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In Vivo Characterization of 6ß-Naltrexol, an Opioid Ligand with Less Inverse Agonist Activity Compared to Naltrexone and Naloxone in Opioid Dependent Mice

KIRSTEN M. RAEHAL, JOHN J. LOWERY, CASTIGLIANO M. BHAMIDIPATI, RYAN M. PAOLINO, JENNIFER R. BLAIR, DANXIN WANG, WOLFGANG SADÉE AND EDWARD J. BILSKY

Department of Pharmacology, University of New England College of Osteopathic Medicine, Biddeford, Maine 04005 (J.J.L., C.M.B., R.M.P., J.R.B., E.J.B); Department of Pharmacology, College of Medicine and Public Health, The Ohio State University, Columbus, Ohio 43210 (K.M.R., D.W., W.S.).

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Corresponding Author: Dr. Edward Bilsky

Department of Pharmacology

University of New England College of Osteopathic Medicine

11 Hills Beach Road

Biddeford, Maine 04005

Tel: 207.283.0170, x2707

Fax: 207.294.5931

Email: ebilsky@une.edu

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Abstract

The mu opioid receptor displays basal signaling activity, which appears to be enhanced by exposure to opioid agonists. This study assesses the *in vivo* pharmacology of the putative "neutral" antagonist 6ß-naltrexol in comparison to other ligands with varying efficacy, such as naloxone, an inverse agonist in the opioid dependent state. ICR mice were used to generate full antagonist dose-response curves for naloxone, naltrexone, nalbuphine and 6ß-naltrexol in blocking acute antinociceptive effects of morphine and precipitating opioid withdrawal in models of physical dependence. 6ß-Naltrexol was roughly equipotent to naloxone and between 4.5 and 10-fold less potent than naltrexone in blocking morphine-induced antinociception and locomotor-activity, showing that 6ß-naltrexol enters the central nervous system. In contrast to naloxone and naltrexone, 6ß-naltrexol precipitated only minimal withdrawal at high doses in an acute dependence model and was ~77-fold and 30-fold less potent than naltrexone and naloxone, respectively, in precipitating withdrawal in a chronic dependence model. 6ß-Naltrexol reduced the inverse agonist effects of naloxone in vitro and in vivo, as expected for a neutral antagonist. Therefore, the pharmacological effects of 6ß-naltrexol differ markedly from those of naloxone and naltrexone in the opioid dependent state. A reduction of withdrawal effects associated with neutral mu opioid receptor antagonists may offer advantages in treating opioid overdose and addiction.

Introduction

Basal signaling/constitutive activity of G-protein coupled receptors is now firmly established largely on the basis of in vitro results (Kenakin, 2004a,b; 2003), with receptor ligands displaying a range of efficacies from full agonists to full inverse agonists. However, for receptor systems that display constitutive activity, many questions remain to be resolved. (a) Do the in vitro observations translate into changes that can be measured in vivo? (b) Are there clinical applications for inverse agonists versus "neutral" antagonists? (c) How is basal receptor activity regulated and how does disease and/or chronic drug exposure alter levels of basal signaling and ligand efficacy? Studies on beta-adrenergic receptors, for example, suggest that these issues may contribute to patient outcomes in diseases such as congestive heart failure and asthma (Maack et al., 2000; Callaerts-Vegh et al., 2004).

We, and others, have established that the mu and delta opioid receptors display basal signaling, which is altered by exposure to opioid agonists (Costa and Herz, 1989; Wang et al., 1994; Wang et al., 2001, 2004; Liu and Prather, 2001; 2002). The opioid system offers advantages in studying the role of basal signaling, including (a) cloned receptors that have been extensively studied, (b) well defined signaling pathways that can be readily measured in cell lines and brain tissue, (c) a large library of ligands that differ in terms of fundamental structures and intrinsic activity, and (d) a number of well characterized physiological endpoints relevant to addiction and chronic pain states.

Our previous in vitro studies indicate that chronic opioid exposure increases the level of basal signaling at the mu opioid receptor. When tested in the opioid dependent state, the classic opioid antagonists naloxone and naltrexone act as inverse agonists in vitro, whereas in opioid naïve cells they do not perturb signaling to any significant extent (Wang et al., 1994; Wang et al.,

2004; 2001). In contrast, the mu opioid receptor antagonist CTAP acts as a neutral antagonist in vitro, while producing a less severe withdrawal than naloxone or naltrexone in opioid dependent animals (Maldonado et al., 1992a; Wang et al., 1994; Bilsky et al., 1996; Cruz et al., 1996; Liu and Prather, 2001; Sterious and Walker, 2003).

An in vitro screen of several naloxone and naltrexone derivatives identified ligands with high affinity for mu and delta opioid receptors and less inverse agonist activity than the parent compounds in opioid dependent cell lines (Wang et al., 2001). We recently reported an initial in vivo characterization of these proposed neutral antagonists, including their ability to precipitate withdrawal in opioid dependent mice, leading to the identification of 6ß-naltrexol as a viable lead compound (Figure 1) (Wang et al., 2004). In this paper, we present a comprehensive study of the in vivo pharmacology of naloxone, naltrexone and 6ß-naltrexol (Figure 1). We have also included the partial opioid agonist nalbuphine for comparison purposes. The results further support the hypothesis that basal signaling at the mu opioid receptor occurs in vivo and that compounds that differ in terms of intrinsic efficacy produce quantifiable differences in the severity of precipitated withdrawal. Our results also indicate that regulation of basal signaling and/or ligand efficacy is differentially regulated in the different mouse brain regions we have examined.

Methods

Animals. Adult, male ICR mice (25-35 g) (Harlan Industries, Cleveland, OH) were used for all experiments. Mice were housed in groups of 4-5 in standard Plexiglas containers with food and water available ad libitum. Animals were maintained in a temperature and humidity controlled colony on a 12-h light/dark cycle (lights on at 07:00). All studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health. The University of New England IACUC committee approved all protocols involving animals.

Drug Solutions and Injections. Morphine sulfate, naloxone, naltrexone and 6ß-naltrexol were obtained through the National Institute on Drug Abuse drug supply program. Nalbuphine was purchased from Sigma-Aldrich (St. Louis MO, USA). All drugs were dissolved in distilled water for intracerebroventricular (i.c.v.) injections and physiological saline (0.9% NaCl) for intraperitoneal (i.p.) and subcutaneous (s.c.) injections. The i.c.v. injections were performed as previously described (Porreca et al., 1984). Briefly, mice were lightly anesthetized with ether, and a 5 mm incision was made along the midline of the scalp. An injection was made using a 25-µL Hamilton syringe at a point 2 mm caudal and 2 mm lateral from bregma. The injection was made using a 27-gauge needle at a depth of 3 mm in a volume of 5 µL. The i.p. and s.c. injections were administered using a 1-mL syringe with a 30-gauge needle at a volume of 10 ml/kg body weight.

Tail-Flick Assay. Antinociception was assessed using the 55˚C warm-water tail flick assay. The latency to the first sign of a rapid tail-flick was used as the behavioral endpoint (Jannsen et al., 1963). Each mouse was tested for baseline latency by immersing its tail in the water bath and recording the time to response. Mice typically reacted within 1-2 seconds at this temperature,

with any mice having a baseline latency of greater than 5 seconds being eliminated from further testing. A maximal score was assigned to mice not responding in 15 seconds to avoid tissue damage. The percentage antinociception was calculated as: (test latency-control latency)/(15-control latency) x 100.

Antinociception Studies for Determining Antagonist Potencies. Antagonist potencies were determined by administering vehicle or various doses of the test compound i.c.v. or i.p. 10 minutes prior to an A_{90} dose of morphine (20 nmol, i.c.v.). Mice were then tested 20 minutes later (time of morphine peak effect) in the 55°C tail-flick assay. Percent inhibition of the morphine effect was determined for each mouse and an ID_{50} value (and 95% confidence interval) was calculated using linear regression (FlashCalc software, Dr. Michael Ossipov, University of Arizona, Tucson, AZ, USA).

Antinociception Studies for Determining Duration of Antagonist Effects. The duration of antagonist effects for each compound was estimated by pretreating mice with equieffective i.p. doses of naltrexone (1 mg/kg), naloxone (10 mg/kg) or 6ß-naltrexol (10 mg/kg) at various times prior to a s.c. injection of an antinociceptive A_{90} dose of morphine (30 mg/kg). Mice were tested 20 minutes later in the tail-flick assay.

Antinociceptive Studies to Assess Partial Agonist Effects. Doses of naloxone, naltrexone, 6ß-naltrexol or nalbuphine were administered i.p. to mice. Mice were then tested at various times after injection in the 55°C and 50°C tail-flick assay. In addition, doses of each compound were also tested in the acetic acid writhing assay as previously described (Wells et al., 2001). Antagonists were given i.p. 20 minutes prior to an i.p. injection of 0.6% acetic acid. Mice were videotaped for 20 minutes and the session was scored for number of writhes by an observer blinded to the experimental treatments.

Locomotor Studies for Determining Antagonist Potencies. Locomotor-activity was measured using an activity monitoring system (Coulbourn Instruments) and Truscan software. Each of the eight chambers consists of 10”W x 10”L x 16”H arena surrounded by Plexiglas walls. The floor consists of a removable plastic drop pan that is cleaned between sessions. A sensor ring surrounds the arena on the bottom outside edges of the four sides of the chamber and contains 16 infrared beams that transect both the length and the width of the chambers on all four sides (beam spacing = 0.6 inches, resolution of 32 x 32 squares). Measurement of the animals’ position is determined every 100 milliseconds and the software calculates a number of parameters related to aspects of locomotor-activity. The total distance traveled by each mouse was used as the primary measure of activity, with the data summed into 5-minute intervals (bins). Mice were habituated to the chamber for 30 minutes and then administered two injections within a one minute time span. The first injection was given i.p. and consisted of vehicle or various doses of the test compounds. The second injection was given s.c. and was either vehicle or a fixed dose of morphine (30 mg/kg). This dose of morphine was previously determined to produce robust stimulation of forward locomotion with stereotypic circling around the perimeter of the arena and Straub tail. Mice were immediately placed back into the chambers and monitored for an additional 90 minutes. Sessions were divided into 24 5-minute bins with the distance traveled calculated for each mouse. The time period from 20 to 50 minutes after the injections was chosen for analysis as this was a time when morphine was reaching its peak effect and when all antagonists were producing maximal blockade. For calculation of ID_{50} values, data were expressed as a percent of controls. The control morphine effect was calculated by taking the mean distance traveled by the morphine control and subtracting the mean distance traveled by the vehicle control. The data for each individual mouse receiving a dose of the test compound

was then expressed as a percentage of this control. ID_{50} values (and 95% confidence interval) were calculated using linear regression (FlashCalc software).

Locomotor Studies for Determining Onset of Antagonist Blockade. To estimate the functional pharmacokinetic parameters of onset of antagonist effect we used a modified version of the locomotor assay described above. The total distance traveled was summed into one-minute intervals to give better temporal resolution of the drug effects. Following a 30-minute habituation, mice received an injection of morphine sulfate (30 mg/kg, s.c.) and were immediately placed back into the chambers for an additional 30 minutes. Mice then received an i.p. injection of vehicle or equieffective doses of naltrexone (1 mg/kg), naloxone (10 mg/kg) or 6ß-naltrexol (10 mg/kg) and placed back into the chambers for an additional 60 minutes.

Physical Dependence Studies. Mice received acute or chronic exposure to morphine using a variety of protocols in an effort to produce increasingly severe levels of physical dependence. These protocols are summarized in Table 1. For the pellet implantation, mice were lightly anesthetized with ether and a small opening was cut into the back (scapular region) using surgical scissors. A single morphine (25 or 75 mg) or placebo pellet was inserted s.c. towards the lumbar region of the mouse and the wound was closed with two wound clips. The ability of each test compound to precipitate opioid withdrawal was assessed by administering i.p. doses at various times after the last morphine injection (or at 72 hr in the case of the morphine pellets). Immediately after the injection, mice were placed into a clear Plexiglas cylinder with a filter paper bottom. Mice were videotaped for 20 minutes and the tapes analyzed by a trained observer who was blinded to all experimental treatments. Various measures were recorded for each mouse including vertical jumping, wet dog shakes, paw tremors, number of fecal boli, presence of diarrhea, and piloerection (Yano and Takemori, 1977; Sepúlveda et al., 1999). Each nominal sign

was either present or absent and the number of paw tremors, body shakes, vertical jumps, and fecal boli for each animal was recorded.

In Vitro Competition Studies. Mice were made physically dependent to morphine using a 3-day escalating dosing regimen (Table 1). Four hours after the last injection, the animals were sacrificed and the whole brain was rapidly removed and dissected, with tissues individually frozen in liquid nitrogen. Tissues were later thawed and tissue homogenates were incubated at 30°C for 30 minutes with concentrations of the test compounds (agonists, antagonists, inverse agonists) in the [^{35}S]GTPγS assay as previously described (Wang et al., 2004) in the presence of 10μM GDP. For the competition study, increasing concentrations of naloxone were added to the membrane homogenate with or without a fixed concentration of 6ß-naltrexol (10 μM).

In Vivo Competition Studies. Mice were made physically dependent to morphine using a 3-day escalating dosing regimen (Table 1). Four hours after the last injection, mice were injected i.p. with increasing doses of 6ß-naltrexol followed one minute later by an i.p. injection of a fixed dose of naloxone (10 mg/kg). Mice were then immediately placed into a Plexiglas observation cylinder and videotaped for 20 minutes. Vertical jumping was the primary index of withdrawal severity.

Statistical Analysis. Dose-response curves were analyzed as described above using FlashCalc software. Most of the other antinociceptive, locomotor and physical dependence data were analyzed using analysis of variance (ANOVA) followed by Games/Howell post-hoc analysis. The nominal data were analyzed using chi-square. In all cases, significance was established at the $p<0.05$ level.

Results

We have previously reported on the relative potencies of i.p. naloxone, naltrexone and 6ß-naltrexol to block the antinociceptive effects of an A_{90} i.c.v. dose of morphine (Wang et al., 2001). Table 2 provides a summary of these potencies along with antagonist potencies of i.c.v. and i.p. nalbuphine against a fixed A_{90} antinociceptive dose of morphine in the 55°C tail-flick assay. The antagonist potencies of each of the tested compounds are expressed as ID_{50} values (inhibitory dose 50%) with 95% confidence limits. The rank order of i.c.v. antagonist potencies was naltrexone > 6ß-naltrexol = naloxone ≥ nalbuphine. The rank order of i.p. antagonist potencies was naltrexone = nalbuphine ≥ 6ß-naltrexol = naloxone. None of the compounds produced significant antinociception in the 55°C tail-flick assay when administered alone at doses up to 60 mg/kg i.p. (data not shown). We also tested for agonist activity in the 50°C tail-flick and acetic acid writhing assays. Of the four compounds, only nalbuphine produced significant antinociceptive activity with ~30% MPE in the lower temperature tail-flick assay and ~90% suppression of writhing induced by 0.6% acetic acid (data not shown).

To further determine the potencies of each of the compounds to antagonize a CNS mediated effect of morphine, mice were pretreated with saline or increasing doses of each antagonist (i.p.) followed by a second injection of saline or a dose of morphine (30 mg/kg, s.c.). This dose of morphine strongly stimulates forward locomotion in ICR mice and produces a full antinociceptive effect in the 55°C tail-flick assay. Complete dose-response curves for all four antagonists were run, with the naltrexone, naloxone and 6ß-naltrexol graphs depicted in Figure 2 (panels A-C, respectively). Due to the relatively short duration of action of naloxone and naltrexone, a time window from 20-50 minutes post antagonist/morphine injections was chosen for analysis of total distance traveled for each group. This represented a time period when both

the morphine and antagonist effects were near maximal and relatively stable. The dose-response curves and ID_{50} values for each compound are summarized in Figure 2D and Table 2, respectively. The rank order of potency of the compounds was naltrexone > naloxone =6ß-naltrexol > nalbuphine.

To determine if any of the test compounds produced changes in locomotor-activity, separate control groups were run for each antagonist. Mice were injected i.p. with saline or the highest dose of each antagonist tested in the previous experiment. Thirty minutes later, mice were placed into the locomotor chambers for a thirty-minute session. This protocol was used to take advantage of the higher levels of activity mice display when initially placed in a novel environment (i.e., it has greater sensitivity for detecting drug effects that may either decrease or increase levels of activity). The results of these experiments are depicted in Figure 3. The ANOVA indicated that there was a significant main effect for the treatment with an $F(4,35)=3.29$, $p=0.02$. Post-hoc analysis with a Games/Howell test set at the 5% significance level did not indicate statistically significant differences between the groups, though the comparisons between the naltrexone/6ß-naltrexol and the naloxone/6ß-naltrexol groups approached significance.

Complete dose-response curves for each antagonist to precipitate withdrawal jumping were generated in two models of opioid physical dependence (Figure 4). Panel A represents the data from an acute dependence model (single dose of morphine, 100 mg/kg, s.c., -4hr), and panel B shows results from a more severe chronic dependence model (morphine pellet, 75 mg, s.c., -72hr). In both models, increasing doses of naloxone or naltrexone resulted in a greater number of vertical jumps. The severity of withdrawal was significantly greater with the chronic versus acute morphine exposure, with greater maximal jumping and a characteristic leftward shift in the

naloxone and naltrexone dose response curves. In the acute dependence model, higher doses of naltrexone produced signs of withdrawal that may have interfered with the expression of jumping. Approximately 80% of the mice treated with the 30 and 60 mg/kg doses of naltrexone displayed behaviors that suggested severe intestinal cramping (writhing, shaking of the hind quarters, etc.) that were not observed with any of the other compounds at the doses tested. In the chronic model, the lower doses of naltrexone needed to elicit maximal jumping did not produce similar behaviors, though diarrhea became a prominent sign of withdrawal in the naltrexone and naloxone groups. Overall mortality during precipitation of withdrawal with the higher doses of naloxone and naltrexone averaged 20-30%.

In contrast to the effects of naltrexone and naloxone, 6ß-naltrexol and nalbuphine both precipitated significantly less jumping in the acute models of physical dependence at doses up to 100 mg/kg. In the chronic model of physical dependence, the two compounds precipitated significant jumping with 6ß-naltrexol able to elicit as many vertical jumps as naltrexone and naloxone at a dose of 30 mg/kg. If a half-maximal level of jumping is set at 90 jumps/20 minutes, then the doses of naltrexone, naloxone and 6ß-naltrexol required to elicit a 50% response are 0.09, 0.29 and 6.9 mg/kg, respectively. Nalbuphine also precipitated robust jumping in the chronic dependence model with a maximal number of jumps of approximately 64 at 30 mg/kg. A higher dose of 60 mg/kg resulted in significant mortality. In contrast, we did not observe any mortality with the highest doses of 6ß-naltrexol tested during the 20 min withdrawal session.

Additional experiments were performed to look at the effects of varying the level of opioid physical dependence on the severity of antagonist-precipitated withdrawal as indexed not only by vertical jumping but by other classic measures as well. We initially chose three dosing regimens

that involved x3 daily injections of s.c. morphine, with the rationale being that injectable morphine would offer more consistent tissue levels of drug when the precipitation of withdrawal was performed 4 hrs after the last injection of morphine. Pilot studies indicated that prolonging the duration of exposure from 3 to 6 days without further escalation of dose resulted in less physical dependence than with the 3 day regimen. We therefore chose to use a 72 hr s.c. implantation of a 25 mg pellet of morphine for the highest level of physical dependence. This regimen resulted in less mortality during the 72 hr induction phase compared to a 75 mg pellet, while inducing a greater level of physical dependence versus the three-day escalating dose regimen.

As expected, there was a correlation between the level of physical dependence and the number of vertical jumps precipitated by a fixed dose of each antagonist. A 3x2 ANOVA indicated significant main effects for both morphine exposure (mild, moderate and severe) and drug (naltrexone and 6ß-naltrexol), $F(2,68)=6.14$, $p<0.01$ an $F(1,68)=8.35$, $p<0.01$, respectively. The interaction between the two variables approached criteria for statistical significance, $F(2,68)=2.94$, $p=0.06$. With the mild dosing regimen, a comparison of the naltrexone and 6ß-naltrexol groups yielded a $t(18)=1.37$, $p=0.19$. At the two greater exposure levels, 6ß-naltrexol precipitated significantly less jumping behavior compared to naltrexone (p's <0.01).

Similar results were observed with the paw tremor measure (Figure 5B), though the data indicate that there was a ceiling effect with this measure (i.e., the moderate and severe dependence regimens resulted in roughly similar number of paw tremors within each particular treatment group). The ANOVA yielded an $F(2,68)=3.4$, $p<0.05$ and an $F(1,68)=5.48$, $p<0.05$, for the factors of treatment and drug. The only significant difference between the naltrexone and 6ß-naltrexol groups occurred at the severe level of morphine exposure, $t(32)=2.11$, $p<0.04$. For the

"wet-dog" shakes (Figure 5C), the ANOVA yielded an $F(2,68)=5.91$, $p<0.01$ and $F(1,68)=0.01$, $p=0.93$ for the factors of treatment and drug. Post-hoc analysis indicated that the moderate and severe levels of morphine exposure resulted in greater numbers of antagonist-precipitated "wet-dog" shakes compared to the mild exposure group with no differences observed between 6ß-naltrexol and naltrexone. The interaction between the two variables did not meet criteria for significance, $F(2,68)=1.48$, $p=0.23$.

As a crude index of gastrointestinal activity, the number of fecal boli was video recorded immediately after the withdrawal session (Figure 5D). Quantification of the number of feces indicated a similar pattern to the jumping and paw tremor graphs in that (a) there is a correlation between the number of fecal boli and the level of morphine exposure, and (b) the 6ß-naltrexol groups had fewer feces present compared to the naltrexone group at a given level of morphine exposure. The ANOVA had F values for the main factors of treatment and drug of 25.58 and 8.74, p's<0.001 and 0.005, respectively. Post-hoc analysis indicated significantly less feces at the moderate and severe morphine exposure levels (p's = 0.01).

With further reference to the measures of gastrointestinal activity, diarrhea was observed in some of the groups. This made the quantification of the fecal boli more difficult, with a best estimate made by an observer blinded to the experimental treatments for loose stools, with attention paid to the amount of material, its consistency and its location relative to other fecal output. The percent of animals having diarrhea (watery feces that soaked into the filter paper or smears) was also recorded. For the 6ß-naltrexol group, the % of animals having diarrhea was 0, 0 and 41% (7/17) for the mild, moderate and severe dependence paradigms. For the naltrexone groups it was 40 (4/10), 30 (3/10) and 65% (11/17), respectively.

The next set of experiments addressed the question of whether 6ß-naltrexol was able to enter the central nervous system to reverse a CNS effect of morphine within the twenty minute time period that was used for the dependence assays. We used a modified locomotor procedure to assess this with one-minute intervals rather than the five-minute bins (Figure 6). All experimental groups had a similar pattern of habituation (0-30 minutes) and induction of the morphine effect (30-60 minutes). Mice receiving a saline injection at 60 minutes continued to display a strong morphine locomotor effect for the remainder of the session (60-120 minutes). In contrast, all four test compounds (equieffective doses in antagonizing morphine antinociception) quickly reversed the locomotor stimulating effects of morphine, reducing the morphine effect by >90% within 10 minutes (Figure 6). When the groups were expressed as a percent of their 60 min pre-injection activity, the initial reductions in distance traveled were approximately linear (correlation values were 0.90, 0.82, 0.97 and 0.90 for naltrexone, naloxone, 6ß-naltrexol and nalbuphine). The calculated time to 50% reduction of the morphine effect (and 95% confidence limits) were 3.1 (2.6-3.7), 3.26 (3.0-3.6), 5.1 (4.5-5.9) and 5.2 (4.2-6.5) minutes, respectively.

From the locomotor studies depicted in Figure 2, it appeared that the antagonist effects of naloxone and naltrexone began to wear off within the 90 minutes after the injection of the drugs. The 55°C tail-flick assay was used to further assess the duration of action of these compounds (Figure 7). Mice were pretreated with equieffective i.p. doses of naltrexone (1 mg/kg), naloxone (10 mg/kg) or 6ß-naltrexol (10 mg/kg) at various times prior to an injection of an A_{90} antinociceptive dose of morphine (30 mg/kg, s.c.). Mice were tested 20 minutes later at the time of morphine peak effect. All three drugs completely blocked the antinociceptive effects of morphine when administered 30 minutes prior to morphine administration. Naltrexone and naloxone pretreatment between 1.5-3.0 hours was ineffective in blocking morphine

antinociception, indicating that the effects of the antagonist were of relatively short duration. In contrast, the duration of action of 6ß-naltrexol was at least 6 hours, with a return to control levels between 12-24 hours.

Further experiments tested the hypothesis that neutral antagonists can competitively antagonize the effects of inverse agonists. We tested naloxone, 7-benzylidenenaltrexone (BNTX), and 6ß-naltrexol in brain and spinal cord tissue harvested from saline or morphine treated mice using the [^{35}S]-GTPγS binding assay. The results for the various tissues and treatments are summarized in Table 3. In tissues harvested from vehicle-treated mice, BNTX acted as an inverse agonist in most of the regions examined (with the exception being the cerebellum), extending our previous findings with this compound in whole brain homogenates (Wang et al., 2004). Maximal inverse effect was 14.5% of control in untreated animals ($p<0.001$ compared to control tissues). In striatal, brainstem and spinal cord tissues collected from opioid dependent mice (moderate degree of dependence), naloxone and naltrexone also acted as an inverse agonist (maximum inverse effect 9% of control; $p<0.01$), whereas 6ß-naltrexol did not significantly alter [^{35}S]-GTPγS binding (Table 3). The relatively small size of the inverse effect is a result of large background noise in heterogeneous tissues, using the [^{35}S]-GTPγS binding assay. We were unable to detect inverse agonist effects of naloxone, naltrexone and 6ß-naltrexol in cortical or cerebellar tissue collected from opioid dependent mice.

To assess the relative magnitude of the observed inverse effects, we compared the effects of DAMGO, morphine, 6ß-naltrexol, naloxone and BNTX on [^{35}S]-GTPγS binding in brainstem/midbrain homogenates under identical conditions (10 μM GDP) from untreated and morphine (25 mg pellet for 3 days) pretreated mice (Figure 8). The effects of DAMGO and morphine were blunted by the pretreatment with morphine, whereas the effects of BNTX were

enhanced and the inverse agonist effects of naloxone emerged. Importantly, the magnitude of the agonist effect of morphine (~+15%) was similar to the magnitude of the inverse agonist effects of naloxone (~-10%). The trend towards blunted agonist effects and enhanced inverse agonist effects further increased with a 75 mg morphine pellet treatment for 3 days; data not shown). Therefore, in the morphine-dependent state, agonist and inverse agonist effects of morphine and naloxone, respectively, are of similar magnitude.

Given the importance of opioid receptors in the locus coeruleus and periaqueductal gray matter in mediating opioid withdrawal (Maldonado et al., 1992b) and the results summarized in Table 3, we chose to use brainstem/midbrain tissue for 6ß-naltrexol competition studies. Moreover, the brainstem/midbrain homogenates were primarily sensitive to mu opioid receptor agonists, but not to delta and kappa selective opioid agonists, so that any contribution from opioid receptors other than the mu opioid receptor is minimized. In control tissues, naloxone produced a small increase in [^{35}S]-GTPγS binding at high nM and low µM concentrations (Figure 9A). In contrast, naloxone acted as an inverse agonist in tissues harvested from morphine treated mice (Figure 9A), as shown previously. 6ß-Naltrexol did not produce significant effects on [^{35}S]-GTPγS binding in brainstem tissues harvested from either saline or morphine treated mice (Figure 9B). Additional concentration-response curves were run with naloxone using brainstem/midbrain tissue from morphine pretreated mice in the absence or presence of 10µM 6ß-naltrexol (Figure 9C). The co-incubation of 6ß-naltrexol produced a large rightward shift in the naloxone concentration-response curve (EC_{50} values for control and 6ß-naltrexol curves of 190 ± 40 nM and ~9,000 nM, respectively). In similar studies, the same concentration of 6ß-naltrexol was also able to produce a ~24-fold rightward shift in the concentration response curve for BNTX (data not shown).

For the in vivo competition studies, the acute physical dependence model was chosen because of previous work demonstrating minimal withdrawal jumping with doses of 6ß-naltrexol up to 30 mg/kg. All mice received naloxone (10 mg/kg, i.p.) one minute after receiving an i.p. injection of saline or increasing doses of 6ß-naltrexol. The co-injection of 6ß-naltrexol dose-relatedly decreased naloxone-induced withdrawal jumping (Figure 8D), $F(4,45)=3.85$, $p<0.01$. The 10 and 30 mg/kg doses of 6ß-naltrexol were significantly different from the naloxone control (p's < 0.01 and 0.001, respectively).

Discussion

The current study extended our characterization of 6ß-naltrexol by comparing its pharmacology to nalbuphine (partial agonist) and naloxone and naltrexone (inverse agonists) (Traynor et al., 2002; Walker et al., 2004). We established the potency of the compounds to block acute effects of morphine (antinociception and locomotor-activity). Naltrexone was ~3-10-fold more potent than naloxone and 6ß-naltrexol. Nalbuphine had roughly similar antagonist potencies in the antinociceptive assays but was significantly less potent in blocking locomotor-activity. None of the antagonists stimulated activity by themselves when tested at the highest dosing levels. We did observe mild stimulation following a 10 mg/kg dose of nalbuphine (125% above saline controls versus >350% w/morphine). This could have accounted for the relatively low potency in this assay. None of the test compounds produced antinociception in the 55°C tail-flick assay. Agonist effects of nalbuphine were apparent in the 50°C tail-flick and acetic acid writhing assays. 6ß-Naltrexol did not produce observable agonist effects, clearly distinguishing it from nalbuphine.

In the dependence assays, vertical jumping was used as the primary withdrawal measure as it is one of the most reliable measures of withdrawal severity, correlating with dose and duration of opioid exposure and the dose of naloxone injected (Blasig et al., 1973; Buccafusco et al., 1984; Kest et al., 2002). In the acute dependence assay, naloxone and naltrexone precipitated significant withdrawal jumping at doses that blocked acute effects of morphine. Higher doses (30 and 60 mg/kg) elicited further increases in withdrawal jumping, with naltrexone producing behaviors that may interfere with jumping behavior. These behaviors were not observed in opioid naïve animals. In contrast, the neutral antagonist 6ß-naltrexol and the partial agonist

nalbuphine both failed to precipitate withdrawal jumping at 10 mg/kg, doses that block morphine antinociception and locomotor-activity.

In the chronic dependence model, naltrexone and naloxone precipitated jumping at doses 30-60 times lower than in the acute dependence model, consistent with previous results (Way et al. 1969). 6ß-Naltrexol was 25- and 77-fold less potent than naloxone and naltrexone in precipitating withdrawal. This is in contrast to its antagonist potencies against morphine antinociception (equipotent to naloxone and 5-10-fold less potent than naltrexone). The significantly lower potency of 6ß-naltrexol in eliciting withdrawal is consistent with the hypothesis that suppression of basal mu opioid receptor signaling by an inverse agonist contributes to withdrawal. At very high doses 6ß-naltrexol was able to precipitate as severe a withdrawal as that of naloxone and naltrexone, even though mu opioid receptor sites are presumably saturated at lower doses. 6ß-Naltrexol may act as a competitive antagonist in displacing morphine from mu opioid receptors, whereas naloxone acts noncompetitively as an inverse agonist on basal mu opioid receptor activity. Alternatively, 6ß-naltrexol could have acted with low potency at a mu opioid receptor conformation specifically involved in dependence, to which naloxone has high affinity. Additional studies are needed to resolve these questions.

With respect to withdrawal, nalbuphine resembles 6ß-naltrexol. Opioid agonists with low efficacy can precipitate withdrawal in patients that are physically dependent (Preston et al., 1989; Preston et al., 1990). This presumably occurs by displacing morphine from opioid receptors. Moreover, dependent subjects go through a protracted withdrawal syndrome as agonist drug levels decrease over time, indicating that there are compensatory systems that become uninhibited as drug effects wear off (Gonzalez et al., 2004). We propose that the intrinsic inverse

efficacy of naloxone and naltrexone in suppressing basal/constitutive mu opioid receptor activity contributes to the potent drug-induced withdrawal effect (Sadée et al., 2005).

We addressed the question of how varying the level of physical dependence affects antagonist-induced withdrawal. We used moderate doses of naltrexone and 6ß-naltrexol to avoid ceiling or basement effects. Naltrexone elicited an increased severity of withdrawal with each of the measures, whereas an equivalent dose of 6ß-naltrexol produced less severe withdrawal in most measures. This was particularly pronounced with increasing degree of dependence for vertical jumping (robust measure of dependence). The number of fecal pellets (crude measure of gastrointestinal motility/overactivity) and the incidence of diarrhea showed similar patterns.

A ceiling effect may have been reached with the paw tremor measure, as there was little further increase in the number of tremors from the moderate to severe exposure levels. At moderate levels of morphine exposure, 6ß-naltrexol precipitated more wet-dog shakes than naltrexone, but this did not meet criteria for statistical significance. This behavior has been labeled a recessive sign of morphine withdrawal, being predominant when dependence is at a moderate level, and decreasing during more severe withdrawal when dominant signs (jumping) predominate (Blasig et al., 1973, Valverde et al., 1992). This is consistent with 6ß-naltrexol eliciting less severe withdrawal.

We have previously reported on blood and brain levels of naltrexone and 6ß-naltrexol following systemic injection into mice (Wang et al., 2004). In vivo studies addressing functional pharmacokinetics provide additional data on the CNS penetration and duration of action of 6ß-naltrexol. Naloxone and naltrexone are relatively short acting antagonists in rodents (Berkowitz et al., 1975). In contrast, 6ß-naltrexol has a much longer duration of action. The reversal of morphine-induced locomotor-activity indicated that all four compounds quickly act to block

CNS effects of morphine. It is unlikely that these actions are due to non-specific effects, as none of the compounds significantly altered baseline levels of activity. 6ß-naltrexol (10 mg/kg i.p.) can quickly reverse the respiratory depressant effects of morphine (200 mg/kg). These rescue doses work within one minute to prevent lethality. Furthermore, in mice receiving a 25 mg pellet of morphine, the time to first jump in mice that expressed this behavior was not significantly different between the four compounds tested. Collectively, the data indicate that the observed differences in withdrawal severity between inverse agonists, "neutral" antagonists, and partial agonist, are not due to differences in penetration rates/onset of antagonist actions.

A neutral antagonist should block the effects of an inverse agonist. We tested this hypothesis by measuring interactions between naloxone and 6ß-naltrexol in membrane homogenates. Brainstem/midbrain tissue harvested from morphine treated mice yielded readily detectable inverse agonist effects on GTPγS binding for naloxone and BNTX. Direct comparison between the magnitude of agonist and inverse agonist effects, under identical conditions including relatively low GDP levels, indicated that the inverse effects are of the same order of magnitude as agonists effects in the morphine-dependent state. Owing to the difficulty in measuring basal levels of GTPγS binding in heterogeneous brain tissues, the maximal effects we have observed are ~10% for naloxone and ~20% for BNTX. This compares favorably to inverse agonist effects measured in cerebral membrane homogenate for the histamine H3 receptor (maximally 5%), where inverse agonists are clearly distinct from a neutral antagonist (Gbahou et al., 2003; Rouleau et al., 2002). We conclude that the observed inverse agonist effects at mu opioid receptors in brain tissue are potentially significant, and reliably detectable above background noise.

The magnitude of the inverse effect on GPCR coupling does not necessarily reflect the magnitude of any physiological/pharmacological response, as the mu opioid receptor appears to be differentially regulated and coupled in different tissues (Table 3). BNTX acts as an inverse agonist in all brain tissues tested except the cerebellum while naloxone and naltrexone fail to display inverse effects in hippocampus and cortex, regions relatively rich with mu opioid receptors. The reasons for these differential effects remain to be elucidated. It is possible that coexpression of various opioid receptors leads to heterodimers that have different properties/sensitivity to inverse agonists (unpublished results). Alternatively, different brain regions express different levels of regulatory proteins, such as calmodulin, that affect basal mu opioid receptor signaling.

As a neutral antagonist, 6β-naltrexol should block both agonists and inverse agonists. Addition of 6ß-naltrexol shifted the dose-response curve of naloxone (Figure 9) and BNTX (not shown) to the right. This supports the notion that naloxone acts at mu opioid receptors as an inverse agonist suppressing basal activity. The in vivo competition experiments with 6ß-naltrexol provide additional evidence. These results fulfill an important criterion supporting the hypothesis that 6ß-naltrexol acts at mu opioid receptors as a neutral antagonist capable of suppressing the effects of both an agonist (morphine) and an inverse antagonist (naloxone).

In summary, we have provided a quantitative evaluation on the in vivo pharmacology of 6ß-naltrexol, a putative neutral antagonist. By determining the potency of each compound to block acute effects of morphine and to precipitate withdrawal in various opioid dependence models, we were able to estimate the therapeutic index of 6ß-naltrexol. If the mouse studies extend into humans, it may be possible to reverse life-threatening effects of an opioid overdose while precipitating significantly less withdrawal than comparable doses of naloxone. The longer

duration of action of 6ß-naltrexol may be clinically significant in that it would provide prolonged protection from opioid reintoxication. Clinical application may include not only reversal of overdoses in opioid addicts, but also in situations where opioids are intended to provide short-acting sedation/analgesia (Barsan et al., 1989). Furthermore, 6ß-naltrexol may be better tolerated than naltrexone in recovering opioid addicts due to its neutral antagonist profile.

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Footnotes

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B. Reprint requests: Dr. Edward Bilsky, Department of Pharmacology, University of New England College of Osteopathic Medicine, 11 Hills Beach Road, Biddeford, Maine 04005 USA. E-mail: ebilsky@une.edu

Legends for Figures

Figure 1. Chemical structures of naloxone, naltrexone, 6ß-naltrexol and nalbuphine.

Figure 2. Antagonist potencies of the test compounds to block morphine induced locomotor-activity. Mice were habituated to the locomotor chambers for 30 minutes and then received an i.p. injection of saline or doses of an antagonist followed by a s.c. injection of saline or morphine (30 mg/kg). Mice were immediately placed back into the chambers for an additional 90 minutes of monitoring. Dose and time-response curves are shown for naltrexone (panel A), naloxone (panel B) or 6ß-naltrexol (panel C). Panel D summarizes the dose-response curves (see text for details).

Figure 3. Control locomotor-activity experiments using a modified activity monitoring protocol to determine if the test compounds produced significant changes in forward locomotion by themselves. Mice were injected i.p. with saline or a high dose of each test compound thirty minutes prior to being placed into the locomotor chambers for a thirty minute session. Panel A represents the time-course divided into 5 minute intervals and Panel B is the total distance traveled for each group in the 30 minute session.

Figure 4. Precipitated opioid withdrawal in mice made dependent to morphine in an acute (panel A) or chronic (panel B) dependence model. Mice received an i.p. injection of the test compound and were immediately placed into Plexiglas cylinders for a 20 min videotaped observation period.

Figure 5. Four different measures of antagonist-precipitated opioid withdrawal are depicted in mice that received mild, moderate or severe morphine-dependence treatments. The withdrawal measures included vertical jumping (panel A), paw tremors (panel B), body "wet-dog" shakes (panel C) and number of fecal boli (panel D). A ** indicates $p<0.01$ and * $p<0.05$ for comparison between naltrexone and 6ß-naltrexol.

Figure 6. Time-course of reversal of morphine-induced locomotor-activity. Mice were habituated to the locomotor chambers for 30 minutes and then received an injection of morphine (30 mg/kg, s.c.) and monitored for an additional 30 minutes. At t=60 minutes mice received an i.p. injection of vehicle or an equieffective antagonist dose of the test compound and then returned to the chambers for an additional 60 minutes of monitoring.

Figure 7. Duration of antagonist blockade of morphine antinociception by the test compounds. Mice were pretreated with equieffective antagonist doses i.p. at various times prior to an injection of a 30 mg/kg s.c. dose of morphine. Antinociception was assessed at the time of morphine peak effect (20 minutes) in the 55°C tail-flick assay.

Figure 8. Effects of agonists and antagonists on 35S-GTPγS binding in brainstem/midbrain homogenates, in untreated and morphine treated mice (escalating s.c. doses for 3 days). All ligand concentrations were 10 μM. Lack of effect of 6ß-naltrexol in morphine-pretreated samples indicates that the homogenization and washing procedure after sacrifice of the animals eliminated residual morphine from the pellet implantation. A *** indicates $p<0.001$ for comparison between morphine and vehicle treated groups.

Figure 9. Competition studies between naloxone and 6ß-naltrexol. Panels A and B represent the concentration-response curves for naloxone and 6ß-naltrexol in brainstem tissue collected from vehicle and morphine treated mice. Changes in basal ^{35}S-GTPγS binding are used as an index of inverse agonist activity. Panel C measures the inverse agonist effects of naloxone in brainstem tissue harvested from mice previously pretreated with morphine in the absence (control) or presence of a fixed concentration of 6ß-naltrexol (10 μM). Panel D represents an in vivo competition study in mice previously exposed to morphine with vertical jumping used as an index of withdrawal severity. A fixed dose of naloxone (10 mg/kg, i.p.) was given immediately after an i.p. injection of vehicle or increasing doses of 6ß-naltrexol. A **indicates $p<0.01$ and *** $p<0.001$ versus naloxone controls.

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Table 1. Morphine exposure protocols.

Severity	Day 1	Day 2	Day 3	Notes
Acute	100 mg/kg x1	-----	-----	Precipitate @ 4hr
Mild	30 mg/kg x3			Precipitate Day 2, a.m.
Moderate	30 mg/kg x3	60 mg/kg x3	100 mg/kg x3	Precipitate Day 4, a.m.
Severe	25 or 75 mg pellet	-->	-->	Precipitate @ 72 hr

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Table 2. Antagonist potencies of the test compounds against fixed doses of morphine. Numbers in parentheses are the 95% confidence limits.

Drug	Tail-Flick (nmol i.c.v.)	Tail-Flick (mg/kg, i.p.)	Locomotor-activity (mg/kg, i.p.)
Naltrexone	0.26 (0.15-0.46)	0.22 (0.11-0.43)	0.007 (0.004-0.013)
Naloxone	3.43 (1.73-6.79)	1.09 (0.66-1.80)	0.029 (0.018-0.046)
6ß-Naltrexol	2.32 (1.31-4.04)	1.0 (0.58-1.69)	0.0735 (0.016-0.338)
Nalbuphine	7.81 (5.77-10.58)	0.55 (0.28-1.06)	2.82 (1.70-4.69)

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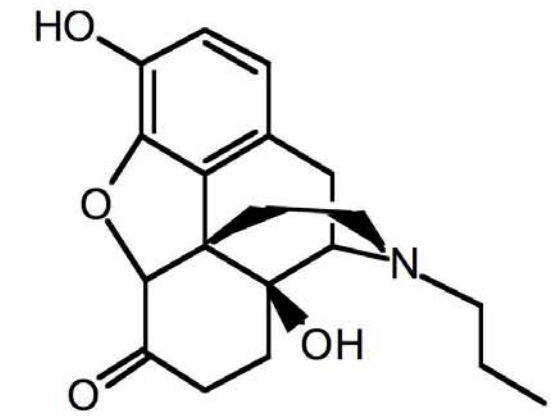
Table 3. [^{35}S]GTPγS assay with test compounds in selected brain regions from mice pretreated with vehicle or morphine (100 mg/kg twice daily for 3 days). Values are expressed as % basal GTPγS binding (* p<0.05, ** p<0.01, *** p<0.001 versus control)

Treatment	Striatum	Brainstem-midbrain	Cortex	Spinal Cord	Cerebellum
Vehicle					
Control	99.4 ± 2.8	100 ± 2.3	100 ± 1.9	99.9 ± 4.3	100.0 ± 5.0
BNTX	86.6 ± 4.1***	85.5 ± 3.2***	89.0 ± 2.8***	87.0 ± 2.4***	95.5 ± 2.3
Morphine					
Control	100.0 ± 0.9	100.0 ± 2.4	99.9 ± 7.1	99.9 ± 4.8	99.9 ± 3.4
Naltrexone	93.8 ± 0.2*	93.8 ± 2.2**	101.0 ± 2.4	90.2 ± 5.4*	97.5 ± 0.8
Naloxone	92.9 ± 0.2*	94.3 ± 2.0**	100.3 ± 2.1	88.8 ± 3.1**	97.3 ± 4.3
6ß-Naltrexol	103.5 ± 7.7	100 .0 ± 1.6	99.7 ± 2.8	95.1 ± 7.7	98.9 ± 3.9



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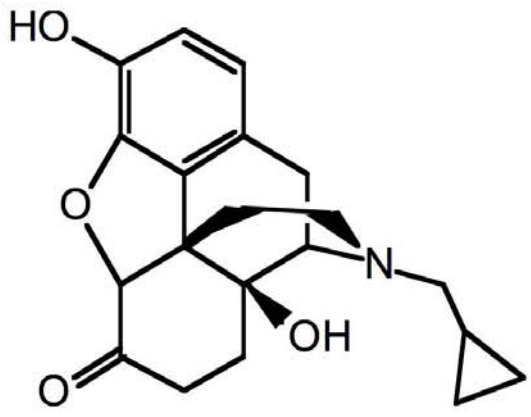

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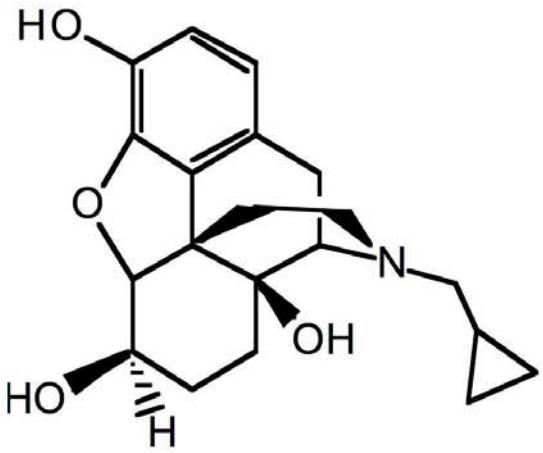

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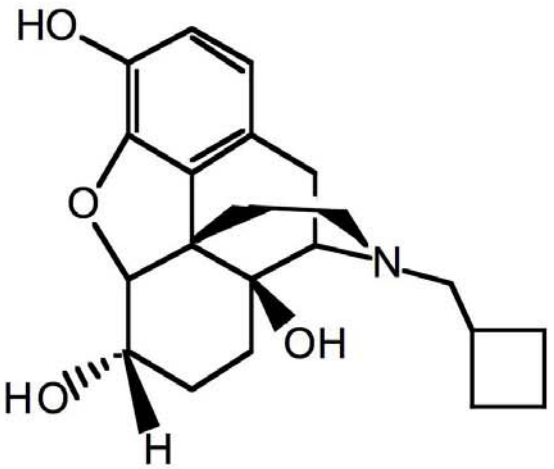
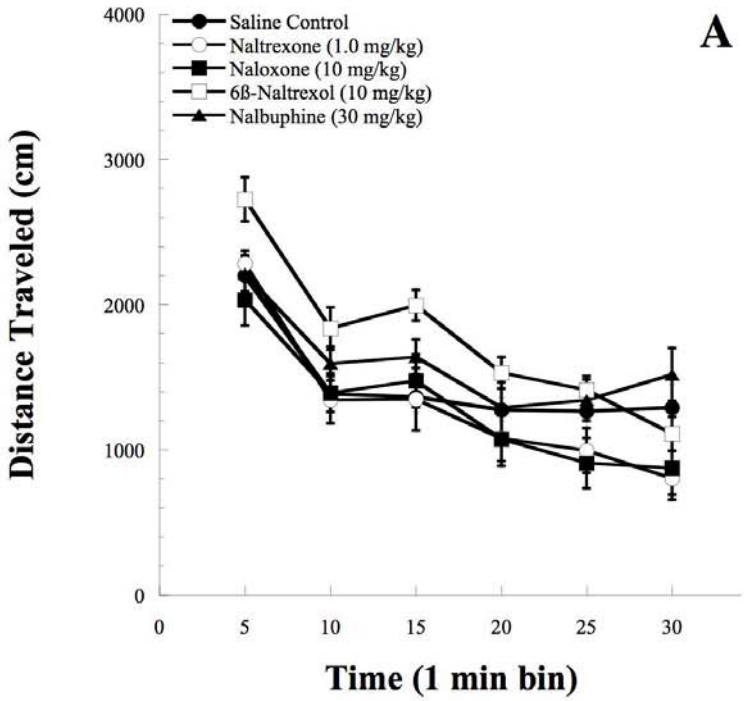
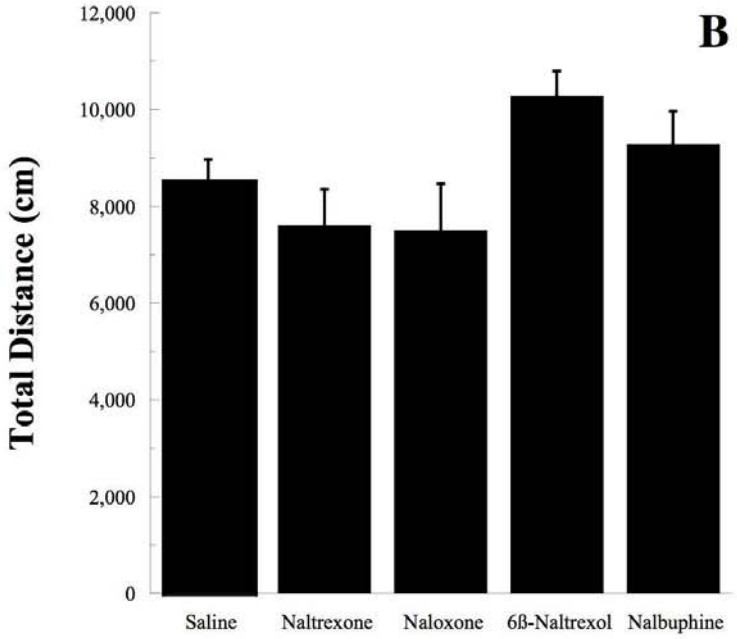


Nalbuphine

Figure 1.

Figure 2.





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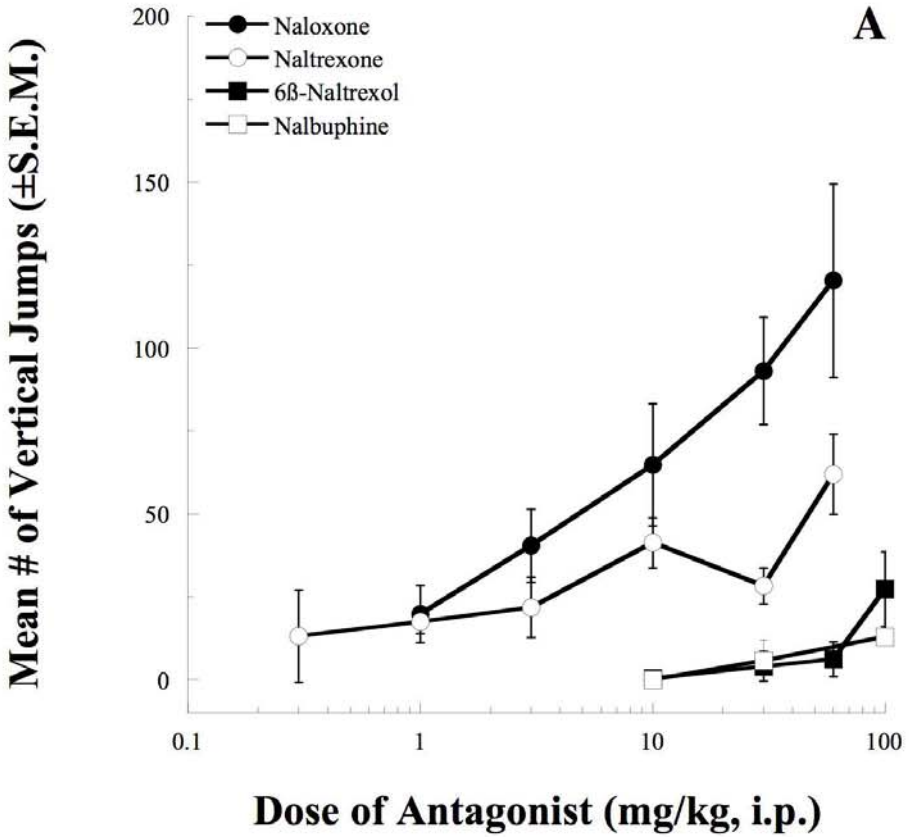

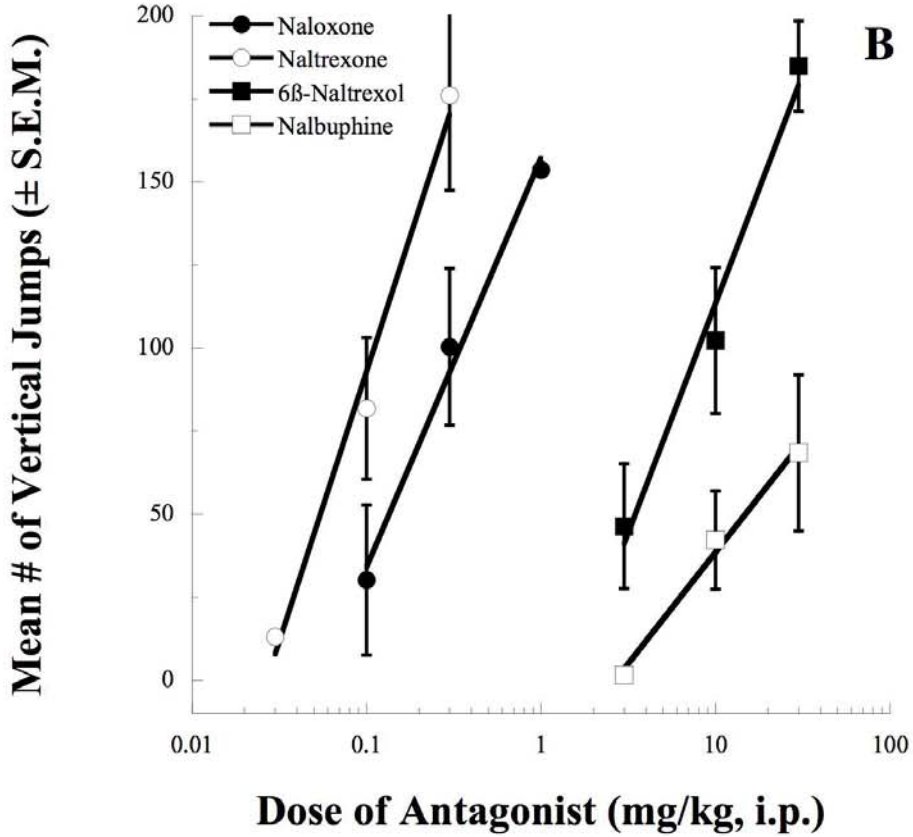

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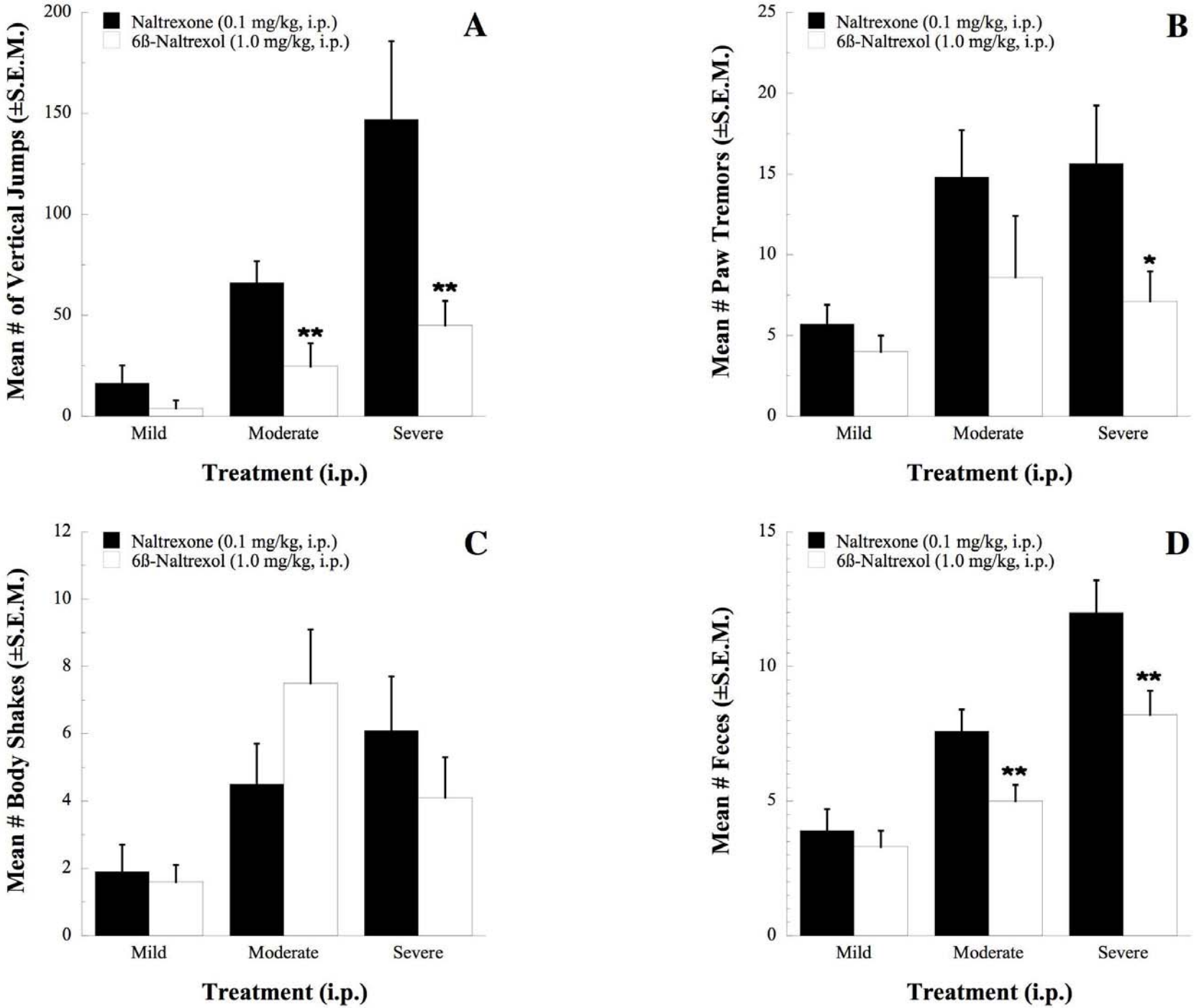


Figure 5.

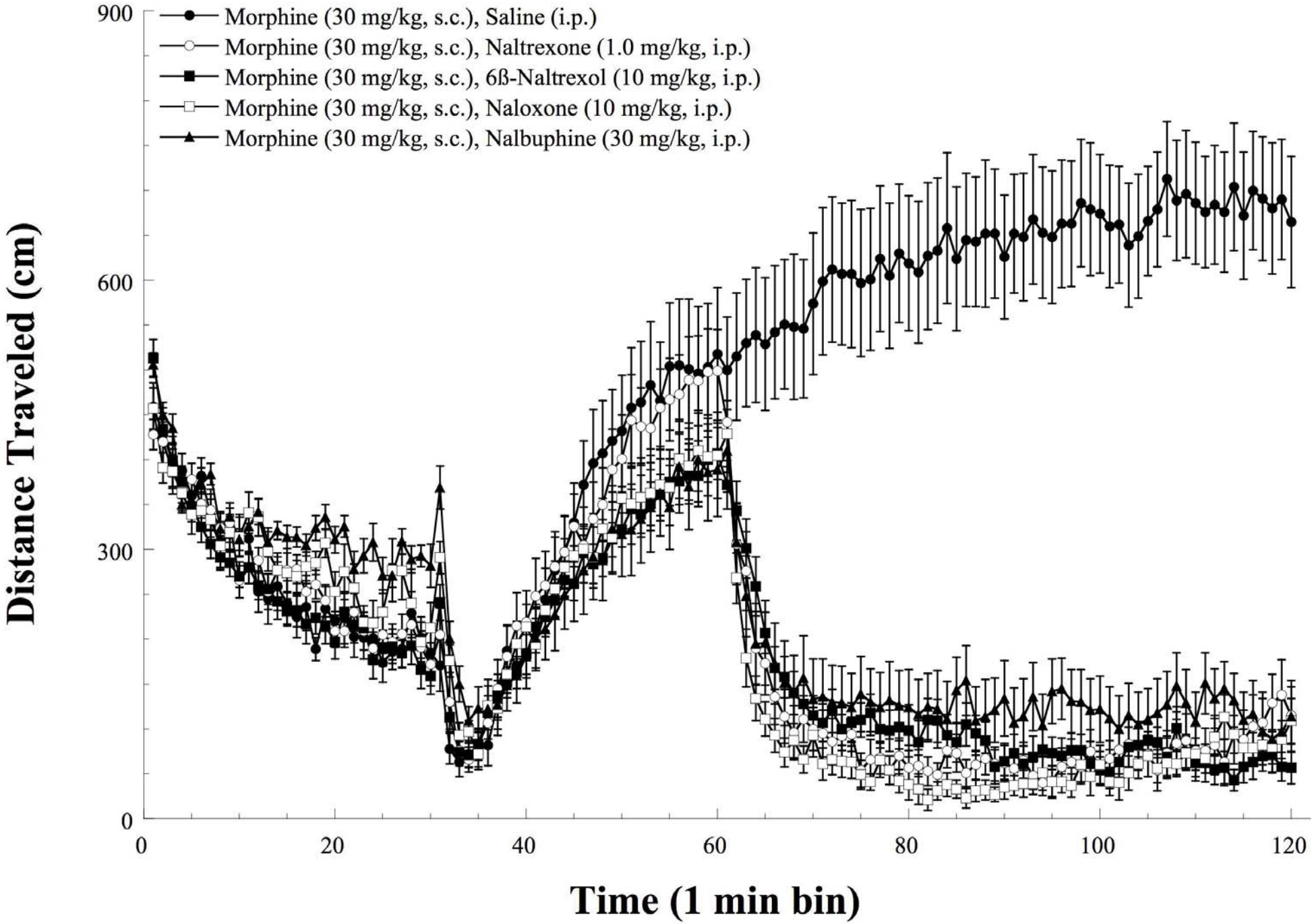


Figure 6.

Figure 7.

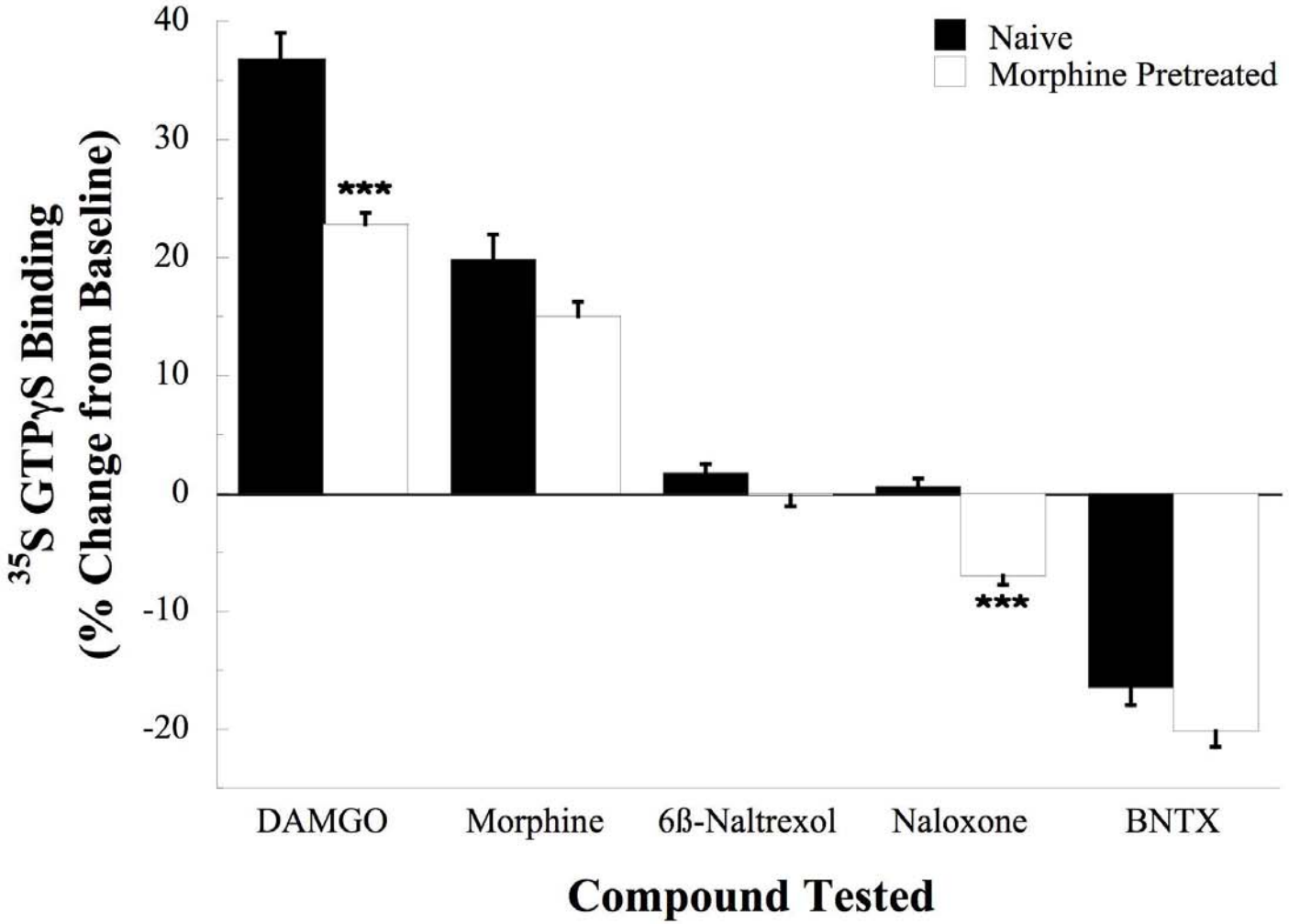
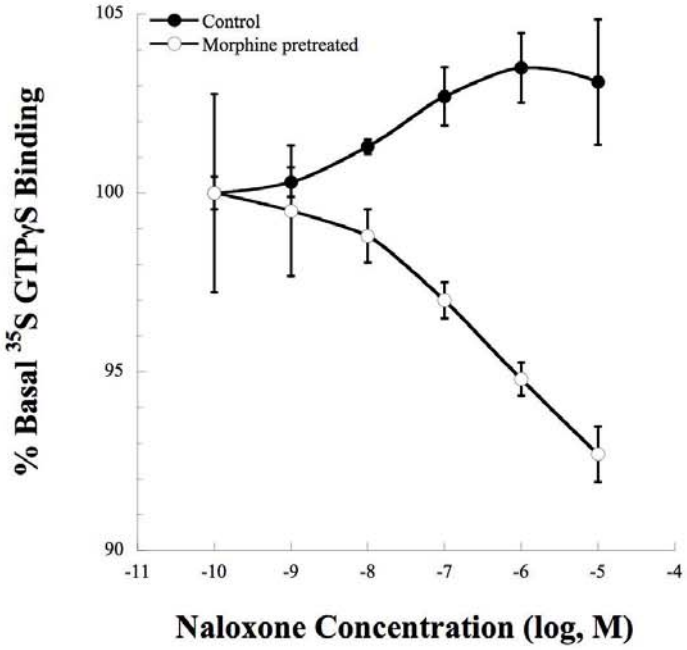
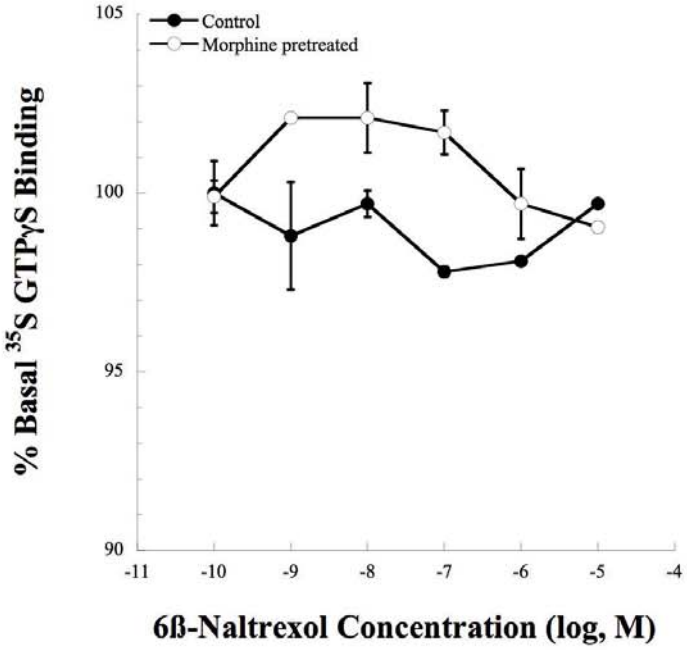
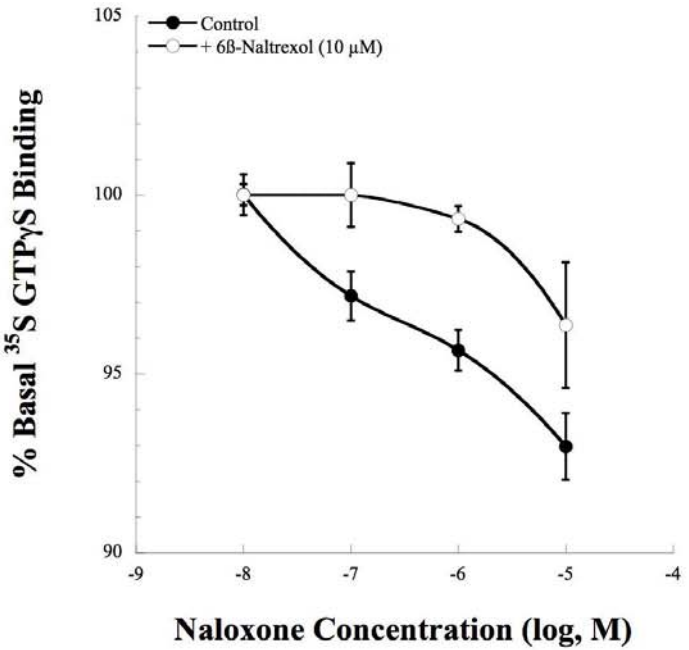


Figure 8.







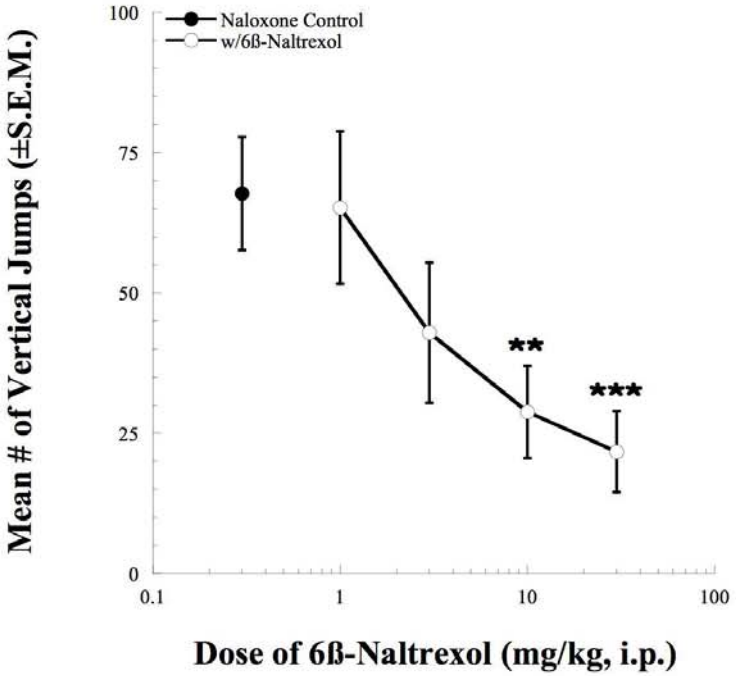


Figure 9.