambers was recorded using a camera system.

Conditioning sessions were conducted twice a day, beginning at 8:00 am and 3:00 pm, for four days (Calcagnetti and Schechter, 1992). Rats were treated with nicotine tartrate (0.6 mg/kg) or saline s.c. and confined to one of the two conditioning chambers for 30 min. The nicotine dose was selected from published dose–response studies (Berrendero et al., 2002; Fudala and Iwamoto, 1986). One-half of the animals were conditioned with nicotine during the morning conditioning session and saline during the afternoon session and the other half were conditioned with saline during the morning conditioning session and nicotine during the afternoon session. They were randomly assigned to the two conditioning chambers; half of the rats were conditioned with nicotine in the striped chamber and saline in the white chamber and half were conditioned with nicotine in the white chamber and saline in the striped chamber. Control animals were treated with saline (1 ml/kg) s.c. before each of the eight conditioning sessions.

Rats were tested on day five approximately 24 h after the final conditioning session. The sliding doors separating the entrance chamber from the conditioning chambers were opened, each animal was placed in the entrance chamber and allowed to move freely between the two conditioning chambers for 15 min and the amount of time spent in each chamber was recorded. Data are presented as the difference in the amount of time rats spent in the two conditioning chambers during place preference testing.

2.2.3. Acquisition

To study the effect of Gly-Gln on the acquisition of a conditioned place preference to nicotine, rats were treated with Gly-Gln (100 nmol) or saline i.c.v. immediately before each conditioning session. The animals were randomly assigned to one of four treatment groups: Gly-Gln+nicotine, saline +nicotine, Gly-Gln+saline and saline+saline. They were treated with Gly-Gln (100 nmol) or saline i.c.v. and, two min later, they were given nicotine (0.6 mg/kg) or saline s.c. and confined to the appropriate conditioning chamber for 30 min. On day five, each rat was placed in the entrance chamber, allowed to freely explore the two conditioning chambers for 15 min and the amount of time spent in each chamber was recorded.

2.2.4. Expression

To study the effects of Gly-Gln and related peptides on the expression of a conditioned place preference, rats were conditioned with nicotine (0.6 mg/kg) or saline s.c. for four days. On the test day, they were given a single dose of Gly-Gln (3, 30 or 100 nmol), glycyl-D-glutamine (Gly-D-Gln; 100 nmol) or saline i.c.v. 5 min before place preference testing. Rats were then placed in the entrance chamber, allowed to explore the two conditioning chambers for 15 min and the amount of time spent in each chamber was recorded.

2.2.5. Cyclo(Gly-Gln)

Gly-Gln does not cross the blood–brain barrier (Himmelseher et al., 1996; Vazquez et al., 1992) and must be injected centrally. To circumvent this necessity, we tested whether cyclo (Gly-Gln), a cyclic Gly-Gln derivative (Unal et al., 1997), would inhibit expression of nicotine place preference after either i.c.v. or i.p. administration. Rats were conditioned with nicotine (0.6 mg/kg) or saline s.c. for four days. On day five they were pretreated with cyclo(Gly-Gln) (100 nmol) or saline i.c.v. 5 min before place preference testing or cyclo(Gly-Gln) (25 mg/kg) i.p. 30 min before place preference testing. They were then allowed to freely explore the two conditioning chambers and the amount of time spent in each chamber was recorded.

2.3. Conditioned place aversion

2.3.1. Nicotine

A conditioned place aversion to nicotine was induced by treating nicotine dependent rats with the nicotinic receptor antagonist mecamylamine. Nicotine dependence was produced by implanting an Alzet osmotic minipump with a flow rate of 1 μl/h (Model 2001, Durect Corporation, Cupertino, CA) s.c. between the two scapulas under halothane anesthesia. The osmotic minipump delivered nicotine tartrate at a rate of 9 mg/kg/day (Suzuki et al., 1996). Controls were implanted with an osmotic minipump containing saline.

To produce a conditioned place aversion, two conditioning sessions were conducted on the seventh day of nicotine treatment beginning at 8:00 a.m. and 3:00 p.m.. During the morning conditioning session, rats were treated with saline s.c. and were confined in one of the two conditioning chambers for 60 min. During the afternoon conditioning session they were treated with mecamylamine hydrochloride (1 mg/kg) s.c. and were confined in the other conditioning chamber for 60 min. Control animals received saline before both the morning and afternoon conditioning sessions. The following day each rat was placed in the entry chamber and allowed to move freely between the two conditioning chambers for 15 min and the amount of time spent in each chamber was recorded.

To test whether Gly-Gln inhibits the acquisition of a conditioned place aversion, nicotine dependent rats were treated with Gly-Gln (100 nmol) or saline i.c.v. 2 min before they received mecamylamine during conditioning. Controls were injected with Gly-Gln (100 nmol) or saline i.c.v. and were treated with saline s.c., instead of mecamylamine, during place conditioning. They were tested the following day as described above.

2.3.2. U50,488

U50,488-induced place aversion was conducted using a similar protocol except that rats were conditioned with U50,488 for three days (Skoubis et al., 2005; Suzuki et al., 1993). During each morning conditioning sessions rats were pretreated with Gly-Gln (100 nmol) or saline i.c.v. and, 2 min later, they were injected with saline s.c. and placed in one of the conditioning chambers for 30 min. During the afternoon conditioning sessions they were pretreated with Gly-Gln (100 nmol) or

saline i.c.v. 2 min before they were given U50,488 (1 mg/kg) s.c. and confined to the other conditioning chamber for 30 min. On day 4, the animals were allowed to move freely between the two conditioning chambers for 15 min and the amount of time spent in each chamber was recorded.

2.4. Drugs

Glycyl-glutamine, nicotine tartrate, mecamylamine hydrochloride, U50,488 (*trans*-1*S*,2*S*)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl] benzeneacetamide hydrochloride) and halothane were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Glycyl-D-glutamine and cyclo(Gly-Gln) were obtained from Bachem Bioscience, Inc. (King of Prussia, PA, USA).

2.5. Data analysis

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

3. Results

3.1. Conditioned place preference

3.1.1. Acquisition

Rats conditioned with nicotine tartrate (0.6 mg/kg s.c.) for four days spent significantly more time in the nicotine-paired chamber than in the saline-paired chamber during place preference testing (Fig. 1). A lower dose of nicotine (0.4 mg/kg s.c.) also produced a significant place preference response (180±55 s; $n=5$). Saline treated control animals did not display a preference for either chamber (Fig. 1). These data confirm that nicotine produces a conditioned place preference at an appropriate dose as shown previously (Berrendero et al., 2002; Calcagnetti and Schechter, 1994; Dewey et al., 1999; Fudala and Iwamoto, 1986; Risinger and Oakes, 1995).

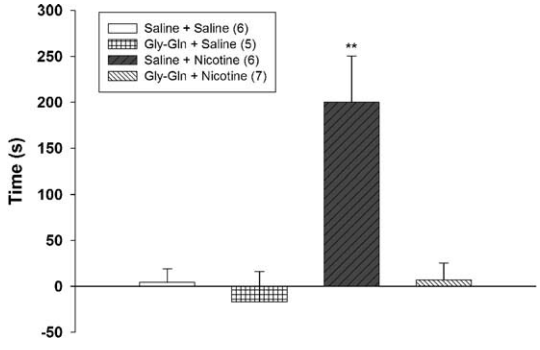

Fig. 1. Glycyl-glutamine inhibits acquisition of a conditioned place preference to nicotine. Rats were conditioned with Gly-Gln (100 nmol) or saline i.c.v. followed, 2 min later, by nicotine tartrate (0.6 mg/kg) or saline s.c. The columns represent the difference in the amount of time animals spent in the two conditioning chambers during place preference testing. The numbers in parentheses indicate the number of animals in each group. Data were analyzed with analysis of variance followed by the Newman–Keuls test. $^{**}P<0.01$ differs from saline treated control animals.

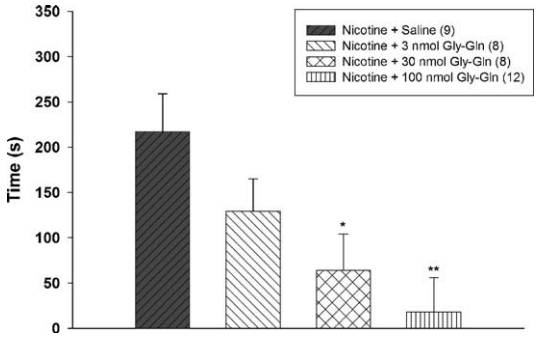

Fig. 2. Gly-Gln inhibits expression of nicotine-induced conditioned place preference. Rats were conditioned with nicotine tartrate (0.6 mg/kg; s.c.) and were given the indicated dose of Gly-Gln or saline i.c.v. 5 min before place preference testing. Data represent the difference in the amount of time spent in the two conditioning chambers during place preference testing and were analyzed with analysis of variance followed by Newman–Keuls test. $^{*}P<0.05$ and $^{**}P<0.01$ differ from nicotine+saline conditioned animals.

Pretreatment with Gly-Gln (100 nmol i.c.v.) inhibited the acquisition of a conditioned place preference to nicotine significantly [$F(1,20)=8.1$, $P=0.01$] (Fig. 1). Animals pretreated with Gly-Gln before conditioning with nicotine spent approximately the same amount of time in the two chambers during testing as did saline-conditioned animals. Control animals treated with Gly-Gln, but not nicotine, did not show a preference for either chamber during place preference testing (Fig. 1).

3.1.2. Expression

To test whether Gly-Gln would inhibit expression of a pre-established conditioned place preference to nicotine, rats were conditioned with nicotine tartrate (0.6 mg/kg) or saline s.c. for four days and given a single dose of Gly-Gln (3–100 nmol) or saline i.c.v. 5 min before place preference testing on day five. Glycyl-glutamine inhibited expression of a pre-established place preference to nicotine significantly [$F(3,33)=5.1$, $P<0.01$] (Fig. 2). The response was dose-dependent and was not reproduced by the Gly-Gln stereoisomer Gly-D-Gln (100

Table 1
Gly-D-Gln does not inhibit expression of a nicotine place preference

Treatment	Time (s)
Saline+saline (6)	9±16
Nicotine+saline (9)	217±42[a]
Saline+Gly-Gln (5)	7±17
Nicotine+Gly-Gln (12)	18±38
Saline+Gly-D-Gln (4)	6±36
Nicotine+Gly-D-Gln (7)	207±25[a]

Rats were conditioned with nicotine tartrate (0.6 mg/kg) or saline s.c. for four days and were given a single injection of saline, Gly-Gln (100 nmol) or Gly-D-Gln (100 nmol) i.c.v. 5 min before place preference testing on day five. Numbers in parentheses indicate the number of animals in each group. The data represent the time spent in the nicotine-paired chamber minus the time spent in the vehicle-paired chamber and were analyzed by Student's *t*-test. [a]$P<0.01$ differs from saline treated control animals.

nmol) (Table 1). Neither Gly-Gln nor Gly-D-Gln had any effect on place preference responding when given alone to rats conditioned with saline, rather than nicotine (Table 1). Together, these experiments show that Gly-Gln inhibits both the acquisition and expression of a nicotine conditioned place preference but does not produce either place preference or aversion when given to otherwise untreated animals.

3.1.3. Cyclo(Gly-Gln)

Fig. 3 shows that cyclo(Gly-Gln) administration also inhibited the expression of a pre-established nicotine place preference significantly [$F(2,34)=8.8$, $P<0.001$]. Intracerebroventricular cyclo(Gly-Gln) (100 nmol) treatment suppressed place preference responding to approximately the same extent as an equimolar i.c.v. dose of Gly-Gln (Figs. 2 and 3). After i.p. administration, cyclo(Gly-Gln) (25 mg/kg) inhibited expression of nicotine place preference significantly (Fig. 3) but it had no effect on place preference responding when given to rats conditioned with saline in lieu of nicotine. These data show that cyclo(Gln-Gln) inhibits the expression of a conditioned place preference to nicotine and extend earlier evidence that cyclo (Gln-Gln) is capable of reproducing the central effects of the linear dipeptide after peripheral administration (Unal et al., 1997).

3.2. Conditioned place aversion

Conditioned place aversion was used to investigate whether Gly-Gln inhibits withdrawal in nicotine dependent rats. Rats conditioned with mecamylamine (1 mg/kg s.c.) spent significantly less time in the mecamylamine-paired chamber than in the saline-paired chamber during place preference testing (Fig. 4) as shown previously (Watkins et al., 2000; Suzuki et al., 1996). Mecamylamine administration did not produce either a place preference or place aversion in control animals that were treated chronically with saline instead of nicotine for seven days (Fig. 4).

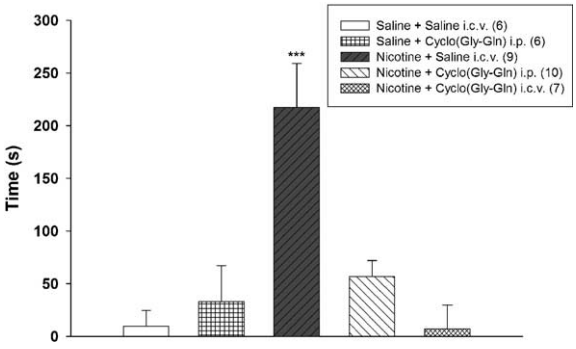

Fig. 3. Cyclo(Gly-Gln) inhibits expression of nicotine-induced conditioned place preference. Rats were conditioned with nicotine tartrate (0.6 mg/kg) or saline s.c. and were treated with cyclo(Gly-Gln) (100 nmol) or saline i.c.v. 5 min before place preference testing or with cyclo(Gly-Gln) (25 mg/kg) i.p. 30 min before testing. The columns represent the difference in the amount of time animals spent in the two conditioning chambers during place preference testing and were analyzed by analysis variance followed by Newman–Keuls test. ***$P<0.001$ differs from nicotine+saline treated animals.

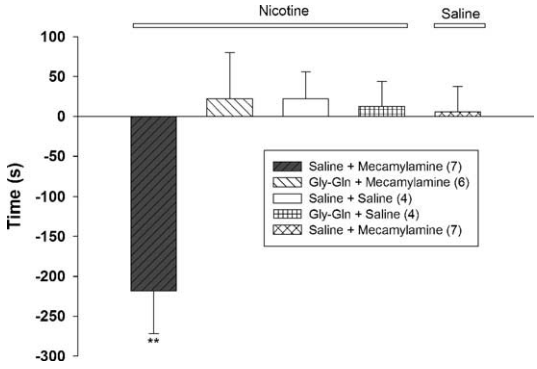

Fig. 4. Gly-Gln inhibits mecamylamine-induced conditioned place aversion in nicotine dependent rats. Rats were administered nicotine tartrate (9 mg/kg per day) or saline for seven days with an osmotic minipump. On day seven they were conditioned with Gly-Gln (100 nmol) or saline i.c.v. followed, 2 min later, by mecamylamine (1 mg/kg) or saline s.c. and were tested the following day. Data represent the time spent in the mecamylamine-paired chamber minus the time spent in the saline-paired chamber during place preference testing and were analyzed by analysis of variance followed by the Newman–Keuls test. **$P<0.01$ differs from saline treated control animals.

To test whether Gly-Gln would inhibit acquisition of mecamylamine-induced place aversion, nicotine dependent rats were treated with Gly-Gln (100 nmol) or saline i.c.v. 2 min before they were injected with mecamylamine or saline s.c. during place preference conditioning. Glycyl-glutamine pretreatment prevented acquisition of a conditioned place aversion completely [$F(1,17)=5.2$, $P<0.05$] (Fig. 4). The data in Fig. 4 show that rats pretreated with Gly-Gln before conditioning with mecamylamine spent approximately the same amount of time in both conditioning chambers during testing the following day, much like the response of control animals that did not receive mecamylamine. Glycyl-glutamine had no effect on nicotine dependent animals conditioned with saline, instead of mecamylamine (Fig. 4). Glycyl-glutamine thus prevents mecamylamine-induced place aversion in nicotine dependent animals but does not produce place preference or aversion in nicotine dependent rats that did not receive mecamylamine.

Subsequently, we investigated the possibility that Gly-Gln inhibits the behavioral response to aversive stimuli non-selectively, in a manner unrelated to nicotine withdrawal, by testing whether Gly-Gln pretreatment would prevent acquisition of a place aversion to the kappa opioid receptor agonist U50,488 in animals that were not dependent on nicotine (Skoubis et al., 2005; Suzuki et al., 1993). Table 2 shows that a

Table 2
Gly-Gln does not inhibit U50,488-induced conditioned place aversion

Treatment	Preference (s)
Saline+saline (6)	22±34
Gly-Gln+saline (5)	12±32
Saline+U50,488 (8)	−98±43
Gly-Gln+U50,488 (7)	−107±53

Rats were conditioned with Gly-Gln (100 nmol) or saline i.c.v. followed by U50,488 (1 mg/kg) or saline s.c. The data represent the time spent in the U50,488-paired chamber minus the time spent in the saline-paired chamber during place preference testing.

relatively low dose of U50,488 (1 mg/kg s.c.) produced place aversion after three days of conditioning as shown previously (Skoubis et al., 2005; Suzuki et al., 1993). Glycyl-glutamine (100 nmol i.c.v.) pretreatment immediately before each U50,488 conditioning session had no effect whatsoever on the subsequent place aversion displayed by U50,488 treated animals during testing on day four. As shown previously, Gly-Gln did not produce place preference or aversion when given to animals conditioned with saline, rather than U50,488. Together, these data indicate that Gly-Gln inhibits conditioned place aversion caused by mecamylamine-induced nicotine withdrawal but does not influence place aversion induced by U50,488.

4. Discussion

The main findings of this study are that Gly-Gln inhibits the acquisition and expression of nicotine conditioned place preference, a measure of the rewarding or incentive effect of nicotine (Tzschentke, 1998), and prevents mecamylamine-induced conditioned place aversion in nicotine dependent rats, a test of negative motivational or affective aspects of nicotine withdrawal (Suzuki et al., 1996). The data extend earlier evidence that Gly-Gln inhibits morphine place preference and suppresses somatic signs of morphine withdrawal (Cavun et al., 2005) and suggest that Gly-Gln's inhibitory activity is not restricted to opioids, despite its physiological derivation from β-endorphin$_{1-31}$.

Glycyl-glutamine, itself, had no discernible behavioral effect in these experiments. Specifically, it did not produce either place preference or place aversion when administered to rats daily, before conditioning with saline, or acutely, immediately before place preference testing. This means that Gly-Gln's ability to block place preference responding does not result from the countervailing induction of a place aversion. It is also notable that Gly-Gln did not produce place aversion in nicotine dependent animals and so evidently does not precipitate nicotine withdrawal. This differentiates Gly-Gln from naloxone and other opioid receptor antagonists which induce conditioned place aversion in control animals (Mucha and Walker, 1987; Skoubis et al., 2001) and precipitate somatic withdrawal symptoms in nicotine dependent rats (Malin et al., 1993).

Nevertheless, Gly-Gln's ability to prevent nicotine place preference conditioning could be attributable to an effect on memory or other non-specific sensory, motor or behavioral mechanisms. In an earlier study, we found that Gly-Gln had no effect on the acquisition of a place preference to palatable food (Cavun et al., 2005) which makes it unlikely that Gly-Gln inhibits place preference conditioning non-specifically by interfering with memory consolidation or by compromising sensory or motor function. This prior study also showed that Gly-Gln's ability to block morphine place preference conditioning was not attributable to state-dependent learning (Cavun et al., 2005). Hydrolysis and subsequent activation of glycine receptors are also unlikely to explain its inhibitory activity because co-injection of equimolar amounts of glycine and glutamine does not reproduce the physiological (Owen et al., 1997; Unal et al., 1994) or behavioral (Cavun et al., 2005) effects of the intact dipeptide. The observation that Gly-D-Gln failed to inhibit nicotine or morphine (Cavun et al., 2005) place preference responding also implies that Gly-Gln's efficacy is not readily explained by non-specific peptide effects. Nevertheless, it is important to emphasize that Gly-Gln's pharmacological spectrum of activity has not been fully elucidated and alternative explanations for its efficacy can not be ruled out conclusively.

We also tested cyclo(Gly-Gln), a cyclic Gly-Gln analog, and found that it inhibited nicotine place preference responding following either i.c.v. or i.p. administration. This experiment was predicated on an earlier finding that cyclo(Gly-Gln) inhibits morphine-induced hypotension and respiratory depression after both i.c.v. and intra-arterial injection (Unal et al., 1997) and provides additional evidence that cyclo(Gly-Gln) crosses the blood–brain barrier and produces the same pharmacological effects as does the linear dipeptide. These findings are consistent with extensive evidence that cyclo(His-Pro) and other cyclic dipeptides are centrally active and capable of crossing the blood–brain barrier (Banks et al., 1993; Prasad, 1995). Cyclic dipeptides are also absorbed from the small intestine and appear to be relatively resistant to peptidase degradation (Gallo-Torres et al., 1984; Mizuma et al., 1998; Prasad, 1995; Tamura et al., 1996). Although the prospect that a cyclic dipeptide would reproduce the pharmacological effects of a linear peptide seems implausible, it is certainly not unprecedented (Sakurada et al., 1982) and molecular modeling (Unal et al., 1997) and crystallographic analysis (Caira et al., 2002) indicate that Gly-Gln and cyclo(Gly-Gln) are capable of assuming similar conformations. The current data thus provide further evidence that cyclo(Gly-Gln) is capable of reproducing the effects of Gly-Gln following both i.c.v. and i.p. administration and support the feasibility of developing Gly-Gln analogs that are centrally active following peripheral administration.

Administration of a nicotine receptor antagonist to nicotine dependent animals produces somatic withdrawal symptoms and behavioral responses indicative of an aversive response (Malin et al., 1992; Suzuki et al., 1996; Watkins et al., 2000). Nicotine withdrawal also causes somatic withdrawal symptoms in humans although it is generally assumed that craving for nicotine and the dysphoric mood that accompanies nicotine withdrawal play a much more important role in nicotine recidivism than the relatively mild somatic symptoms withdrawal produces (Hughes et al., 1991). Accordingly, we studied nicotine withdrawal by using conditioned place aversion, in which nicotine dependent rats are conditioned to associate the dysphoria produced by mecamylamine precipitated withdrawal with specific environmental cues (Suzuki et al., 1996). We found that Gly-Gln pretreatment blocked acquisition of a conditioned place aversion to nicotine completely. It did not block U50,488-induced place aversion, however, and so its inhibitory activity is probably not due to a generalized suppression of the behavioral response to aversive stimuli. Glycyl-glutamine thus inhibits the rewarding effect of nicotine and apparently interferes with the behavioral expression of nicotine withdrawal.

These experiments were predicated, in part, on earlier evidence that Gly-Gln inhibits voluntary ethanol consumption by P rats selectively bred to consume large amounts of ethanol (Resch et al., 2005; Simpson et al., 1998). Opioid neurons are thought to play an important role in the incentive effects of ethanol and opioid receptor antagonists reduce ethanol drinking in both humans and laboratory animals (Herz, 1997; Volpicelli et al., 1992). Glycyl-glutamine reduced ethanol intake by 50% or more over a 24 h period when injected i.c.v. (Simpson et al., 1998) or directly into the nucleus accumbens (Resch et al., 2005), a brain region importantly involved in reward mechanisms. Once again, these data are consistent with the conclusion that Gly-Gln inhibits the rewarding properties of multiple addictive drugs, not just opioids.

These studies originated from the discovery that Gly-Gln is synthesized endogenously by POMC neurons and its mechanism of action in animal models of nicotine, opioid and ethanol reward and/or withdrawal is not known. Radioligand binding experiments have shown that Gly-Gln is incapable of displacing [^{3}H]-naloxone binding even at quite high concentrations (Unal et al., 1994) and NovaScreen analysis (NIMH/NovaScreen Drug Discovery and Development Program) failed to detect significant displacement of radioligands for opioid, GABA, glutamate, glycine, or a wide range of other receptors and uptake sites (unpublished data). Glycyl-glutamine's ability to inhibit morphine withdrawal symptoms is not reversed by D-serine, an agonist at the NMDA associated glycine binding site, and so its efficacy is not readily explained by blockade of NMDA receptors (Cavun et al., 2005). One might hypothesize that, like other neuropeptides, Gly-Gln interacts with a specific Gly-Gln receptor but this remains to be demonstrated.

Glycyl-glutamine has an interesting and potentially useful pharmacological profile. It essentially eliminates the rewarding effects of morphine, inhibits morphine-induced tolerance, dependence, and withdrawal and attenuates the cardiovascular and respiratory depression opioids produced without compromising opioid analgesia (Cavun et al., 2005; Owen et al., 2000; Unal et al., 1994, 1997). Here we report that Gly-Gln blocks the acquisition and expression of a conditioned place preference to nicotine and inhibits nicotine withdrawal. Glycyl-glutamine thus suppresses the addictive properties of both morphine and nicotine suggesting that it produces its inhibitory effects through a mechanism common to both drugs.

Acknowledgements

The authors thank Anne Crandall, Jaclyn Moore and Kristin Zindell for their technical assistance. This research was supported by a grant (DA018029) from the National Institute on Drug Abuse.

References

Akil, H., Young, E., Watson, S.J., Coy, D.H., 1981. Opiate binding properties of naturally occurring N- and C-terminus modified beta-endorphins. Peptides 2, 289–292.

Bals-Kubik, R., Herz, A., Shippenberg, T.S., 1988. β-Endorphin-(1–27) is a naturally occurring antagonist of the reinforcing effects of opioids. N.S. Arch. Pharmacol. 338, 392–396.

Banks, W.A., Kastin, A.J., Akerstrom, V., Jaspan, J.B., 1993. Radioactively iodinated cyclo(His-Pro) crosses the blood–brain barrier and reverses ethanol-induced narcosis. Am. J. Physiol. Endocrinol. Metab. 264, E723–E729.

Berrendero, F., Kieffer, B.L., Maldonado, R., 2002. Attenuation of nicotine-induced antinociception, rewarding effects, and dependence in μ-opioid receptor knock-out mice. J. Neurosci. 22, 10935–10940.

Caira, M.R., Buyukbingol, E., Adejare, A., Millington, W.R., 2002. Crystal structure of the dipeptide cyclo(glycyl-L-glutamine). Anal. Sci. 18, 1175–1176.

Calcagnetti, D.J., Schechter, M.D., 1992. Reducing the time needed to conduct conditioned place preference testing. Prog. Neuropsychopharmacol. Biol. Psychiatry 16, 969–976.

Calcagnetti, D.J., Schechter, M.D., 1994. Nicotine place preference using the biased method of conditioning. Prog. Neuropsychopharmacol. Biol. Psychiatry 18, 925–933.

Cavun, S., Göktalay, G., Millington, W.R., 2005. Glycyl-glutamine, an endogenous β-endorphin-derived peptide, inhibits morphine conditioned place preference, tolerance, dependence and withdrawal. J. Pharmacol. Exp. Ther. 315, 949–958.

Covey, L.S., Glassman, A.H., Stetner, F., 1999. Naltrexone effects on short-term and long-term smoking cessation. J. Addict. Dis. 18, 31–40.

David, S., Lancaster, T., Stead, L.F., 2001. Opioid antagonists for smoking cessation. Cochrane Database Syst. Rev. CD003086.

Dewey, S.L., Brodie, J.D., Gerasimov, M., Horan, B., Gardner, E.L., Ashby, C. R., 1999. A pharmacologic strategy for the treatment of nicotine addiction. Synapse 31, 76–86.

Dores, R.M., Jain, M., Akil, H., 1986. Characterization of the forms of β-endorphin and α-MSH in the caudal medulla of the rat and guinea pig. Brain Res. 377, 251–260.

Epstein, A.M., King, A.C., 2004. Naltrexone attenuates acute cigarette smoking behavior. Pharmacol. Biochem. Behav. 77, 29–37.

Ezzati, M., Lopez, A.D., 2003. Estimates of global mortality attributable to smoking in 2000. Lancet 362, 847–852.

Fudala, P.J., Iwamoto, E.T., 1986. Further studies on nicotine-induced conditioned place preference in the rat. Pharmacol. Biochem. Behav. 25, 1041–1049.

Gallo-Torres, H.E., Heimer, E.P., Witt, C., Miller, O.N., Meienhofer, J., Cheeseman, C.I., 1984. The in vivo gastrointestinal absorption in rats of the intact cyclo(L-leucylglycine) dipeptide. Can. J. Physiol. Pharmacol. 62, 319–328.

Haynes, L., 1991. β-Endorphin C-terminal peptide evokes arachidonic acid release from cortical neurones. Eur. J. Pharmacol. 192, 397–401.

Herz, A., 1997. Endogenous opioid systems and alcohol addiction. Psychopharmacology 129, 99–111.

Himmelseher, S., Pfenninger, E., Herrmann, P., 1996. Cerebrospinal and plasma amino acid concentrations after administration of i.v. glycyl-glutamine and glycyl-tyrosine containing amino acid solutions in humans. JPEN J. Parenter. Enteral Nutr. 20, 281–286.

Hirsch, M.D., O'Donohue, T.L., 1986. Structural modifications of pro-opiomelanocortin-derived peptides alter their behavioral effects markedly. J. Pharmacol. Exp. Ther. 237, 378–385.

Hughes, J.R., Gust, S.W., Skoog, K., Keenan, R.M., Fenwick, J.W., 1991. Symptoms of tobacco withdrawal. A replication and extension. Arch. Gen. Psychiatry 48, 52–59.

Krishnan-Sarin, S., Meandzija, B., O'Malley, S., 2003. Naltrexone and nicotine patch smoking cessation: a preliminary study. Nicotine Tob. Res. 5, 851–857.

Lerman, C., Patterson, F., Berrettini, W., 2005. Treating tobacco dependence: state of the science and new directions. J. Clin. Oncol. 23, 311–323.

Loh, Y.P., 1992. Molecular mechanisms of β-endorphin biosynthesis. Biochem. Pharmacol. 44, 843–849.

Malin, D.H., Lake, J.R., Newlin-Maultsby, P., Roberts, L.K., Lanier, J.G., Carter, V.A., Cunningham, J.S., Wilson, O.B., 1992. Rodent model of nicotine abstinence syndrome. Pharmacol. Biochem. Behav. 43, 779–784.

Malin, D.H., Lake, J.R., Carter, V.A., Cunningham, J.S, Wilson, O.B., 1993. Naloxone precipitates nicotine abstinence syndrome in the rat. Psychopharmacology 112, 339–342.

Mizuma, T., Masubuchi, S., Awazu, S., 1998. Intestinal absorption of stable cyclic dipeptides by the oligopeptide transporter in rat. J. Pharm. Pharmacol. 50, 167–172.

Mucha, R.F., Walker, M.J.K., 1987. Aversive property of opioid receptor blockade in drug-naive mice. Psychopharmacology 93, 483–488.

Nemeth-Coslett, R., Griffiths, R.R., 1986. Naloxone does not affect cigarette smoking. Naloxone does not affect cigarette smoking. Psychopharmacology 89, 261–264.

Nicolas, P., Li, C.H., 1985. β-Endorphin-(1–27) is a naturally occurring antagonist to etorphine-induced analgesia. Proc. Natl. Acad. Sci. U. S. A. 82, 3178–3181.

Owen, M.D., Gürün, M.S., Zaloga, G.P., Millington, W.R., 1997. Glycyl-L-glutamine [β-endorphin$_{30\text{-}31}$] attenuates hemorrhagic hypotension in conscious rats. Am. J. Physiol. 273, R1598–R1606.

Owen, M.D., Unal, C.B., Callahan, M.F., Triveda, K., York, C., Millington, W. R., 2000. Glycyl-glutamine inhibits the respiratory depression, but not the antinociception, produced by morphine. Am. J. Physiol. Regul., Integr. Comp. Physiol. 279, 1944–1948.

Parish, D.C., Smyth, D.G., Normanton, J.R., Wolstencroft, J.H., 1983. Glycyl glutamine, an inhibitory neuropeptide derived from β-endorphin. Nature 306, 267–270.

Prasad, C., 1995. Bioactive cyclic dipeptides. Peptides 16, 151–164.

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

Resch, G.E., Shridharani, S., Millington, W.R., Garris, D.R., Simpson, C.W., 2005. Glycyl-L-glutamine in nucleus accumbens reduces ethanol intake in alcohol preferring (P) rats. Brain Res. Brain Res. 1058, 73–81.

Risinger, F.O., Oakes, R.A., 1995. Nicotine-induced conditioned place preference and conditioned place aversion in mice. Pharmacol. Biochem. Behav. 51, 457–461.

Rukstalis, M., Jepson, C., Strasser, A., Lynch, K.G., Perkins, K., Patterson, F., Lerman, C., 2005. Naltrexone reduces the relative reinforcing value of nicotine in a cigarette smoking choice paradigm. Psychopharmacology 180, 41–48.

Sakurada, S., Sakurada, T., Jin, H., Sato, T., Kisara, K., Sasaki, Y., Suzuki, K., 1982. Antinociceptive activities of synthetic dipeptides in mice. J. Pharm. Pharmacol. 34, 750–751.

Simpson, C.W., Resch, R.E., Millington, W.R., Myers, R.D., 1998. Glycyl-L-glutamine injected centrally suppresses alcohol drinking in P rats. Alcohol 16, 101–107.

Skoubis, P.D., Matthes, H.W., Walwyn, W.M., Kieffer, B.L., Maidment, N.T., 2001. Naloxone fails to produce conditioned place aversion in mu-opioid receptor knock-out mice. Neuroscience 106, 757–763.

Skoubis, P.D., Lam, H.A., Shoblock, J., Narayanan, S., Maidment, N.T., 2005. Endogenous enkephalins, not endorphins, modulate basal hedonic state in mice. Eur. J. Neurosci. 21, 1379–1384.

Slusher, B.S., Thomas, A., Paul, M., Schad, C.A., Ashby, C.R., 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, A.I., Funder, J.W., 1988. Proopiomelanocortin processing in the pituitary, central nervous system, and peripheral tissues. Endocr. Rev. 9, 159–179.

Sutherland, G., Stapleton, J.A., Russell, M.A., Feyerabend, C., 1995. Naltrexone, smoking behavior and cigarette withdrawal. Psychopharmacology 120, 418–425.

Suzuki, T., Funada, M., Narati, M., Misawa, M., Nagase, H., 1993. Morphine-induced place preference in the CXBK mouse: characteristics of mu opioid receptor subtypes. Brain Res. 602, 45–52.

Suzuki, T., Ise, Y., Tsuda, M., Maeda, J., Misawa, M., 1996. Mecamylamine-precipitated nicotine-withdrawal aversion in rats. Eur. J. Pharmacol. 314, 281–284.

Tamura, K., Lee, C.P., Smith, P.L., Borchardt, R.T., 1996. Effect of charge on oligopeptide transporter-mediated permeation of cyclic dipeptides across Caco-2 cell monolayers. Pharm. Res. 13, 1752–1754.

Tzschentke, T.M., 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.

Unal, C.B., Owen, M.D., Millington, W.R., 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, C.B., Owen, M.D., Millington, W.R., 1997. Cyclo(Gly-Gln) inhibits the cardiorespiratory depression produced by β-endorphin and morphine. Brain Res. 747, 52–59.

Vazquez, J.A., Raghunath, M., Adibi, S.A., 1992. Uptake and hydrolysis of glycylglutamine at the blood–brain barrier. Metabolism 41, 121–124.

Volpicelli, J.R., Alterman, A.I., Hayashida, M., O'Brien, C.P., 1992. Naltrexone in the treatment of alcohol dependence. Arch. Gen. Psychiatry 49, 876–880.

Walters, C.L., Cleck, J.N., Kuo, Y.C., Blendy, J.A., 2005. μ-Opioid receptor and CREB activation are required for nicotine reward. Neuron 46, 933–943.

Watkins, S.S., Stinus, L., Koob, G.F., Markou, A., 2000. Reward and somatic changes during precipitated nicotine withdrawal in rats: centrally and peripherally mediate effects. J. Pharmacol. Exp. Ther. 292, 1053–1064.

Wong, G.Y., Wolter, T.D., Croghan, G.A., Croghan, I.T., Offord, K.P., Hurt, R. D., 1999. A randomized trial of naltrexone for smoking cessation. Addiction 94, 1227–1237.

Zakarian, S., Smyth, D.G., 1982. Distribution of β-endorphin-related peptides in rat pituitary and brain. Biochem. J. 202, 561–571.

Zarrindast, M.R., Faraji, N., Rostami, P., Sahraei, H., Ghoshouni, H., 2003. Cross-tolerance between morphine- and nicotine-induced conditioned place preference in mice. Eur. J. Pharmacol. 74, 363–369.