

Opioid antagonists differ according to negative intrinsic efficacy in a mouse model of acute dependence

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1 The purpose of the present study is to compare the capacity of opioid antagonists to elicit withdrawal jumping in mice following two acute pretreatment doses of the opioid agonist morphine. Antagonists that precipitate vigorous withdrawal jumping across both morphine treatment doses are hypothesized to be strong inverse agonists at the μ -opioid receptor, whereas antagonists that elicit withdrawal jumping in mice treated with the high but not the low dose of morphine are hypothesized to be weak inverse agonists.

2 Male, Swiss-Webster mice (15–30 g) were acutely treated with 56 or 180 mg kg⁻¹ morphine 4 h prior to injection with naloxone, naltrexone, diprenorphine, nalorphine, or naloxonazine. Vertical jumping, paw tremors, and weight loss were recorded. Naloxone, naltrexone, and diprenorphine produced withdrawal jumping after 56 and 180 mg kg⁻¹ morphine pretreatment. Nalorphine and naloxonazine produced moderate withdrawal jumping after 180 mg kg⁻¹ morphine pretreatment, but failed to elicit significant withdrawal jumping after 56 mg kg⁻¹ morphine pretreatment. Nalorphine and naloxonazine blocked the withdrawal jumping produced by naloxone. All antagonists produced paw tremors and weight loss although these effects were generally not dose-dependent.

3 Taken together, these findings reveal a rank order of negative intrinsic efficacy for these opioid antagonists as follows: naloxone = naltrexone \geq diprenorphine > nalorphine = naloxonazine. Furthermore, the observation that nalorphine and naloxonazine blocked the naloxone-induced withdrawal jumping provides additional evidence that nalorphine and naloxonazine are weaker inverse agonists than naloxone.

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Abbreviations: ANOVA, analysis of variance; BNTX, 7-benzylidenenaltrexone; β -CNA, β -chloronaltrexamine; CTAP, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂; s.e.m., standard error of the mean

Introduction

Chronic morphine exposure has been postulated to produce a state of constitutively active μ -opioid receptors in a number of cell lines (Wang *et al.*, 1994; 1999; 2000; Burford *et al.*, 2000; Liu & Prather, 2001). These investigators suggest that chronic morphine exposure shifts the balance from normal, agonist-sensitive μ -opioid receptors to a greater proportion of constitutively active, agonist-independent μ -opioid receptors. The actions of this constitutively active μ -opioid receptor are balanced by concomitantly upregulated adenylate cyclase (cAMP) activity. Agonists such as DAMGO ([D-Ala², N-Me-Phe⁴, Gly³-ol]-enkephalin) and morphine decrease cAMP accumulation. Traditional antagonists, such as naloxone, naltrexone, β -chloronaltrexamine (β -CNA), 7-benzylidenenaltrexone (BNTX), and diprenorphine, further increase cAMP accumulation (cAMP overshoot) in morphine-treated cells. Naloxone, naltrexone, β -CNA, and diprenorphine were labeled inverse agonists because these antagonists produced effects opposite to those of agonists. Other antagonists such as 6 β -naltrexol, 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) have been identified as possible neutral antagonists since these compounds failed to alter cAMP levels in this upregulated cAMP system. Furthermore, CTAP blocked the effects of both naloxone and morphine in these cells (Wang *et al.*, 1994; 2001; Bilsky *et al.*, 1996; Liu & Prather, 2001).

Conceivably, μ -opioid receptor antagonists in this scheme could be classified on a continuum of partial to full negative intrinsic efficacy under certain conditions in the same way μ -opioid receptor agonists are classified on a continuum of partial to full positive intrinsic efficacy. Data are very limited in functional assays that distinguish among antagonists in regards to negative intrinsic efficacy. *In vivo*, CTAP, CTOP, 6 β -naltrexol, and β -CNA appear to be selective μ -opioid receptor antagonists in nondependent subjects (Gulya *et al.*, 1988; Kramer *et al.*, 1989; Adams *et al.*, 1994; Porter *et al.*, 2002; Sterious & Walker, 2003; Wang *et al.*, 2004). However, the pharmacological profiles for CTAP, CTOP, and 6 β -naltrexol are unique in morphine-dependent mice in that these antagonists failed to produce significant withdrawal jumping, one of the most characteristic signs of opiate withdrawal, whereas naloxone and naltrexone produce striking withdrawal

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jumping (Gulya *et al.*, 1988; Wang *et al.*, 1994; 2001; 2004). Interestingly, CTAP blocked the withdrawal jumping in mice produced by naloxone (Wang *et al.*, 1994; Bilsky *et al.*, 1996). Yet, in rats dependent on morphine, CTAP produced moderate withdrawal effects (Maldonado *et al.*, 1992). In morphine-treated, electrically stimulated isolated guinea-pig ileum, CTAP produced effects similar to naloxone (Mundey *et al.*, 2000). The discrepancies between these studies may be due to different dependence states of the preparations.

A common and reliable method for studying opioid dependence is the precipitated withdrawal jumping assay in mice. In this assay, mice are either implanted with a morphine pellet (Cowan, 1976; Miyamoto & Takemori, 1993) or injected with a single high dose of morphine (Smits, 1975; Wiley & Downs, 1979; Sofuoglu *et al.*, 1990). After a specified time, an antagonist, usually naloxone, is administered and withdrawal jumping is observed within 15 min (Huidobro & Maggiolo, 1965; Way *et al.*, 1969). The more dependent the mice, that is, the higher the morphine pretreatment dose, the lower the dose of naloxone required to elicit the withdrawal jumping (Way *et al.*, 1969).

In the present study, five opioid antagonists were compared for their capacity to produce withdrawal jumping as well as paw tremors and weight loss, two additional signs of opioid withdrawal (Fernandez-Espejo *et al.*, 1995; Broseta *et al.*, 2002) in mice injected with a high pretreatment dose of 180 mg kg⁻¹ morphine or a low pretreatment dose of 56 mg kg⁻¹ morphine. Antagonists that produce withdrawal jumping after pretreatment with both high and low morphine doses are hypothesized to be strong inverse agonists possessing high negative intrinsic efficacy. On the other hand, antagonists that produce withdrawal jumping in mice treated with high but not low doses of morphine are hypothesized to be weaker inverse agonists possessing lower negative intrinsic efficacy. Finally, to further test the hypothesis that antagonists may differ along a continuum of negative intrinsic efficacy, alleged weaker inverse agonists were injected to block withdrawal jumping produced by stronger inverse agonists. It is hypothesized that a compound with less negative intrinsic efficacy would block the effects of a compound with greater negative intrinsic efficacy.

Methods

Subjects

Male, Swiss Webster mice ($N=1308$) weighing 15–30 g (Ace Animals, Inc., Philadelphia, PA, U.S.A.) were group-housed in a colony room maintained under a 12-h light–dark cycle. Water and food were freely available in the home cages. All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

Apparatus

Ten 4L Pyrex[®] graduated beakers (Corning Incorporated, Corning, NY, U.S.A.) were used during the precipitated withdrawal jumping. A CS-2000 scale (OHAUS, Forham Park, NJ, U.S.A.) was used to weigh the mice. A hand-held

VWR counter (VWR International, West Chester, PA, U.S.A.) was utilized for counting jumps.

Procedure

The procedures used in these experiments were modified from Way *et al.* (1969) and Yano & Takemori (1977). Groups of 10–11 mice were injected subcutaneously (s.c.) with either 56, 100, or 180 mg kg⁻¹ morphine. After 3 h and 40 min, the mice were weighed and placed into 4000 ml Pyrex[®] graduated beakers for a 20 min habituation period. After the habituation period ended, the mice were injected with a single dose of antagonist, s.c. The number of jumps and paw tremors were recorded for 15 min. Jumping was defined as all four paws simultaneously off the floor. Paw tremors were defined as one or both paws shaking in a lateral fashion. At the end of the observation period, the mice were weighed again. During antagonist combination experiments, the mice were injected with either 56 or 100 mg kg⁻¹ morphine. After 3 h and 40 min, the mice were injected with a dose of naltrexone, nalorphine, naloxonazine, or saline, s.c., and placed into the beakers. Jumping and the number of paw tremors were counted during the 20 min habituation period. After 20 min, naloxone was injected and the number of jumps and paw tremors were recorded.

Drugs

The following compounds were used: diprenorphine hydrochloride (HCl), morphine sulfate, nalorphine HCl, naloxone HCl, naltrexone HCl (generously supplied by National Institute on Drug Abuse, Rockville, MD, U.S.A.), and naloxonazine HCl (Sigma Biochemicals, Natick, MA, U.S.A.). All compounds were dissolved in saline and prepared to deliver each injection in a volume of 0.1–0.4 ml. Doses are expressed as the forms listed above. Saline was injected in a volume of 1 ml kg⁻¹ of body weight.

Data analysis

The percentage of mice jumping or exhibiting paw tremors was calculated by dividing the number of mice exhibiting at least one withdrawal jump or one bout of paw tremors within the 15 min observation period by the total number of mice in the group. To determine the % of body weight loss, the weight loss at the conclusion of the experiment was divided by the average weight of the group prior to the morphine injection and multiplied by 100. Most experiments were performed in triplicate and data points from multiple experiments were averaged together to construct dose–response curves. Data for withdrawal jumping, paw tremors, and weight loss were analyzed by two-way analysis of variance using antagonist dose and pretreatment condition as factors (GraphPad Prism v. 4.0, San Diego, CA, U.S.A.). If an antagonist failed to produce dose-dependent effects, the doses were averaged, resulting in a single value for a given behavioral effect (paw tremors and weight loss) and an unpaired *t*-test was performed. Significance was set at $P<0.05$. Dose–response curves for withdrawal jumping were analyzed by linear regression and ED₅₀ values and 95% CL were determined (PharmToolsPro, v1.1.27, Philadelphia, PA, U.S.A.).

Results

Control experiments

Saline produced no jumping or paw tremors in mice after 4 h pretreatments of either 56 or 180 mg kg⁻¹ morphine. These mice lost 1.2 and 0.7 g, or approximately 3 and 2% of their body weight, respectively (Table 2). Doses of 56 and 100 mg kg⁻¹ naloxone produced no jumping or paw tremors in mice after 4 h pretreatments of saline. These mice lost 0.4 and 0.7 g, or approximately 1–2% of their body weight (data not shown). Pretreatment of 180 mg kg⁻¹ morphine produced lethality in approximately 12% of the mice during the studies.

Naloxone experiments

Naloxone (1.0–100 mg kg⁻¹). produced dose-dependent ($F_{3,3} = 14.09$, $P < 0.03$) withdrawal jumping after 4 h pretreatment of 56 and 180 mg kg⁻¹ morphine with ED₅₀ values of 38 and 4.3 mg kg⁻¹, respectively (Figure 1; Table 1). Naloxone was significantly more potent in eliciting withdrawal jumping after a pretreatment of 180 than 56 mg kg⁻¹ morphine

Table 1 ED₅₀ values for opioid antagonists to precipitate withdrawal jumping in mice after 4 h pretreatment of either 56, 100, or 180 mg kg⁻¹ morphine

Antagonist	Morphine pretreatment (mg kg ⁻¹)	ED ₅₀ values (mg kg ⁻¹) (95% CL)
Naloxone	56	38 (13–100)
	100	25 (4.2–150)
	180	4.3 (2.3–66)
Naltrexone	56	7.6 (2.5–25)
	180	2.1 (0.094–9.1)
Diprenorphine	56	2.3 (0.43–15)
	180	0.70 (0.033–520)
Nalorphine	56	NA ^a
	180	26 (13–99)
Naloxonazine	56	NA ^a
	180	50 (11–5100)

^aMaximum % of mice jumping was less than 50%.

($F_{1,3} = 11.38$, $P < 0.04$). A dose of 100 mg kg⁻¹ naloxone produced maximal withdrawal jumping in 91 and 90% of the mice after 4 h pretreatment of 56 and 180 mg kg⁻¹ morphine, respectively. Naloxone elicited paw tremors in 51 and 36% of the mice and produced 6.4 and 5.2% weight loss after 4 h pretreatment of 56 and 180 mg kg⁻¹ morphine, respectively (Table 2). However, the effects of naloxone on paw tremors and weight loss were not dose-dependent or different across treatment conditions.

Naltrexone experiments

Naltrexone (0.1–100 mg kg⁻¹). produced dose-dependent ($F_{6,6} = 12.49$, $P < 0.004$) withdrawal jumping after 4 h pretreatment of 56 and 180 mg kg⁻¹ morphine with ED₅₀ values of 7.6 and 2.1 mg kg⁻¹, respectively (Figure 1; Table 1). These ED₅₀ values were not significantly different between the two pretreatment conditions, however. A dose of 100 mg kg⁻¹ naltrexone produced maximal withdrawal jumping in 100% of the mice after 4 h pretreatment of 56 and 180 mg kg⁻¹ naloxone. Naltrexone was the only antagonist to dose-dependently elicit paw tremors ($F_{1,6} = 4.55$, $P < 0.04$) in mice after 56 and 180 mg kg⁻¹ morphine (Table 2). The average percentages of mice exhibiting paw tremors across naltrexone doses were 49 and 51% after a 4 h pretreatment of 56 and 180 mg kg⁻¹ morphine, respectively. Naltrexone produced 5.7 and 4.7% weight loss after 4 h pretreatment of 56 and 180 mg kg⁻¹ morphine, respectively, although this effect was not dose-dependent or different across treatment conditions.

Diprenorphine experiments

Diprenorphine (0.01–56 mg kg⁻¹). produced dose-dependent ($F_{5,5} = 6.11$, $P < 0.034$) withdrawal jumping after 4 h pretreatment of 56 and 180 mg kg⁻¹ morphine with ED₅₀ values of 2.3 and 0.7 mg kg⁻¹, respectively (Figure 1; Table 1). These ED₅₀ values were not significantly different between the two pretreatment conditions, however. A dose of 56 mg kg⁻¹ diprenorphine produced maximal withdrawal jumping in approximately 90% of the mice after 4 h pretreatment of 56 and 180 mg kg⁻¹ morphine. Diprenorphine elicited paw tremors in 27 and 23% of the mice after pretreatment with 56 and 180 mg kg⁻¹ morphine, respectively; these effects were not dose-dependent or different across treatment conditions

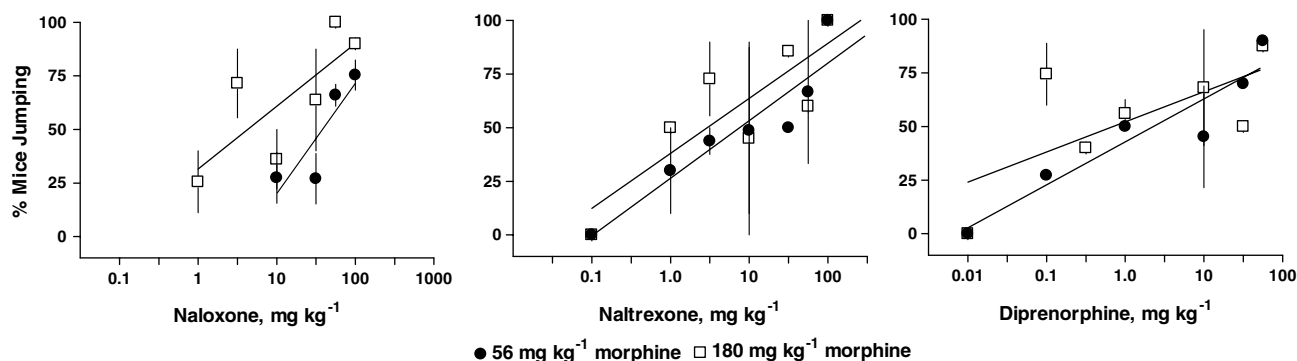


Figure 1 Effects of naloxone, naltrexone, and diprenorphine on withdrawal jumping in mice pretreated 4 h earlier with either 56 or 180 mg kg⁻¹ morphine. Each point on the dose–response curve represents 1–3 experiments consisting of 7–11 mice each. Ordinate: % of mice exhibiting withdrawal jumping. Abscissa: dose of antagonist, in mg kg⁻¹. Vertical bars represent the s.e.m. across experiments.

(Table 2). Although the effects of diprenorphine on body weight loss were not dose-dependent, the percentage of body weight lost produced by diprenorphine after 180 mg kg^{-1} morphine (4.2%) was significantly less than after 56 mg kg^{-1} morphine (6.9%) morphine ($P < 0.008$).

Nalorphine experiments

Nalorphine ($1.0\text{--}56 \text{ mg kg}^{-1}$) produced withdrawal jumping in approximately 40 and 70% of the mice after 4 h pretreatment of 56 and 180 mg kg^{-1} morphine, respectively (Figure 2). There was not a significant effect of nalorphine dose or morphine pretreatment condition. The ED_{50} value for nalorphine to elicit withdrawal jumping after 4 h pretreatment of 180 mg kg^{-1} morphine was 26 mg kg^{-1} ; an ED_{50} could not be determined for nalorphine after 4 h pretreatment of 56 mg kg^{-1} morphine (Table 1). Nalorphine elicited paw tremors in 11 and 12% of the mice and produced 6.8 and 5.5% weight loss after 4 h pretreatment of 56 and 180 mg kg^{-1} morphine, respectively (Table 2). However, the effects of nalorphine on paw tremors and weight loss were not dose-dependent or different across treatment conditions.

Naloxonazine experiments

Naloxonazine ($0.1\text{--}100 \text{ mg kg}^{-1}$) produced withdrawal jumping in approximately 37 and 50% of the mice after 4 h pretreatment of 56 and 180 mg kg^{-1} morphine, respectively

(Figure 2). There was not a significant effect of naloxonazine dose or morphine pretreatment condition. The ED_{50} value for naloxonazine to elicit withdrawal jumping after 4 h pretreatment of 180 mg kg^{-1} morphine was 50 mg kg^{-1} ; an ED_{50} could not be determined for naloxonazine after 4 h pretreatment of 56 mg kg^{-1} morphine (Table 1). Naloxonazine elicited paw tremors in 48 and 21% of the mice after pretreatment with 56 and 180 mg kg^{-1} morphine, respectively; these effects were not dose-dependent or different across treatment conditions (Table 2). Although the effects of naloxonazine on body weight loss were not dose-dependent, the percentage of body weight lost produced by naloxonazine after 180 mg kg^{-1} morphine (6.3%) was significantly greater than after 56 mg kg^{-1} (3.1%) morphine ($P < 0.04$).

Combination experiments

In mice treated with 56 mg kg^{-1} morphine 4 h prior to testing, saline pretreatment failed to significantly alter the naloxone dose-response curve (ED_{50} 79 mg kg^{-1} (51–122), data not shown) relative to naloxone alone (Table 2). In some of the combination experiments, 100 mg kg^{-1} was chosen over 180 mg kg^{-1} morphine because nalorphine, naloxonazine, and naltrexone precipitated a reasonable amount of withdrawal jumping after the mice were pretreated with 180 mg kg^{-1} morphine (Figures 1 and 2). The morphine dose was titrated to 100 mg kg^{-1} to ensure nalorphine and naloxonazine produced jumping in 50% or less of the mice. A dose of

Table 2 Average values across antagonist doses for the % mice exhibiting paw tremors and the % of body weight loss after 4 h pretreatment of either 56 or 180 mg kg^{-1} morphine

Antagonist	Paw tremors (% mice \pm s.e.m.)		Weight loss (% body weight \pm s.e.m.)	
	56 mg kg^{-1} Morphine	180 mg kg^{-1} Morphine	56 mg kg^{-1} Morphine	180 mg kg^{-1} Morphine
Saline	0	0	3.2	1.9
Naloxone	51 ± 4.5	36 ± 9.1	6.4 ± 1.8	5.2 ± 0.37
Naltrexone	49 ± 11	51 ± 5.2	5.7 ± 0.61	4.7 ± 0.43
Diprenorphine	27 ± 9.7	23 ± 9.2	6.9 ± 0.66	4.2 ± 0.51^a
Nalorphine	11 ± 7.1	12 ± 5.8	6.8 ± 0.31	5.5 ± 0.75
Naloxonazine	48 ± 16	21 ± 10	6.3 ± 0.76	3.1 ± 0.50^a

^aSignificantly different from 56 mg kg^{-1} morphine ($P < 0.05$).

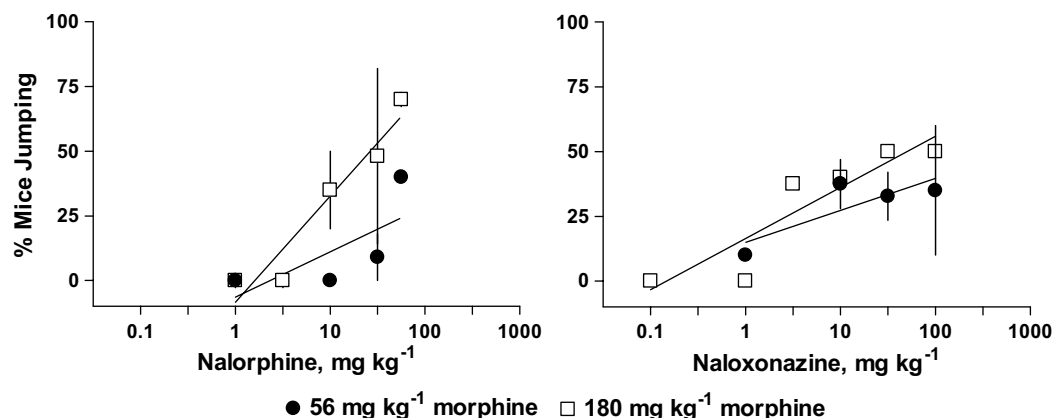


Figure 2 Effects of nalorphine and naloxonazine on withdrawal jumping in mice pretreated 4 h earlier with either 56 or 180 mg kg^{-1} morphine. Each point on the dose-response curve represents 1–4 experiments consisting of 7–11 mice each. See Figure 1 for other details.

1.0 mg kg⁻¹ naltrexone alone precipitated withdrawal jumping in 25% ($N=10$) of the mice pretreated with 56 mg kg⁻¹ morphine (Figure 1). Doses of 1.0 and 10 mg kg⁻¹ naltrexone alone precipitated withdrawal jumping in 50% ($N=10$) and 40% ($N=10$) of the mice pretreated with 100 mg kg⁻¹ morphine (data not shown). Twenty min pretreatments of 1.0 or 10 mg kg⁻¹ naltrexone failed to significantly alter naloxone precipitated withdrawal in mice injected with either 56 or 100 mg kg⁻¹ morphine 4 h prior to testing (Figure 3). Naltrexone pretreatments did not alter paw tremors or the % body weight loss produced by naloxone in mice pretreated with either 56 or 100 mg kg⁻¹ morphine.

Pretreatments of nalorphine (3 and 30 mg kg⁻¹) and naloxonazine (10 and 32 mg kg⁻¹) for 20 min blocked naloxone

precipitated withdrawal in mice injected with either 56 or 100 mg kg⁻¹ morphine 4 h prior to testing (Figure 4). A dose of 3 mg kg⁻¹ nalorphine produced a four-fold shift to the right in the naloxone dose-response curve after mice received a 4 h pretreatment of 56 mg kg⁻¹ morphine. A higher dose of nalorphine completely eliminated the capacity of 56 and 100 mg kg⁻¹ naloxone to produce withdrawal jumping. A dose of 10 mg kg⁻¹ naloxonazine failed to alter the naloxone dose-response curve after a 4 h pretreatment of 56 mg kg⁻¹ morphine (data not shown). However, a higher dose of 32 mg kg⁻¹ naloxonazine shifted the naloxone dose-response curve two-fold to the right after a 4 h pretreatment of 56 mg kg⁻¹ morphine. To further characterize the capacity of naloxonazine to block naloxone, 10 or 32 mg kg⁻¹ naloxonazine was administered 15 min prior to naloxone in mice after a 4 h pretreatment of 100 mg kg⁻¹ morphine. The ED₅₀ for naloxone to precipitate withdrawal jumping in mice pretreated with 100 mg kg⁻¹ morphine was 25 mg kg⁻¹ (Table 2). Doses of 10 and 32 mg kg⁻¹ naloxonazine shifted the naloxone dose-response curve 6- and 15-fold to the right ($P<0.05$). Nalorphine did not alter paw tremors or weight loss produced by naloxone. Naloxonazine significantly increased the percentage of mice exhibiting paw tremors after naloxone from 51% (Table 2) to 94% ($P<0.008$) in mice pretreated with 56 mg kg⁻¹ morphine. Although 10 and 32 mg kg⁻¹ naloxonazine increased the percentage of mice exhibiting paw tremors after naloxone from 38% to 80% and 73%, respectively, in mice pretreated with 100 mg kg⁻¹ morphine, these differences were not significant. Naloxonazine pretreatments did not alter the % body weight loss produced by naloxone in mice pretreated with either 56 or 100 mg kg⁻¹ morphine.

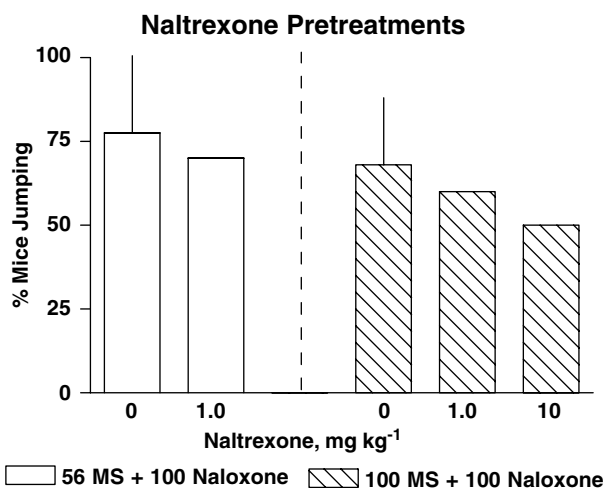


Figure 3 Effects of 1.0 or 10 mg kg⁻¹ naltrexone pretreatments on naloxone-precipitated withdrawal jumping in mice pretreated 4 h earlier with either 56 (open bars) or 100 mg kg⁻¹ (striped bars) morphine. Naltrexone was administered 20 min prior to naloxone. Bars above 0 represent control naloxone-precipitated jumping from Figure 1. Variance is expressed as s.t.d. Combination naltrexone and naloxone bars represent a single experiment in 10 mice. Ordinate: % of mice exhibiting withdrawal jumping. Abscissa: dose of naltrexone pretreatment, in mg kg⁻¹.

Discussion

In the present study, naloxone and naltrexone elicited robust withdrawal jumping, paw tremors, and weight loss in mice that were injected with 56 or 180 mg kg⁻¹ morphine 4 h prior to testing. These findings are in agreement with approximately 30 years of research using similar acute dependence procedures in mice. For example, other investigators have precipitated

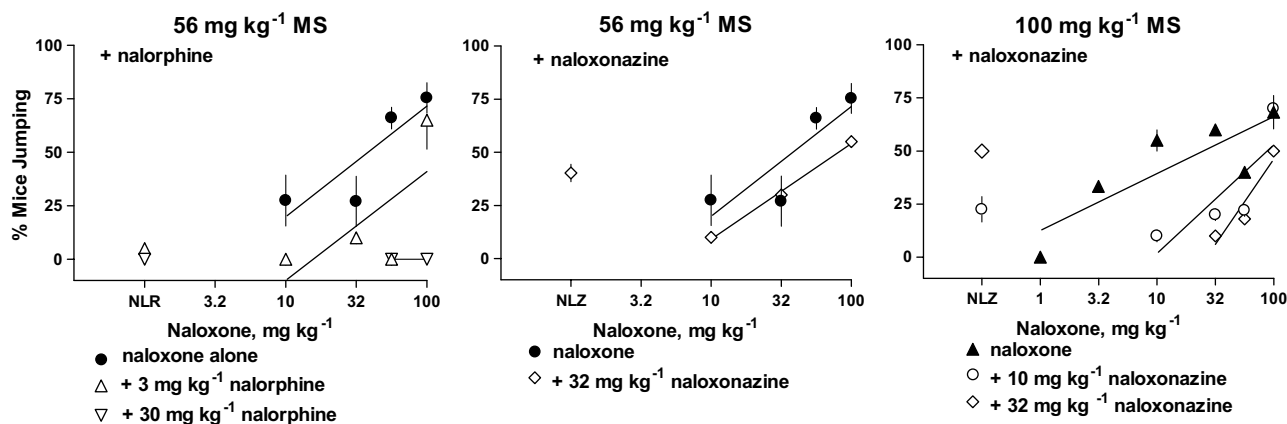


Figure 4 Effects of nalorphine and naloxonazine pretreatments on naloxone-precipitated withdrawal jumping in mice pretreated 4 h earlier with either 56 or 100 mg kg⁻¹ morphine. Nalorphine and naloxonazine were administered 20 min prior to naloxone. Control naloxone data from Figure 1. Each point in the combination experiment represents 1–3 experiments consisting of 7–11 mice. Points above NLR and NLZ represent the effects of nalorphine ($N=30$) and naloxonazine ($N=30$ –44) alone in the combination experiments. See Figure 1 for other details.

withdrawal jumping in mice treated with acute doses of 4–100 mg kg⁻¹ of morphine and tested doses of 1–128 mg kg⁻¹ naloxone (Kosersky *et al.*, 1974; Smits, 1975; Sofuoglu *et al.*, 1990). Similar pretreatment times of 3–5 h were used (Wiley & Downs, 1979) in these studies although shorter pretreatment times were required after much lower pretreatment doses of morphine (Smits, 1975; Linseman, 1977). Lower doses of naloxone and naltrexone produce withdrawal jumping in mice when higher pretreatment doses of morphine are injected (e.g., Way *et al.*, 1969). In the present study, naloxone and naltrexone were eight- and three-fold more potent, respectively, when 180 mg kg⁻¹ morphine was injected as compared to 56 mg kg⁻¹ morphine. The observation that the precipitated withdrawal jumping is dependent on the dose of agonist and antagonist used as well the time between these injections indicates that this is a robust behavioral response sensitive to pharmacological manipulations.

Using this versatile assay, five opioid antagonists were compared for their potency and effectiveness to produce withdrawal jumping. The rank order of potency for the five antagonists to produce withdrawal jumping after pretreatment with 180 mg kg⁻¹ morphine was diprenorphine > naltrexone > naloxone ≥ nalorphine = naloxonazine. This rank order of potency is in general agreement with the binding affinity of these antagonists at the μ -opioid receptor (Toll, 1992; Lee *et al.*, 1999). One exception was naloxonazine, which exhibits higher binding affinity than nalorphine (Johnson & Pasternak, 1984); yet, in the present study, the two compounds were approximately equipotent. The five antagonists were also distinguishable based on their capacity or effectiveness to elicit withdrawal jumping. In the present study, naloxone and naltrexone produced withdrawal jumping in most mice after treatment with either 56 or 180 mg kg⁻¹ morphine. Diprenorphine also produced withdrawal jumping in a majority of mice; however, the slopes of the dose–response curves were shallow. Nalorphine and naloxonazine produced withdrawal jumping in approximately half the mice after a pretreatment dose of 180 mg kg⁻¹ morphine. When a lower treatment dose of 56 mg kg⁻¹ morphine was injected, nalorphine and naloxonazine were essentially ineffective. Therefore, based on the capacity to produce withdrawal jumping in mice pretreated with morphine, the rank order of effectiveness was naloxone = naltrexone ≥ diprenorphine > nalorphine ≥ naloxonazine.

In cell culture, it has become clear that opioid antagonists can differ along a continuum of inverse agonism at δ receptors (e.g., Costa & Herz, 1989) and more recently at μ -opioid receptors (e.g., Wang *et al.*, 2004). Naloxone, naltrexone, β -CNA, BNTX, and diprenorphine are proposed inverse agonists because these compounds produce effects opposite to those of agonists *in vitro*, especially in morphine-treated cells (Wang *et al.*, 2001; 2004; Brillet *et al.*, 2003). Other antagonists such as 6 β -naltrexol, CTOP, CTAP, and nalorphine are proposed neutral antagonists *in vitro* (Wang *et al.*, 1994; 2001). *In vivo*, 6 β -naltrexol, CTOP, CTAP, and nalorphine failed to produce significant withdrawal jumping, whereas naloxone and naltrexone produce striking withdrawal jumping in morphine-dependent mice (Gulya *et al.*, 1988; Wang *et al.*, 1994; 2001; 2004; present study). However, in rats dependent on morphine, CTAP produced moderate withdrawal effects (Maldonado *et al.*, 1992) and enhanced adenylyl cyclase activity *in vitro* similar to inverse agonists (Szűcs *et al.*,

2004). Similarly, nalorphine can precipitate some withdrawal jumping under higher dependence conditions such as morphine pellet implantation (Cowan, 1976). Interestingly, naltrexone, CTAP, nalorphine, nalbuphine, and morphine withdrawal shared discriminative-stimulus effects in pigeons maintained on daily morphine injections (Walker *et al.*, 2004). Yet, under other *in vitro* and *in vivo* conditions, nalorphine and nalbuphine, but not naltrexone, produce μ -opioid agonist effects (Young *et al.*, 1992; Walker *et al.*, 2001; Gharagozlou *et al.*, 2003). However, the observation that nalorphine is a partial agonist under some assay conditions probably does not account for the antagonism of naloxone-precipitated withdrawal jumping under the present assay conditions since early studies demonstrated that low doses of agonists given shortly before naloxone actually increases withdrawal jumping (Bläsing *et al.*, 1976) and decreases the naloxone ED₅₀ to produce jumping (Cheney *et al.*, 1972). Therefore, classification of antagonists as partial agonists, inverse agonists, or neutral antagonists depends on the conditions of a given assay, as would be predicted from receptor theory (Kenakin, 2004).

The observation in the present study that all five antagonists produced at least some degree of withdrawal jumping in mice pretreated with 180 mg kg⁻¹ but not 56 mg kg⁻¹ morphine is analogous to relative efficacy differences detected between high- and low-efficacy agonists in certain assays. For example, when a noxious stimulus is low, both high- and low-efficacy agonists produce antinociception. However, when the noxious stimulus is increased, only the higher efficacy agonists possess enough intrinsic efficacy to produce an antinociceptive effect (Shaw *et al.*, 1988; Walker *et al.*, 1993; Cook *et al.*, 2000). Correspondingly, inverse agonists possessing stronger negative intrinsic efficacy, such as naloxone and naltrexone, precipitate withdrawal jumping after both high and low morphine pretreatment doses. Inverse agonists possessing weaker negative intrinsic efficacy, such as nalorphine and naloxonazine, only precipitate withdrawal jumping when the mice were highly dependent, that is, after high pretreatment doses of morphine. Intrinsic efficacy is a fluid property depending on the experimental parameters and the state of the receptor (Fathy *et al.*, 1999; Yang & Lanier, 1999) although the rank order of negative intrinsic efficacy should remain the same for a group of inverse agonists across assays if intrinsic efficacy is the relevant parameter that distinguishes among the compounds. For example, nalorphine will precipitate more withdrawal jumping under higher dependence conditions such as chronic treatment (Cowan, 1976; France & Morse, 1989) than observed in the present study. Nevertheless, naloxone and naltrexone still produce more pronounced withdrawal effects than nalorphine under any dependence condition.

Theoretically, just as low efficacy agonists block the antinociceptive effects of high-efficacy agonists when the stimulus intensity is high (Walker *et al.*, 1993; Cook *et al.*, 2000), neutral antagonists or weak inverse agonists should block the actions of strong inverse agonists when the stimulus intensity is low. Indeed, proposed weak inverse agonists nalorphine and naloxonazine blocked naloxone-induced withdrawal jumping in mice treated with either 56 or 100 mg kg⁻¹ morphine. Similarly, CTAP and nalorphine blocked the effects of naloxone and morphine *in vitro* (Wang *et al.*, 1994; 2001; Liu & Prather, 2001) as well as *in vivo* (Wang *et al.*, 1994;

Bilsky *et al.*, 1996; present study). However, pretreatments of strong inverse agonist naltrexone failed to alter naloxone precipitated withdrawal jumping in mice pretreated with either 56 or 100 mg kg⁻¹ morphine. Therefore, antagonism of naloxone-precipitated jumping was only observed by proposed neutral antagonists or weak inverse agonists such as CTAP, nalorphine, and naloxonazine (Bilsky *et al.*, 1996; present study). Taken together, these data suggest that nalorphine, naloxonazine, CTAP and 6 β -naltrexol would possess less negative intrinsic efficacy than naloxone and naltrexone. Theoretically, to block the effects of a strong inverse agonist, a compound would need to possess less negative intrinsic efficacy than the strong inverse agonist. Practically, however, a weaker inverse agonist would only be observed to block a stronger inverse agonist in an assay in which the weak inverse agonist does not elicit a strong effect.

Although these antagonists possess slightly different selectivities for multiple opioid receptors (Gillan & Kosterlitz, 1982; Geary & Wooten, 1985; Goldstein & Naidu, 1989; Mansour & Watson, 1993), affinity alone does not appear responsible for the observed results. For example, the antagonist with the highest affinity for the μ -opioid receptor, diprenorphine (Lee *et al.*, 1999), was not the most effective antagonist to elicit jumping. Naloxonazine binds reversibly to both subtypes of μ -opioid receptors shortly after administration, but binds irreversibly to μ_1 -opioid receptor subtype after 24 h (Hahn *et al.*, 1982; Ling *et al.*, 1985a). Clearly, naloxonazine's antagonism of naloxone-precipitated jumping indicates that the two compounds are competing at similar opioid receptors whether reversibly or irreversibly. *In vivo*, naloxonazine has complex effects that may depend on the species tested. For example, the antinociceptive, gastrointestinal propulsion, and respiratory effects of naloxonazine appear to be mediated by subtypes of μ -opioid receptors in rodents (Ling *et al.*, 1985a,b; Heyman *et al.*, 1988), but not necessarily rhesus monkeys (Gatch *et al.*, 1996). Despite these subtle differences in binding profiles, a reasonable hypothesis remains that the differential capacity of naloxonazine to elicit withdrawal jumping relative to naloxone may be related to the disparity between negative intrinsic efficacies for the two compounds.

Another finding in the present study indicates that some measures of opioid withdrawal in the acute dependence model may be more sensitive indicators of inverse agonism or negative intrinsic efficacy than others. For example, all antagonists produced some degree of dose-dependent withdrawal jumping generally related to the acute pretreatment dose of morphine. Although all antagonists consistently produced some degree of body weight loss and paw tremors in mice acutely treated with morphine, these effects were not dose-dependent and not necessarily related to the morphine treatment dose. In earlier studies of morphine dependence,

withdrawal symptoms such as jumping were most readily observed after injection with strong opioid antagonists and labeled 'dominant' withdrawal symptoms. Other opioid compounds, such as nalorphine, were less effective than naloxone in producing jumping (Bläsing *et al.*, 1973; 1976). On the other hand, other withdrawal signs, such as wet dog shakes and writhing, were more likely to appear under more moderate morphine dependence conditions, later in the observation period (Bläsing *et al.*, 1973), or when withdrawal jumping was blocked by other compounds such as benzodiazepines (Maldonado *et al.*, 1991; Valverde *et al.*, 1992). These other symptoms were labeled 'recessive' withdrawal symptoms and appeared to have a somewhat reciprocal relationship with the occurrence of withdrawal jumping (Bläsing *et al.*, 1973). In the present acute dependence study, the mice were mildly to moderately dependent given the observed levels of withdrawal jumping, paw tremors, and weight loss. Interestingly, the highest frequency of paw tremors (a possible 'recessive' withdrawal symptom) was observed when naloxonazine blocked naloxone-precipitated withdrawal jumping. Overall, in our studies, withdrawal jumping was the most robust and sensitive behavioral index of inverse agonism or negative intrinsic efficacy. Future behavioral studies with combinations of other weak and strong inverse agonists will allow us to further delineate the possible reciprocal relationship of paw tremors and withdrawal jumping.

Taken together, these findings suggest that antagonists may vary according to negative intrinsic efficacy, as indicated by their differential capacity to produce withdrawal jumping and the capacity of some weaker (nalorphine and naloxonazine) but not strong inverse agonists (naltrexone) to block the effects produced by naloxone in mice acutely treated with morphine. Conceivably, μ -opioid receptor antagonists may be classified on a continuum of partial to full negative intrinsic efficacy under certain conditions in the same way that μ -opioid receptor agonists are classified on a continuum of partial to full positive intrinsic efficacy. The observations in the present series of experiments contribute to recent efforts by other investigators (Bilsky *et al.*, 1996; Wang *et al.*, 2004) to provide an *in vivo* systematic classification of antagonists in relation to negative intrinsic efficacy. Additional and complementary *in vitro* and *in vivo* experiments that vary assay conditions are required to make definitive conclusions regarding the relationship of inverse agonism, negative intrinsic efficacy, and opioid dependence.

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