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Pharmacological Selectivity of CTAP in a Warm Water Tail-Withdrawal Antinociception Assay in Rats

Caren L. Steinmiller* and Alice M. Young^{#,✉}

*Department of Psychiatry and Behavioral Neuroscience, Wayne State University, Detroit MI 48202

[#]Departments of Pharmacology and Neuroscience, Texas Tech University Health Sciences Center, Lubbock TX 79430 and Psychology, Texas Tech University, Lubbock TX 79409

Abstract

Rationale—To facilitate *in vivo* characterization of the *mu* antagonist CTAP, the present study characterized CTAP selectivity *in vivo*

Objectives—CTAP, the classical antagonist naltrexone, the *kappa*-selective antagonist nor-BNI, and the *delta*-selective antagonist naltrindole were compared as antagonists of representative *mu*, *kappa*, and *delta* agonists in a warm water tail-withdrawal assay.

Methods—Male Sprague-Dawley rats were pretreated with CTAP (0.01 to 10.0 µg, *ICV*), naltrexone (0.1 to 10 mg/kg *SC*; 0.1 to 10 µg *ICV*), nor-BNI (1 mg/kg *SC*), or naltrindole (0.01 to 1 µg, *ICV*) and tested with cumulative doses of agonist in 50° C or 55° C tail-withdrawal assays.

Results—At 55° C, morphine and DAMGO produced dose-dependent antinociceptive effects that were antagonized by CTAP or naltrexone (*SC* or *ICV*) in a surmountable, dose-dependent manner. Neither *kappa* agonists (bremazocine, spiradoline, U69,593; all *SC*) nor the *delta* agonist DPDPE (*ICV*) produced antinociception at 55° C, but all produced full antinociception at 50° C. CTAP did not antagonize effects of spiradoline, U69,593, or DPDPE, whereas nor-BNI produced insurmountable antagonism of effects of *kappa* agonists, and naltrindole produced surmountable antagonism of effects of DPDPE. Apparent pA₂ estimates for naltrexone, CTAP, and naltrindole agreed with published estimates, although Schild slopes diverged from predictions for simple competitive antagonism.

Conclusions—CTAP produces dose-dependent antagonism selective for *mu* agonist effects in a standard 55° C tail withdrawal antinociceptive assay.

Keywords

Antinociception; CTAP selective drug antagonism; opioid analgesics

Introduction

Studies with competitive antagonists are a key tactic to identify functional roles of *mu* opioid systems. The classical opiate antagonists naloxone and naltrexone have high affinity for *mu* receptors, but only 3 to 100-fold selectivity for *mu* relative to *kappa* or *delta* opioid receptors (Magnan et al. 1982; Emmerson et al. 1994). Beta-funaltrexamine and naloxonazine are much more selective as *mu* antagonists (Zadina et al. 1997; Sakurada et al. 2000), but both are

✉Correspondence should be addressed to AMY: Department of Pharmacology and Neuroscience, Texas Tech University Health Sciences Center, 3601 4th Street STOP 6592, Lubbock TX 79430, Email address: alice.young@ttuhsc.edu, Phone: (806) 743-2425 x253, FAX: (806) 743-2744

irreversible ligands, with long-lasting effects in functional assays. Three octapeptides, CTP (D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH₂), CTOP (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂), and CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂), show promise as competitive *mu*-selective antagonists.

In binding assays, CTP, CTOP and CTAP show high affinity and exceptional selectivity for *mu* receptors (Pelton et al. 1986; Kazmierski et al. 1988). In functional assays in guinea pig ileum (GPI) and mouse vas deferens (MVD), CTOP and CTAP exert potent antagonist actions selective for *mu* receptors (Kramer et al. 1989; Mulder et al. 1991). CTP exerts potent and selective *mu* antagonism in the GPI but also agonist actions at *delta* opioid and/or somatostatin receptors in the MVD (Shook et al. 1987). *In vivo*, all three octapeptides display little agonist activity but rather act as competitive *mu* antagonists in noxious thermal antinociception assays (Guyla et al. 1988; Kramer et al. 1989). Of the three, CTAP shows the most promise as a useful selective competitive *mu*-antagonist devoid of intrinsic activity, displaying potent and highly selective *mu* receptor binding *in vitro* and *in vivo* (Gulya et al. 1986; Kazmierski et al. 1988) and functional antagonism in bioassays (Kramer et al. 1989).

Only limited information is available, however, about the ability of CTAP to antagonize *delta* or *kappa* receptor agonist effects *in vivo*. In radiant-heat tail-flick assays in mice and warm water tail-withdrawal and hotplate assays in rats, CTAP acts as a potent antagonist of antinociceptive effects of *mu* agonists (Kramer et al. 1989; He and Lee 1998; Sterious and Walker 2003), but also as an antagonist of antinociceptive effects of the *delta* agonist DPDPE in mice (Kramer et al. 1989; He and Lee 1998). Blockade of DPDPE effects may not reflect *delta* antagonist actions, however, as antagonism of DPDPE appeared noncompetitive in the hotplate assay (Kramer et al. 1989) and DPDPE effects in the tail-flick assay may be mediated via spinal *mu* opioid receptors (*cf* Matthes et al. 1996; Sora et al. 1997).

In order to characterize the selectivity of CTAP as an antagonist *in vivo*, we compared the ability of CTAP to act as an antagonist to antinociceptive effects of *mu*, *kappa* or *delta* opioid receptor agonists in a warm water tail-withdrawal assay in rats. Control experiments compared effects of CTAP to those of the classical antagonist naltrexone, the *kappa*-selective antagonist nor-binaltorphimine, and the *delta*-selective antagonist naltrindole. In addition, given the different chemical compositions of *mu* agonists (alkaloid versus peptide) and the constraints on their routes of administration, CTAP and naltrexone were examined for their ability to antagonize effects of morphine and DAMGO given *ICV*. The 55° C warm water tail-withdrawal assay was chosen because it readily identifies morphine-like narcotics (Janssen et al. 1963) and has greater sensitivity to analgesic actions of *mu* agonists as compared to *kappa* or *delta* agonists, which are relatively inactive (Adams et al. 1990; Adams et al. 1994) unless stimulus intensity is decreased. In light of this, a 50° C tail-withdrawal assay was used to assess antagonist effects against *kappa* or *delta* agonists.

Methods and Materials

Subjects

Male Sprague-Dawley rats (N=75; Harlan Breeding Laboratories, Indianapolis, IN) were housed individually in suspended cages with *ad libitum* water under a 12 hr light-dark cycle (0630 - 1830) in a temperature-(70 ± 5° C) and humidity-controlled (40-60%) room. Rats weighed approximately 300 g at the start of experiments and were fed a 15 to 18 g ration of Purina chow daily to allow growth to 330 - 380 g (mean of 350 g) and then maintenance of that weight. Rats were maintained in accordance with guidelines of the Institutional Animal Care and Use Committees of Wayne State University and Texas Tech University Health Sciences Center and The Guide for the Care and Use of Laboratory Animals (<http://www.nap.edu/readingroom/books/labrats/>).

Apparatus

Prior to initiation of studies, rats were trained to lie quietly in Harvard Apparatus rodent restraint tubes in a quiet room. Tap water was heated to 75° C in a Precision model 181 water bath; water temperatures of 40°, 50°, or 55° C were obtained by mixing heated and tap water in a Thermos® flask (diameter = 8 cm). Water temperature was measured by a Sontek model BAT-12 with a Bailey/Sontek Type T thermocouple. Tail-withdrawal latencies were observed visually and recorded with a hand-operated digital stopwatch (resolution = 1/100 sec).

Surgery

Rats were anesthetized with pentobarbital (25 mg/kg, *IP*) and ketamine (5 mg/kg, *IM*), and given atropine (0.4 mg/kg, *SC*) to reduce bronchial secretions. Each rat was placed in a small animal stereotaxic apparatus (David Kopf Instruments), and a 5.5 mm stainless steel cannula (22 gauge; Plastic Products, Inc.) was implanted in the left ventricle (coordinates: AP = -0.8; ML = 1.5; DV = -3.6 from bregma; Paxinos and Watson 1986). Cannula patency was verified one week after surgery and at approximately six-week intervals thereafter. A cannula was considered patent if a rat drank within 1 min and consumed at least 14 g of water in 0.5 h after 8 µg angiotensin II, *ICV*. Once a cannula tested as non-patent, a rat was not used for further *ICV* tests. Placement was verified by *ICV* injection of 1 µl India ink followed by euthanasia and gross dissection of coronal slices at the level of the lateral ventricles.

Tail-withdrawal Procedure

A cumulative dose test procedure, in which tail-withdrawal latency was measured in short successive trials (Walker et al. 1994), was used to estimate potency and maximal effects of agonists. A rat was weighed, placed into a restraint tube with tail hanging freely, and the distal 5 to 10 cm of the tail was immersed in water of the desired temperature. To prevent tissue damage, a cut-off time of 15 or 20 s was imposed at the 55° or 50° C temperature, respectively. If the rat did not withdraw its tail before the cut-off time elapsed, the experimenter removed the stimulus. At the start of every test, a temperature of 50° or 55° C was presented, followed, to control for tail withdrawal independent of water temperature, by two to four presentations of 40° C water at 2 min intervals. A rat that did not withdraw the tail within the cut-off interval on two out of four presentations of 40° C qualified for a test.

After qualifying for the test, the rat was removed from the restraint tube, injected with vehicle or an appropriate antagonist, and returned to the tube. After a 15 min pretreatment period (20 min for bremazocine), tail withdrawal latencies were measured at 55° C (or 50° C). At the end of the 5-min test period, a second injection was given. Cumulative doses of agonist were injected before this and all subsequent trials, with doses increased by 0.25 to 0.50 log units. Trials continued until a limit of cut-off time solubility, *ICV* volume, or antagonist duration was reached, or until another behavior (*i.e.*, convulsions) interfered with measurement.

Tests were conducted approximately every 7 days. At the outset, all rats received at least three tests of morphine, *SC*, at 55° C to determine reproducibility of effects, at least one test of consecutive vehicle injections to assess changes over successive trials, and a test of morphine after pretreatment with 0.1 mg/kg naltrexone, *SC*. Thereafter, tests of agonists alone alternated with tests preceded by injection of antagonists: Naltrexone (0.01, 0.1, 1.0, or 10.0 mg/kg, *SC*; 0.1, 1.0 or 10 µg/rat *ICV*; -15 min), CTAP (0.1, 1.0, or 10 µg, *ICV*; -15 min), nor-binaltorphimine (nor-BNI; 1.0 mg/kg, *SC*; -24 h), or naltrindole (1.0 µg, *ICV*; -15 min). In pilot experiments (not shown), all antagonists except nor-BNI were active within 15 min, and naltrexone had the shortest duration of action, which decayed after 90 min. Agonist dose-effect functions that required more than 6 trials (90 min) were determined by tests of overlapping dose ranges. Each rat participated in 12-20 tests over 6 months or until loss of cannula patency

and/or euthanasia for reasons unrelated to the experiment. Periodic tests of morphine alone were conducted to ascertain changes in agonist sensitivity.

Tests

To assess the ability of CTAP (*ICV*) to antagonize antinociceptive effects of *mu*, *kappa*, or *delta* agonists, effects of the *mu* agonists morphine (*SC* and *ICV*) and DAMGO (*ICV*), the *kappa*-selective agonists spiradoline, bremazocine, and U69,593 (all *SC*), and the *delta*-selective agonist DPDPE (*ICV*) were determined alone and after *ICV* pretreatment with CTAP. Representative agonists were also tested after *SC* or *ICV* pretreatment with the prototypic opioid antagonist naltrexone. Rats were randomly assigned to groups ($N = 5$ to 11), and each group received multiple tests either of CTAP and agonist, or naltrexone (*SC*, then *ICV*) and agonist. To the extent possible, agonists were studied in the following order: morphine (*SC*, then *ICV*), DAMGO; morphine, DAMGO, spiradoline; morphine, bremazocine, U69,593, morphine, DPDPE. To verify that *kappa* or *delta* agonists were indeed susceptible to a selective antagonist, rats tested with these agonists received a final test that utilized a single administration of a selective *kappa* or *delta* antagonist (nor-BNI or naltrindole).

Data analysis

Latencies were converted to percent maximum effect by the formula:

$$\%MPE = \frac{\text{test latency} - \text{control latency}}{\text{cut-off latency} - \text{control latency}} \times 100,$$

using the control latency measured at the beginning of each cumulative dose test. Each rat served as its own control.

All dose-response curves for individual rats, in absence or presence of antagonist, were fitted using the semilogarithmic form of the logistic dose-response equation:

$$E = E_{\min} + \frac{(E_{\max} - E_{\min}) \cdot 10^{(\text{Log}[A] \cdot h)}}{10^{(\text{Log}[ED_{50}] \cdot h)} + 10^{(\text{Log}[A] \cdot h)}}$$

where E is % MPE. Because equilibrium conditions cannot be ensured *in vivo*, analyses focused on changes in agonist potency to produce 50% MPE (Christopoulos et al. 1999), and $E_{\min}$ and $E_{\max}$ were set at 0 and 100. A is the dose of agonist (in mg/kg or $\mu\text{g}/\text{rat}$), ED_{50} is the agonist dose causing 50% MPE, and h is the Hill slope. For each test, $\log ED_{50}$ values were averaged across subjects and a 95% CL calculated with the t statistic. To assess magnitude and dose-dependency of antagonist effects, a potency ratio was calculated for each rat by dividing the ED_{50} in the presence of antagonist by the ED_{50} in the absence of antagonist.

For experiments in which agonists produced $\geq 50\%$ MPE when combined with antagonists, antagonist potencies were assessed with three procedures, with antagonist doses converted to moles/kg. First, Schild analyses of potency ratios for individual rats were used to derive apparent *in vivo* estimates of pA_2 , the negative logarithm of the mole/kg dose of antagonist that increases, by a factor of 2, the agonist dose required to produce a 50% MPE (Arunlakshana and Schild 1959; Tallarida 1982); fits of regression lines with unconstrained or -1 slopes were compared by F-test. Then, two alternative analyses were conducted because, although repeated tests of morphine (*SC*) revealed few time-dependent changes in sensitivity, parallel time controls were not conducted. Therefore, data were re-analyzed with two nonlinear techniques that do not require dose ratios. In the first (Lew and Angus 1995), agonist ED_{50} values and antagonist doses were used to fit the following equation

$$-\text{Log}[ED_{50}] = -\text{log}\left([B]^{\text{Schildslope}} + 10^{-pA_2} \cdot \text{Schildslope}\right) - P,$$

where B = antagonist dose, in moles/kg, and P is a fitting constant equal to the logarithm of the ratio of the control agonist EC₅₀ to the estimated pA₂/SchildSlope (Christopoulos *et al.* 1999). To test conformity to a model of competitive antagonism, the fit of this equation was compared by F-test to its fit when SchildSlope = 1. In the second nonlinear technique (Motulsky and Christopoulos 2003), % MPE data for each set of agonist-antagonist combinations were fit simultaneously to a modified Gaddum/Schild equation

$$E = E_{\min} + \frac{(E_{\max} - E_{\min})}{1 + \left\{ 10^{\text{Log}[ED_{50}]} \left[1 + (\text{Log}[B] / 10^{-pA_2})^{\text{schildslope}} \right] / \text{Log}[A] \right\}^h}$$

with variables as above. Note that in the absence of antagonist (Log[B]=0), this equation is the four-parameter logistic equation used to fit agonist dose-response data. Parallelism of agonist dose-response curves in presence and absence of antagonist was assessed by F-test, which compared a fit in which the h parameter was shared among each set of curves with a fit in which each curve was allowed its own h factor. F tests also tested whether the Schild slope for each set of curves differed from unity. For the rare sets of curves for which this was not the case, the curves were refitted with the SchildSlope parameter constrained to a value of 1. All data and statistical analyses were performed with GraphPad Prism (version 4.03 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com). P<0.05 was considered significant.

Drugs

Morphine sulfate, CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂), and naltrexone hydrochloride were obtained from the National Institute on Drug Abuse, Rockville, MD. DAMGO was purchased from Sigma Chemical Co., St. Louis, MO. Spiradoline mesylate, bremazocine, U69,593 (5alpha, 7alpha, 8beta-N-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl] benzeneacetamide), nor-binaltorphimine, DPDPE, and naltrindole were purchased from Research Biochemicals, Inc., Natick, MA. Drugs administered SC were dissolved in physiological saline, except for spiradoline, which was dissolved in sterile water. Solutions were prepared to administer each SC injection in a volume of 0.1 to 2.0 ml/100 g body weight, and injections were administered in the dorsal flank. Drugs administered ICV were dissolved in sterile water and were delivered *via* Hamilton syringe at a rate of 1 µl over 30 to 60 sec. Solutions were prepared to administer each ICV injection in a volume of 1.0 to 4.0 µl per injection, with the total volume per test not to exceed 20 µl.

RESULTS

Stability and reproducibility of agonist sensitivity

To assess reproducibility of tail withdrawal latencies, all rats received multiple tests of morphine, SC, before and after cannula implantation, and tests of consecutive vehicle injections (data not shown). These controls indicated that the potency of morphine decreased 2- to 3-fold over the first two or three tests but remained stable thereafter, and that sensitivity to antinociceptive effects of morphine differed among groups of rats. Tests of multiple vehicle injections demonstrated that the repeated injection procedure itself did not change latencies. Additionally, no differences were observed in maximal effects of morphine or its potency before or after surgical implantation of cannulae. Pretreatment with 0.1 mg/kg naltrexone increased the morphine ED₅₀ by 10 fold, indicating robust antagonism of antinociceptive effects of morphine in all rats.

Antagonism of *mu* agonist effects

When given SC, morphine produced dose-dependent antinociceptive effects in the 55° C assay, yielding maximal effects at 10.0 to 32 mg/kg (Figures 1 and 2, left). When given ICV, morphine

(center) and DAMGO (right) also produced dose-dependent effects, yielding maximal effects at 10 to 32 $\mu\text{g}/\text{rat}$. As expected, naltrexone given *SC* or *ICV* exerted surmountable, dose-dependent antagonism of antinociceptive effects of *mu* agonists, producing dose-related increases in doses of morphine or DAMGO required for effect, but no loss of maximal effect. Quantitative comparisons (Table 1) suggested that naltrexone potency varied with route of administration. First, naltrexone was more potent when given *ICV* than when given *SC*. Second, when given *ICV*, naltrexone was more potent against *mu* agonists given *ICV* than *SC*. These trends were evident independent of analysis, but comparisons utilizing dose ratios (*i.e.*, Schild analyses; Table 1, left) yielded larger confidence intervals than did comparisons using non-linear analyses of ED_{50} values (Table 1, center) or families of dose-effect functions (Table 1, right). In only one analysis was antagonism directly proportional to naltrexone dose (*i.e.*, described by a Schild slope of -1, indicative of simple competitive interaction), but all analyses yielded slopes indicative of antagonism.

ICV administration of CTAP also produced surmountable, dose-dependent antagonism of antinociceptive effects of morphine and DAMGO at 55° C (Figure 1, bottom). Quantitative comparisons (Table 1) suggested that CTAP was more potent as an antagonist of central than of peripheral morphine, accompanied, at only the highest dose, by a loss of maximal morphine effect. CTAP was more potent as an antagonist of morphine given *ICV* than of DAMGO given *ICV*, but latter data were highly variable. Antagonism was directly proportional to CTAP dose (*i.e.*, described by a Schild slope of -1) in only three instances, and in only one instance for all analyses of a combination, but all analyses yielded slopes indicative of antagonism.

Antagonism of *kappa* agonist effects

Kappa agonists did not exert antinociceptive effects at 55° C (not shown), so stimulus intensity was decreased to 50° C, at which all exerted full antinociceptive effects. Antinociceptive effects of peripherally administered spiradoline or U69,593 were not antagonized by any dose of CTAP, whereas those of bremazocine were antagonized only by the highest dose of CTAP (Figure 2, middle). Effects of bremazocine and spiradoline, but not those of U69,593, were antagonized by doses of naltrexone (Figure 2, top) higher than those effective against effects of *mu* agonists. Nor-BNI (1.0 mg/kg, *SC* -24 h; Figure 2, bottom) produced profound antagonism of antinociceptive effects of all *kappa* agonists.

As a check of antagonist actions of CTAP or naltrexone at 50° C, each was tested against morphine, given *SC*. Morphine elicited full antinociceptive effects, and both naltrexone (*SC*) and CTAP exerted surmountable antagonism. Naltrexone potency was lower at 50° C than at 55° C, whereas CTAP potency was slightly higher (Table 1). As a final test of whether rats tested with *kappa* agonists were sensitive to CTAP antagonism of *mu* effects, all were tested with morphine after pretreatment with 10 μg CTAP. CTAP increased the morphine ED_{50} by approximately 10-fold, yielding a pA_2 estimate similar to that from tests with a full range of CTAP doses.

Antagonism of *delta* agonist effects

DPDPE produced no antinociceptive effects at 55° C but full effects at 50° C. CTAP did not antagonize the antinociceptive effects of DPDPE at any dose (Figure 4). When given *SC*, naltrexone produced dose-dependent antagonism of DPDPE effects, with a potency lower than against *mu* agonists. Naltrindole, *ICV*, dose-dependently antagonized effects of DPDPE, and all analyses yielded similar potency estimates (Table 1).

Comparisons among analyses

Apparent *in vivo* pA_2 estimates for antagonists that produced surmountable antagonism of agonist effects were calculated by Schild analysis and by two non-linear techniques (Table 1).

Few analyses yielded Schild slopes of -1, but all with Schild slopes different from 0 yielded defined confidence intervals, indicating that our assay conditions were sensitive to dose-dependent effects of antagonists. Schild analysis, which uses dose ratios, yielded larger confidence intervals than did analyses focused on either agonist ED₅₀ doses or families of agonist dose-response functions. The latter analyses, which incorporated the most information from the dose-effect functions, yielded smallest confidence limits for both pA₂ values and slope.

DISCUSSION

CTAP dose-dependently antagonized *in vivo* antinociceptive effects of morphine and DAMGO, but not those of *kappa* or *delta* agonists in a warm water tail-withdrawal assay. Parallel experiments demonstrated that *kappa* and *delta* agonists were sensitive to appropriate selective antagonists, and that CTAP antagonized actions of morphine under assay conditions required for *kappa* or *delta* agonist activity. Taken together, these results suggest that CTAP acts as a selective *mu* opioid antagonist *in vivo*.

Initial experiments assessed antagonist effects of the classical antagonist naltrexone. Naltrexone dose-dependently antagonized antinociceptive effects of *mu*, *delta* and, with exception of U69,593, *kappa* agonists. As expected, potency of SC naltrexone, as assessed by apparent pA₂ values, was higher against *mu* agonists than against *kappa* or *delta* agonists. Potency estimates for SC naltrexone were similar to those reported for antagonism of antinociceptive effects of *mu* agonists in mice or rats (Garner et al. 1997; Sterious and Walker 2003) but lower than values reported for antagonism of effects of *kappa* agonists in mice or primates (Garner et al. 1997; Ko et al. 1998; Powell and Holtzman 1999). When given ICV rather than SC, naltrexone potency increased 30 to 300-fold as an antagonist of either SC morphine (apparent pA₂ values of 9.4 vs. 7.9) or ICV morphine (10.6 vs. 8.2), whereas potency as an antagonist of ICV DAMGO increased 10,000-fold (11.5 vs. 7.2). In contrast, Sterious and Walker (2003) reported no difference in potency of ICV or SC naltrexone as an antagonist of SC morphine under similar assay conditions. The lack of antagonism of effects of the *kappa* agonist U69,593 by even 10 mg/kg naltrexone was unexpected, in light of naltrexone antagonism of similar effects of bremazocine and spiradoline in the present study and reports that naltrexone is an effective antagonist of U69,593 effects in mice and primates (Millan 1989; Ko et al. 1998; Powell and Holtzman 1999) at doses effective against other *kappa* agonists.

CTAP dose-dependently antagonized antinociceptive effects of *mu*-opioid agonists but, with one exception, not of *kappa*- or *delta*-opioid agonists in the warm water tail-withdrawal assay. CTAP was nearly 100-fold more potent than SC naltrexone as an antagonist of SC morphine (pA₂ values of 9.8 vs. 7.9). CTAP was equi-potent to ICV naltrexone as an antagonist of SC morphine (9.8 vs. 9.4), but was 100-fold more potent as an antagonist of ICV morphine (12.4 vs. 10.6). Our results for CTAP were similar to those reported by Sterious and Walker (2003) for the same agonists and routes of administration, indicating greater CTAP potency *in vivo* than found *in vitro* assays such as the guinea pig ileum (Kramer et al. 1989).

The potency differences between naltrexone and CTAP as antagonists of *mu* agonists may arise from several sources. First, CTAP may possess higher affinity for receptors mediating antinociceptive effects of the *mu* agonists tested. Second, route of administration may impact potency estimates. Antinociceptive effects of *mu* opioid agonists are mediated through both spinal and supraspinal *mu* receptor populations (Narita et al 2002; Chow et al. 2004; Goodchild et al 2004), and central administration may increase the probability of drug-receptor interactions, thereby increasing apparent potency. Third, use of a broad range of antagonist doses against all agonists, done to minimize the likelihood of differences across agonists due

to direct effects, may have limited the applicability of the theoretical model. In particular, use of high antagonist doses may have revealed non-*mu* opioid, or even non-opioid, effects of agonists, leading to an underestimate of dose ratios for agonist effects (Zhang et al. 2000). Finally, it is unlikely that drugs were tested under equilibrium conditions. Schild slopes differed from -1, suggesting mechanisms other than one-to-one competition for a single receptor population. This argues caution in using *in vivo* apparent pA_2 values as estimates of affinity (cf Contrera et al. 1986), but does not preclude use of pA_2 values as empirical estimates of antagonist potency (cf Motulsky and Christopoulos 2003).

CTAP did not antagonize antinociceptive effects of the *kappa* agonists spiradoline or U69,593, and antagonized effects of bremazocine at doses 100 to 10,000 times higher than required to antagonize effects of *mu* agonists. *Kappa* agonists did not produce antinociceptive effects at 55° C, confirming reports that *kappa* agonists are less effective than *mu* agonists in the warm water tail-withdrawal assay when noxious stimulation is intense (Tyers 1980; Heyman et al. 1988; Fanselow et al. 1989; Millan 1989; Millan and Colpaert 1990; Adams et al. 1994). When stimulus intensity was reduced to 50° C, all *kappa* agonists elicited maximal antinociceptive effects, which were fully antagonized by the *kappa*-preferring antagonist nor-BNI. Antagonism by nor-BNI was insurmountable at the *kappa* agonist doses tested, and competing behaviors prevented tests of higher doses. CTAP acted to antagonize *mu* agonist effects in rats insensitive to CTAP antagonism of *kappa* agonist effects, ruling out individual differences as a source of inactivity against *kappa* agonists.

CTAP also did not antagonize antinociceptive effects of the *delta*-selective agonist DPDPE. DPDPE did not produce antinociceptive effects at 55° C, but produced full antinociceptive effects at 50° C. These effects were not antagonized by CTAP, but were dose-dependently antagonized by naltrexone at doses 10- to 100-times greater than those required for antagonism of *mu*-agonist effects. Effects of DPDPE were also antagonized by the *delta*-selective antagonist naltrindole, with a potency in agreement with published reports (Crook et al. 1992; Portoghesi et al. 1992; Chakrabarti et al. 1993; Stamidis and Young 1993; Tiseo and Yaksh 1993). In contrast with reports that *ICV* or intrathecal DPDPE may elicit antinociception *via* CTAP-sensitive sites in mice (Kramer et al. 1989; He and Lee, 1998), the potency of naltrindole, reduced potency of naltrexone, and lack of antagonism by CTAP in the present study suggest that antinociceptive effects of *ICV* DPDPE in the 50° C tail-withdrawal assay arose primarily from actions at *delta* sites.

Our analyses of antagonist potency used apparent *in vivo* pA_2 estimates derived from Schild analyses (Arunlakshana and Schild 1959) and two nonlinear techniques (Lew and Angus 1995; Motulsky and Christopoulos 2003). The latter techniques minimize contributions of agonist potency in the absence of antagonist to potency calculations and generate smaller confidence limits, and thus may be preferable for *in vivo* potency estimates. It is important to note that any use of apparent pA_2 values to describe potency of antagonists *in vivo* involves assumptions (Dykstra and Massie 1988) that the antagonist has no agonist effects in the test system, that agonist and antagonist interact in a surmountable, competitive manner, and that both are at equilibrium. Agonist effects were not observed for any antagonist in our assays, and shifts in agonist dose-effect functions were parallel (although rarely exactly proportional to antagonist dose), but our cumulative dosing procedures may not have matched times of peak drug activity.

For an antagonist with affinity for multiple receptor types, differences in relative antagonist potency can differentiate among agonists selective for a particular receptor subtype (Takemori 1973; Dykstra and Massie 1988), suggesting that differences in relative potency as measured by apparent pA_2 analysis are indicative of differences in receptors mediating a biological effect. Similarly, antagonists with high affinity for a receptor population have higher apparent pA_2

values for effects mediated *via* that receptor type as compared to antagonists with lower affinity for that receptor type (Bertalmio and Woods 1987; 1989). The present results suggest that the greater affinity of CTAP for *mu* as opposed to *kappa* or *delta* opioid receptors *in vitro* (Gulya et al. 1986; Kazmierski et al. 1988) results in selectivity of antagonist actions *in vivo*. Biochemical and *in vivo* experiments suggest that CTAP may function as a neutral antagonist, devoid of ability to precipitate abstinence in dependent organisms (Bilsky et al. 1996; Wang et al. 2001). While the present experiments did not evaluate the neutral antagonist profile of CTAP, the clear selectivity of CTAP antagonist actions will enable such characterization.

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Abbreviations

CTAP, Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂; CTP, D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH₂; GPI, guinea pig ileum; MVD, mouse vas deferens.

References

- Adams JU, Holtzman SG. Tolerance and dependence after continuous morphine infusion from osmotic pumps measured by operant responding in rats. *Psychopharmacology* 1990;100:451–458. [PubMed: 2320705]
- Adams JU, Geller EB, Adler MW. Receptor Selectivity of Icv Morphine in the Rat Cold Water Tail-Flick Test. *Drug Alcohol Depend* 1994;35:197–202. [PubMed: 7956748]
- Arunlakshana O, Schild HO. Some quantitative uses of drug antagonists. *Brit J Pharmacol* 1959;14:48–57.
- Bertalmio AJ, Woods JH. Differentiation between mu and kappa receptor-mediated effects in opioid drug discrimination: apparent pA₂ analysis. *J Pharmacol Exp Ther* 1987;243:591–597. [PubMed: 2824753]
- Bertalmio AJ, Woods JH. Reinforcing effect of alfentanil is mediated by mu opioid receptors: apparent pA₂ analysis. *J Pharmacol Exp Ther* 1989;251:455–460. [PubMed: 2553919]
- Bilsky EJ, Bernstein RN, Wang Z, Sadee W, Porreca F. Effects of naloxone and D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂ and the protein kinase inhibitors H7 and H8 on acute morphine dependence and antinociceptive tolerance in mice. *J Pharmacol Exp Ther* 1996;277:484–90. [PubMed: 8613958]
- Chakrabarti S, Sultana M, Portoghese PS, Takemori AE. Differential antagonism by naltrindole-5'-idothiocyanate on [3H]DSLET and [3H]DPDPE binding to striatal slices of mice. *Life Science* 1993;53:1761–1765.
- Christopoulos A, Parsons AM, Lew MJ, El-Fakahany EE. The assessment of antagonist potency under conditions of transient response kinetics. *Eur J Pharmacol* 1999;382:217–227. [PubMed: 10556673]
- Chow LH, Huang EY, Ho ST, Tsai SK, Tao PL. Dextromethorphan potentiates morphine-induced antinociception at both spinal and supraspinal sites but is not related to the descending serotonergic or adrenergic pathways. *J Biomed Sci* 2004;11:717–725. [PubMed: 15591767]
- Contreras ML, Wolfe BB, Molinoff PB. Kinetic analysis of the interactions of agonists and antagonists with beta adrenergic receptors. *J Pharmacol Exp Ther* 1986;239:136–143. [PubMed: 2876092]
- Crook TJ, Kitchen I, Hill RG. Effects of the delta-opioid receptor antagonist naltrindole on antinociceptive responses to selective delta-agonists in post-weanling rats. *Br J Pharmacol* 1992;107:573–576. [PubMed: 1330187]
- Dhawan BN, Cesselin F, Raghubir R, Reisine T, Bradley PB, Portoghese PS, Hamon M. International Union of Pharmacology. XII. Classification of opioid receptors. *Pharmacol Rev* 1996;48:567–92. [PubMed: 8981566]

- Dykstra LA, Massie CA. Antagonism of the analgesic effects of mu and kappa opioid agonists in the squirrel monkey. *J Pharmacol Exp Ther* 1988;246:813–821. [PubMed: 2843630]
- Emmerson PJ, Liu MR, Woods JH, Medzihradsky F. Binding affinity and selectivity of opioids at mu, delta and kappa receptors in monkey brain membranes. *J Pharmacol Exp Ther* 1994;271:1630–7. [PubMed: 7996478]
- Fanselow MS, Calcagnetti DJ, Helmstetter FJ. Role of mu and kappa opioid receptors in conditional fear-induced analgesia: the antagonistic actions of nor-binaltorphimine and the cyclic somatostatin octapeptides Cys2Tyr3Orn5Pen7-amide. *J Pharmacol Exp Ther* 1989;250:825–830. [PubMed: 2570868]
- Garner HR, Burke TF, Lawhorn CD, Stoner JM, Wessinger WD. Butorphanol-mediated antinociception in mice: partial agonist effects and mu receptor involvement. *J Pharmacol Exp Ther* 1997;282:1253–1261. [PubMed: 9316833]
- Goodchild CS, Nadeson R, Cohen E. Supraspinal and spinal cord opioid receptors are responsible for antinociception following intrathecal morphine injections. *Eur J Anaesthesiol* 2004;21:179–185. [PubMed: 15055889]
- Guyla K, Pelton JT, Hruba VJ, Yamamura HI. Cyclic somatostatin octapeptide analogues with high affinity and selectivity toward mu opioid receptors. *Life Sci* 1986;38:2221–2229. [PubMed: 2872570]
- Gulya K, Krivan M, Nyolczas N, Sarnyai Z, Kovacs GL. Central effects of the potent and highly selective mu opioid antagonist D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP) in mice. *Eur J Pharmacol* 1988;150:355–60. [PubMed: 2901358]
- He L, Lee NM. Delta opioid receptor enhancement of mu opioid receptor-induced antinociception in spinal cord. *J Pharmacol Exp Ther* 1998;285:1181–6. [PubMed: 9618421]
- Heyman JS, Williams CL, Burks TF, Mosberg HI, Porreca F. Dissociation of opioid antinociception and central gastrointestinal propulsion in the mouse: studies with naloxonazene. *J Pharmacol Exp Ther* 1988;245:238–243. [PubMed: 2834537]
- Janssen PAJ, Niemegeers CJE, Dony JGH. The inhibitory effect of fentanyl and other morphine-like analgesics on the warm water induced tail withdrawal reflex in rats. *Arzneimittel-Forschung Drug Research* 1963:502–507.
- Kazmierski W, Wire WS, Lui GK, Knapp RJ, Shook JE, Burks TF, Yamamura HI, Hruba VJ. Design and synthesis of somatostatin analogues with topographical properties that lead to highly potent and specific mu opioid receptor antagonists with greatly reduced binding at somatostatin receptors. *J Med Chem* 1988;31:2170–7. [PubMed: 2903246]
- Kramer TH, Shook JE, Kazmierski W, Ayres EA, Wire WS, Hruba VJ, Burks TF. Novel peptidic mu opioid antagonists: pharmacologic characterization in vitro and in vivo. *J Pharmacol Exp Ther* 1989;249:544–51. [PubMed: 2566679]
- Ko MC, Butelman ER, Traynor JR, Woods JH. Differentiation of kappa opioid agonist-induced antinociception by naltrexone apparent pA₂ analysis in rhesus monkeys. *J Pharmacol Exp Ther* 1998;285:518–26. [PubMed: 9580592]
- Lew MJ, Angus JA. Analysis of competitive agonist-antagonist interactions by nonlinear regression. *Trends Pharmacol Sci* 1995;16:328–37. [PubMed: 7491710]
- Magnan J, Paterson SJ, Tavani A, Kosterlitz HW. The binding spectrum of narcotic analgesic drugs with different agonist and antagonist properties. *Naunyn Schmiedeberg Arch Pharmacol* 1982;319:197–205. [PubMed: 6125900]
- Mansour A, Khachaturian H, Lewis ME, Akil H, Watson SJ. Anatomy of CNS opioid receptors. *Trends Neurosci* 1988;11:308–14. [PubMed: 2465635]
- Martin WR, Eades CG, Thompson JA, Huppler RE, Gilbert PE. The effects of morphine- and nalorphine-like drugs in the nondependent and morphine-dependent chronic spinal dog. *J Pharmacol Exp Ther* 1976;197:517–32. [PubMed: 945347]
- Matthes HWD, Malsonado R, Simonin R, Walverde O, Slowe S, Kitchen I, Befort K, Dierich A, Kieffer BL. Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid receptor gene. *Nature* 1996;383:819–823. [PubMed: 8893006]

- Millan MJ, Czlonkowski A, Lipkowski A, Herz A. Kappa-opioid receptor-mediated antinociception in the rat. II. Supraspinal in addition to spinal sites of action. *J Pharmacol Exp Ther* 1989;251:342–350. [PubMed: 2571723]
- Millan MJ, Colpaert FC. Modification of nociception in a model of localized inflammatory pain by long-term administration of naloxone. *Progress in Clinical and Biological Research* 1990;328:331–334. [PubMed: 2154789]
- Motulsky, HJ.; Christopoulos, A. *Fitting Models to Biological Data using Linear and Nonlinear Regression. A Practical Guide to Curve Fitting.* GraphPad Software, Inc.; San Diego CA: 2003. www.graphpad.com
- Mulder AH, Wardeh G, Hogenboom F, Kazmierski W, Hruby VJ, Schoffelman AN. Cyclic somatostatin analogues as potent antagonists at mu-, but not delta- and kappa-opioid receptors mediating presynaptic inhibition of neurotransmitter release in the brain. *Eur J Pharmacol* 1991;205:1–6. [PubMed: 1687463]
- Narita M, Imai S, Itou Y, Yajima Y, Suzuki T. Possible involvement of mu1-opioid receptors in the fentanyl- or morphine-induced antinociception at supraspinal and spinal sites. *Life Sci* 2002;70:2341–2354. [PubMed: 12150199]
- Paxinos, G.; Watson, C. *The rat brain in stereotaxic coordinates.* 2nd edition. Academic Press; Sydney/Orlando, FL: 1986.
- Pelton JT, Kazmierski W, Gulya K, Yamamura HI, Hruby VJ. Design and synthesis of conformationally constrained somatostatin analogues with high potency and specificity for mu opioid receptors. *J Med Chem* 1986;29:2370–5. [PubMed: 2878079]
- Portoghese PS, Sultana M, Nelson WL, Klein P, Takemori AE. Delta opioid antagonist activity and binding studies of regioisomeric isothiocyanate derivatives of naltrindole: evidence for delta receptor subtypes. *J Med Chem* 1992;35:4086–4091. [PubMed: 1331455]
- Powell KR, Holtzman SG. Differential antagonism of the rate-decreasing effects of kappa-opioid agonists by naltrexone and nor-binaltorphimine. *Eur J Pharmacol* 1999;377:21–8. [PubMed: 10448921]
- Sakurada S, Hayashi T, Yuhki M, Fujimura T, Murayama K, Yonezawa A, Sakurada C, Takeshita M, Zadina JE, Kastin AJ, Sakurada T. Differential antagonism of endomorphin-1 and endomorphin-2 spinal antinociception by naloxonazine and 3-methoxynaltrexone. *Brain Res* 2000;881:1–8. [PubMed: 11033087]
- Shook JE, Pelton JT, Kazmierski W, Lemcke PK, Villar RG, Hruby VJ, Burks TF. A cyclic somatostatin analog that precipitates withdrawal in morphine-dependent mice. *NIDA Research Monographs* 1987;76:295–301.
- Sora I, Funada M, Uhl GR. The mu-opioid receptor is necessary for [D-Pen²,D-Pen⁵]enkephalin-induced analgesia. *Eur J Pharmacol* 1997;324:R1–R2. [PubMed: 9145787]
- Stamidis H, Young GA. Mu-delta opioid interactions. III: Differential antagonism of DPDPE-induced increases in morphine EEG and EEG power spectra by DALCE and naltrindole. *Peptides* 1993;14:511–517. [PubMed: 8392719]
- Sterious SN, Walker EA. Potency differences for D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂ as an antagonist of peptide and alkaloid mu-agonists in an antinociception assay. *J Pharmacol Exp Ther* 2003;304:301–309. [PubMed: 12490605]
- Takemori AE. Determination of pharmacological constants: use of narcotic antagonists to characterize analgesic receptors. *Adv Biochem Pharmacol* 1973;8:335–344.
- Tallarida RJ. The use of drug-receptor affinity measures in the differentiation of receptors. *Fed Proc* 1982;41:2323–7. [PubMed: 6122613]
- Tiseo PJ, Yaksh TL. Dose-dependent antagonism of spinal opioid receptor agonists by naloxone and naltrindole: additional evidence for delta-opioid receptor subtypes in the rat. *Eur J Pharmacol* 1993;236:89–96. [PubMed: 8391455]
- Tyers MB. A classification of opiate receptors that mediate antinociception in animals. *Br J Pharmacol* 1980;69:503–512. [PubMed: 6249436]
- Walker EA, Makhay MM, House JD, Young AM. *In vivo* apparent pA₂ analysis for naltrexone antagonism of discriminative stimulus and analgesic effects of opiate agonists in rats. *J Pharmacol Exp Ther* 1994;271:959–68. [PubMed: 7965818]

- Wang D, Raehal KM, Bilsky EJ, Sadee W. Inverse agonists and neutral antagonists at mu opioid receptor (MOR): possible role of basal receptor signaling in narcotic dependence. *J Neurochem* 2001;77:1590–600. [PubMed: 11413242]
- Zadina JE, Hackler L, Ge LJ, Kastin AJ. A potent and selective endogenous agonist for the mu-opiate receptor. *Nature* 1997;386:499–502. [PubMed: 9087409]
- Zhang L, Walker EA, Sutherland J, Young AM. Discriminative stimulus effects of two doses of fentanyl in rats: Pharmacological selectivity and effects of training dose on agonist and antagonist effects of mu opioids. *Psychopharmacology* 2000;148:136–145. [PubMed: 10663428]

Figure 1.

Antagonism of antinociceptive effects of μ agonists by naltrexone or CTAP, as a function of route of administration, in a 55° C tail-withdrawal assay. Ordinates are latency measures, as percent maximum effect; abscissae are cumulative doses of agonist, in mg/kg or μ g/rat. Control points (C) represent injection of vehicle. Each combination was studied once in 5 to 11 rats; points and bars are means and SEM. Top panels present effects of pretreatment with naltrexone (-15 min, SC); middle panels, pretreatment with naltrexone (-15 min ICV); bottom panels, pretreatment with CTAP (-15 min, ICV). The left column presents effects of morphine given SC; the center column, morphine given ICV; the right column, DAMGO given ICV.

Naltrexone, SC

CTAP, ICV

Nor-BNI, SC

Bremazocine, mg/kg SC

Spiradoline, mg/kg SC

U69,593, mg/kg SC

Figure 2.

Antagonism of antinociceptive effects of *kappa* agonists by naltrexone (top, -15 min, SC), CTAP (middle, -15 min, ICV), and nor-BNI (bottom, -24 h, SC) in a 50° C tail-withdrawal assay. The left column presents effects of bremazocine (SC); the center column, spiradoline (SC); the right column, U69,593 (SC). Each combination was studied in 5 to 8 rats. Other details as in Figure 1.

Naltrexone, SC

CTAP, ICV

Naltrindole, ICV

Figure 3.

Antagonism of antinociceptive effects of the *delta* agonist DPDPE by naltrexone (top, -15 min, SC), CTAP (middle, -15 min, ICV), or naltrindole (bottom, -15 min, ICV) in a 55° C tail-withdrawal assay. Each combination was studied in 8 rats. Other details as in Figure 1.

Estimates of *in vivo* pA₂ values and Schild slopes from Schild analyses and nonlinear regression analyses

	Assay (C)	Agonist dose ratios analyzed by Schild analysis (Arumlakshana and Schild, 1959)				Agonist ED ₅₀ doses analyzed by non-linear regression (Lew and Angus, 1995)				Families of agonist dose-response curves analyzed by global non-linear regression (Moutlsky & Christopoulos, 2003)			
		pA ₂ ^a	95% CL	-Slope	95 % CL	pA ₂ ^a	95% CL	-Slope	95 % CL	pA ₂ ^a	95% CL	-Slope	95 % CL
Naltrexone, SC													
Morphine, SC	55°	7.4	7.2 to 7.5	1.0		7.8	7.5 to 8.2	0.75		7.9	7.7 to 8.0	0.73	0.67 to 0.78
Morphine, ICV	55°	8.1	7.7 to 8.5	0.52	0.43 to 0.60	7.4	6.1 to 8.8	0.52	0.27 to 0.76	8.2	8.0 to 8.5	0.49	0.45 to 0.52
DAMGO, ICV	55°	7.5	7.3 to 7.8	0.58	0.50 to 0.66	7.2	6.9 to 7.4	0.68	0.62 to 0.75	7.2	7.0 to 7.3	0.69	0.65 to 0.73
Morphine, SC	50°	6.4	5.3 to 7.5	0.47	0.09 to 0.85	6.2	4.8 to 7.5	0.52	0.05 to 0.99	6.1	5.6 to 6.7	0.51	0.32 to 0.70
DPDPE, ICV	50°	6.1	5.8 to 6.4	0.57	0.43 to 0.71	6.0	5.6 to 6.6	0.57	0.40 to 0.73	6.1	5.8 to 6.3	0.57	0.50 to 0.65
Bremazocine, SC	50°	UN ^b				5.4	3.2 to 7.7			5.3	4.2 to 6.5	0.31	0.06 to 0.68
Spiradoline, SC	50°	UN ^b				4.8	4.2 to 5.5			4.9	4.5 to 5.3		
U69,593, SC	50°	UN ^b				UN ^c				UN ^c			
Naltrexone, ICV													
Morphine, SC	55°	9.3	8.9 to 9.6	0.59	0.47 to 0.72	9.3	8.7 to 10.0	0.52	0.37 to 0.67	9.4	9.1 to 9.7	0.52	0.45 to 0.59
Morphine, ICV	55°	10.2	9.8 to 10.5	0.57	0.48 to 0.66	9.8	8.5 to 11.2	0.52	0.26 to 0.79	10.6	10.3 to 11.0	0.48	0.43 to 0.52
DAMGO, ICV	55°	11.2	10.8 to 11.5	0.48	0.42 to 0.53	11.5	11.1 to 12.0	0.43	0.38 to 0.48	11.5	11.1 to 11.8	0.43	0.40 to 0.47
CTAP, ICV													
Morphine, SC	55°	9.1	8.9 to 9.4			9.8	8.9 to 10.8	0.64	0.38 to 0.89	9.8	9.4 to 10.3	0.62	0.50 to 0.74
Morphine, ICV	50°	12.2	10.7 to 13.7	0.47	0.29 to 0.64	12.5	11.4 to 13.5	0.48	0.37 to 0.59	12.4	11.9 to 12.9	0.48	0.42 to 0.53
DAMGO, ICV	55°	9.8	9.3 to 10.3			9.4	8.7 to 10.1			9.5	9.2 to 9.8		
Morphine, SC	50°	10.8	10.0 to 11.7	0.37	0.24 to 0.50	10.6	10.0 to 11.1	0.52	0.41 to 0.62	10.6	10.3 to 10.9	0.52	0.47 to 0.58
After <i>kappa</i> drugs										10.6	10.1 to 11.0	0.37	0.30 to 0.44
DPDPE, ICV	50°	UN ^b				UN ^c				UN ^c			
Bremazocine, SC	50°	UN ^b				7.7	7.0 to 8.4			7.6	7.3 to 7.9		
Spiradoline, SC	50°	UN ^b				UN ^c				UN ^c			
U69,593, SC	50°	UN ^b				UN ^b				UN ^b			
Naltrindole, ICV													
DPDPE, ICV	50°	10.0	9.9 to 10.2			10.6	10.1 to 11.0			10.6	10.4 to 10.8	0.66	0.60 to 0.72

^aFor values in bold, F-tests demonstrated that Schild slopes did not differ from -1. For all other values, F-tests demonstrated that Schild slopes differed from -1, so data are not consistent with simple competition, but do provide estimates of potencies across routes, agonists, and assay conditions (*i.e.*, water temperatures).

^bUndefined; Schild slope not different from 0.

^cUndefined; confidence limits undefined.