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Comparison of the opioid receptor antagonist properties of naltrexone and 6β-naltrexol in morphine-naïve and morphine-dependent mice

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Abstract

It has been proposed that on chronic morphine treatment the μ-opioid receptor becomes constitutively active, and as a consequence, the opioid withdrawal response arises from a reduction in the level of this constitutively active receptor. In support of this, the putative μ-opioid receptor inverse agonist naltrexone has been shown to precipitate more severe withdrawal behavior in mice than the putative neutral receptor antagonist 6β-naltrexol. In the present study naltrexone and 6β-naltrexol were compared in NIH Swiss mice to test the hypothesis that their differential ability to precipitate withdrawal is due to differences in their *in vivo* opioid receptor antagonist potencies caused by differential access to μ-opioid receptors in the central nervous system and not necessarily by intrinsic differences in their opioid receptor activity. In naïve mice both compounds had similar potencies to antagonize morphine-induced antinociception in the hot plate and warm-water tail-withdrawal assays when measured under equilibrium conditions and afforded similar calculated apparent *in vivo* μ-opioid receptor affinities. In morphine-dependent mice both compounds precipitated withdrawal jumping but naltrexone was between 10- and 100-fold more potent than 6β-naltrexol. A similar potency difference was seen for other withdrawal behaviors. Both naltrexone and 6β-naltrexol at 1 mg/kg reversed antinociception induced by the long-lasting μ-opioid receptor agonist BU72 in the warm-water tail-withdrawal assay, but antagonism by naltrexone was 6-fold more rapid in onset at equal doses. Since the compounds have similar affinity for the μ-opioid receptor *in vivo*, the results suggest that the differences observed between the ability of naltrexone and 6β-naltrexol to precipitate withdrawal in the mouse may be explained by differential onset of receptor antagonist action.

Keywords: Morphine; Withdrawal; Dependence; Naltrexone; 6β-naltrexol; (Mouse)

1. Introduction

Chronic activation of the μ-opioid receptor by morphine and related μ-opioid receptor agonists leads to dependence. This is manifested as a withdrawal response that can be rapidly precipitated in morphine-dependent organisms by administration of an opioid receptor antagonist such as naltrexone. However, recent evidence argues that opioid withdrawal after chronic morphine is not simply due to displacement of morphine from the μ-opioid receptor. Rather it has been suggested that on long-term agonist exposure a constitutively active form of the μ-opioid receptor, that is a receptor which displays basal signaling activity even in the absence of agonist, dominates in the morphine-dependent state and that this form of the receptor is the key in defining the severity of the opioid withdrawal response (Wang et al., 1994; Wang et al., 2004). Consequently, it has been proposed that withdrawal contains a component resulting from a reversal or loss of the level of constitutively active receptor that dominates in the dependent state (Sadee et al., 2005). Based on this model, an inverse opioid receptor agonist that decreases basal signaling of a constitutively active receptor should precipitate a more severe withdrawal response than a neutral opioid receptor antagonist which has no intrinsic effects on signaling and can only precipitate withdrawal by displacement of an opioid receptor agonist. In addition, it has been hypothesized that opioid receptor antagonists with high negative intrinsic efficacy produce withdrawal after treatment with low doses of morphine, whereas compounds with lower negative efficacy produce withdrawal only after more severe morphine treatment (Walker and Sterious 2005).

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Differences in the ability of naltrexone and 6β-naltrexol to precipitate withdrawal in morphine-dependent mice have been observed (Wang et al., 2004; Raehal et al., 2005). In contrast, naltrexone and 6β-naltrexol have similar affinities *in vitro* for the μ-opioid receptor expressed in HEK293 cells (Wang et al., 2001), suggesting a differential ability to displace μ-opioid receptor agonist bound to receptor may not be responsible for the observed differences to precipitate withdrawal. These contradictory results have led to the proposal that naltrexone is a μ-opioid receptor inverse agonist, while 6β-naltrexol is a neutral antagonist, or at least has reduced inverse μ-opioid receptor agonist activity (Raehal et al., 2005). These findings have been supported by *in vitro* experiments demonstrating that naltrexone, but not 6β-naltrexol, reduces basal [^{35}S]GTPγS binding in tissues from mouse brain as a measure of inverse agonist activity (Wang et al., 2001, 2004).

However, at higher doses 6β-naltrexol does precipitate the same degree of withdrawal responses in morphine-dependent mice as those observed following naltrexone (Chatterjie et al., 1975; Fujimoto et al., 1975; Raehal et al., 2005). Moreover, in the monkey we have shown similar actions of naltrexone and 6β-naltrexol to reduce morphine-induced respiratory depression, scratching, and to precipitate acute morphine withdrawal, even though 6β-naltrexol is 100-fold less potent than naltrexone (Ko et al., 2006). Together, these findings support the idea that 6β-naltrexol is a weaker opioid receptor antagonist than naltrexone (Blumberg and Ikeda, 1976) and suggests the differences between the compounds are due to differential potency rather than dissimilar efficacy (Wang et al., 2004; Raehal et al., 2005). Additionally, the partial opioid receptor agonists nalbuphine and nalorphine precipitate withdrawal in morphine-dependent mice (Raehal et al., 2005; Walker and Sterious, 2005) and monkeys (Aceto 1984; Woods and Gmerek, 1985; Valentino et al., 1983) if administered at high enough doses, indicating that even ligands with positive intrinsic activity are capable of precipitating withdrawal. Therefore, a variety of evidence suggests there may be a quantitative difference in potency rather than a qualitative difference in intrinsic activity between the abilities of naltrexone and 6β-naltrexol to precipitate withdrawal.

In this study we examine the hypothesis that the differential abilities of naltrexone and 6β-naltrexol to precipitate withdrawal are due to an apparent difference in their *in vivo* receptor antagonist potencies that is caused by differential access to agonist occupied μ-opioid receptors. To test this hypothesis the two compounds were compared in NIH Swiss mice for their ability to antagonize morphine-induced antinociception and their ability to precipitate behaviors indicative of morphine withdrawal. Rather than compare the abilities of the two compounds to prevent agonist properties of a single dose of morphine (Raehal et al., 2005) we determined the *in vivo* affinities of the compounds under equilibrium conditions. In addition, since the severity of withdrawal depends upon the degree of agonist displacement from μ-opioid receptors, a reduced rate of access of 6β-naltrexol to these receptors would also reduce its predicted ability to precipitate withdrawal. To compare the time course of the two antagonists to access agonist occupied μ-opioid receptors we measured their abilities to reverse an existing antinociception due to the presence of the μ-opioid receptor agonist 17-methyl-3-hydroxy-[5β, 7β, 3′, 5′]-pyrrolidino-2′[S]-phenyl-7α-methyl-6,14-*endo*ethenomorphinan (BU72). This compound produces a long-lasting antinociception that can be rapidly reversed by both naltrexone and naloxone (Neilan et al., 2004). If 6β-naltrexol has a reduced rate of access to central μ-opioid receptors then this will show up as a slower rate of reversal of BU72 mediated antinociception. Unlike humans, rodents do not significantly metabolize naltrexone to 6β-naltrexol (Malspeis et al., 1975) so this should not be a confounding factor when comparing the compounds *in vivo*.

The results confirm similar *in vivo* μ-opioid receptor affinities for naltrexone and 6β-naltrexol, but different *in vivo* potencies to precipitate withdrawal and different rates of displacement of BU72. These data are consistent with similar pharmacodynamics but differential pharmacokinetics of the two antagonists.

2. Methods

2.1. Drugs

Naltrexone HCl and 6β-naltrexol HCl were gifts from the National Institute on Drug Abuse (NIDA, Bethesda, MD) and morphine sulfate was obtained from Mallinckrodt (St. Louis, MO). BU72 (17-methyl-3-hydroxy-[5β, 7β, 3′, 5′]-pyrrolidino-2′[S]-phenyl-7α-methyl-6,14-*endo*etheno morphinan) was a gift from Dr. SM Husbands, University of Bath, Bath, UK. All compounds were dissolved in sterile water and administered s.c. at a volume of 0.01 ml/g.

2.2. Animals

Adult male NIH Swiss or ICR mice, approximately 25–30 g (Harlan, Indianapolis, IN), were used. The NIH Swiss mice were used for most experiments described. The ICR mice were used only in an experiment to compare naltrexone *in vivo* affinity values in a different strain. Mice were housed in groups and were maintained on a 12 h light/dark cycle (lights on at 7 am) with free access to food and water in a temperature-controlled (22.5±1 °C) room. Each mouse was used only once. Experiments were performed during the lights on hours between 10 am and 2 pm. Studies were performed in accordance with the University Committee on the Use and Care of Animals in the University of Michigan, the *Principles of Laboratory Care*, and the *Guide for the Care and Use of Mammals in Neuroscience and Behavioral Research* (National Research Council 2003).

2.3. Antinociception

For antinociceptive studies a cumulative morphine dosing schedule was employed. Each animal first received vehicle or antagonist (i.p or s.c.) and then 30 min later up to 5 doses of morphine (0.32 mg/kg–320 mg/kg, s.c.) in 30 min intervals. For determination of *in vivo* antagonist affinity by Schild analysis (pA_2 values, see Data Analysis, Section 2.5) varying doses (1, 3, 10 mg/kg) of antagonists were used. For determination of antagonist *in vivo* affinities as pK_B values (see Data Analysis, Section 2.5) single 1 mg/kg or 10 mg/kg doses of antagonist were used. We have previously shown that a single administration of naltrexone is effective for at least 6 h in adult male NIH Swiss mice (Neilan et al.,

2004). Groups of five to six mice were used for each treatment. *Hot Plate:* Post-injection (30 min), the mouse was placed on a 55 °C aluminum hot plate. The latency to a prolonged lifting of the forepaws, jumping, or licking of the paws was measured with a cutoff time of 30 s to prevent tissue damage. *Warm-Water Tail-Withdrawal:* Post-injection (30 min) the tail was placed in a warm-water bath at 50 °C and the latency to withdraw the tail was measured, with a cutoff time of 20 s. *Rate of Antagonist Onset*: In naïve mice, 0.32 mg/kg BU72 was administered s.c. 1 h prior to injection of the antagonists 6β-naltrexol (1 or 10 mg/kg, s.c.) or naltrexone (1 mg/kg, s.c.). The warm-water tail-withdrawal assay was performed at varying time points after antagonist injection to assess the ability of the compounds to reverse BU72-induced antinociception.

2.4. Morphine dependence

Naltrexone (0.1 mg/kg) or 6β-naltrexol (1 and 10 mg/kg) were used to precipitate withdrawal in morphine-dependent mice. Two dosing regimens of morphine administration were used to establish morphine dependence. The first used pellet implantation. A single morphine pellet (75 mg) was inserted s.c. in the back of the mouse under halothane anesthesia (Fujimoto et al., 1975; Raehal et al., 2005). Opioid receptor antagonists were administered 72 h after pellet implantation. In the second regime mice received five s.c. 30 mg/kg morphine injections 12 h apart. Opioid receptor antagonists were given 3 h after the last administration of morphine. To assess withdrawal each mouse was placed singly in a Plexiglas box (28 cm long × 18 cm wide × 13 cm high) and allowed to habituate for 15 min. Mice were then given an injection of opioid receptor antagonist, put back into the Plexiglas box and observed for 20 min by a trained experimenter who was blinded to all dosing conditions. The total occurrences and the frequency in 5 min bins were measured for the following behaviors: jumps, wet dog shakes, paw tremors, bouts of grooming behavior including body grooming, scratching, and head washing (Van Wimersma Greidanus et al., 1985; Maldonado et al., 1991). Eight to nine mice were used for each treatment group.

2.5. Data analysis

Results of the antinociception experiments are expressed as the percentage of maximum possible effect (%MPE), where %MPE = (latency to behavioral endpoint − baseline latency)/(cutoff latency − baseline latency) × 100%. For each treatment condition, mean values (mean ± S.E.M.) were calculated from individual values for all behavioral endpoints. Apparent *in vivo* affinities for the antagonists were determined from antagonist-induced shifts in the morphine dose–effect curve as pK_B or pA_2 values. These values are the negative logarithm of the dissociation constant of an antagonist determined under equilibrium conditions and are an indirect measure of an antagonist's affinity for its receptors. pK_B receptor values were calculated using a single dose of antagonist as indicated in the appropriate figure legend or table according to the equation $pK_B = -\log[B/(\text{dose-ratio} - 1)]$, where B equals the opioid receptor antagonist dose in mol/kg and dose-ratio represents the ED_{50} dose in the presence of antagonist divided by the ED_{50} dose in the absence of antagonist (Ko et al., 1998). Apparent pA_2 values were determined from experiments using three different doses of antagonist expressed in mol/kg according to the Schild method (Arunlakshana and Schild, 1959) with slopes constrained to unity. Data were analyzed by ANOVA followed by the Newman–Keuls test for multiple post hoc comparisons. The criterion for significance was set at $P < 0.05$. GraphPad Prism 4.0 (San Diego, CA) was used to analyze all data.

3. Results

3.1. Antinociception

Morphine (s.c.) dose-dependently increased the latency to nociceptive response in the 55 °C hot plate assay (Fig. 1A) with an ED_{50} of 18.4 ± 4.0 mg/kg, and in the 50 °C warm-water tail-

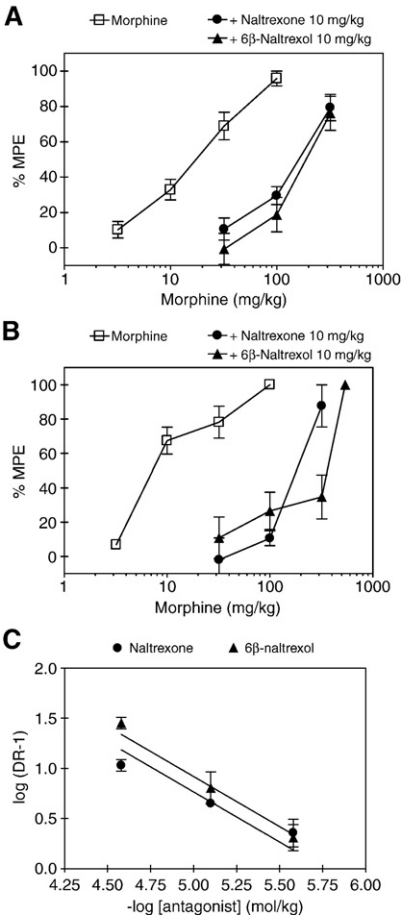

Fig. 1. Morphine-induced antinociception in NIH Swiss mice in (A) the 55 °C hot plate assay in the presence or absence of 10 mg/kg naltrexone or 6β-naltrexol, or (B) the 50 °C warm-water tail-withdrawal assay. While 1, 3, and 10 mg/kg naltrexone or 6β-naltrexol were examined in the warm-water tail-withdrawal assay for the Schild analysis presented in C, only one antagonist dose (10 mg/kg) is shown for clarity. Mice received vehicle, 6β-naltrexol, or naltrexone s.c. 30 min prior to cumulative doses of morphine s.c. in 30 min intervals. Data are expressed as % MPE versus dose of morphine on a logarithmic scale and represent means ± S.E.M. for five-six mice in each group. (C) Schild plots for naltrexone and 6β-naltrexol generated using three doses of each compound (1, 3, and 10 mg/kg) to antagonize morphine in the warm-water tail-withdrawal assay with slopes constrained to unity.

withdrawal assay (Fig. 1B) with an ED_{50} of 12.0±2.3 mg/kg. Pretreatment for 30 min with s.c. 6β-naltrexol or s.c. naltrexone dose-dependently induced rightward shifts in the morphine dose–response curve in both antinociceptive tests. At 10 mg/kg, the two antagonists were equipotent at antagonizing morphine-induced antinociception in the hot plate test affording similar *in vivo* affinities (pK_B values; Table 1). Using antagonist doses of 1, 3, and 10 mg/kg and the warm-water tail-withdrawal assay, similar *in vivo* antagonist affinities were confirmed as pA_2 values calculated by Schild analysis (Fig. 1C; Table 1). The pA_2 for naltrexone (5.8±0.1) is lower than reported following i.p. administration of this antagonist in ND4 Swiss–Webster mice (Garner et al., 1997) so we re-examined antagonism of morphine with 1 mg/kg naltrexone following i.p. rather than s.c. administration but obtained a very similar value (pK_B of 5.7±0.1, Table 1). To determine if this was due to our experimental methodology we repeated the experiment in ICR mice as a different strain. Using these ICR mice and 1 mg/kg naltrexone we did obtain a pK_B for naltrexone against morphine of 7.5±0.2 (data not shown), suggesting a strain effect.

3.2. Morphine dependence

The onset and magnitude of jumping behavior elicited by naltrexone and 6β-naltrexol were compared in morphine pellet-treated mice (Fig. 2). There was a significant main effect for treatment [$F(3,63)=11.6$; $P<0.0001$] and time [$F(3,63)=15.5$; $P<0.0001$] and a significant interaction [$F(9,63)=7.2$; $P<0.0001$]. Naltrexone (0.1 mg/kg) significantly increased the number of jumps 5–20 min after s.c. administration ($P<0.001$). At a dose of 1 mg/kg, 6β-naltrexol was unable to precipitate any withdrawal jumping. However, 10 mg/kg 6β-naltrexol significantly increased the number of jumps ($P<0.01$) such that there was no significant difference between jumping induced by this dose of 6β-naltrexol and 0.1 mg/kg naltrexone across all time points. This indicates a large potency difference between these two compounds to induce withdrawal.

Fig. 3 illustrates the effects of s.c. naltrexone or 6β-naltrexol in eliciting other behavioral withdrawal signs in morphine pellet-treated mice over the 20 min observation period. Naltrexone (0.1 mg/kg) significantly increased jumping behavior and decreased grooming behaviors including body grooming and scratching as compared with vehicle ($P<0.05$). Both doses of 6β-naltrexol (i.e., 1 and 10 mg/kg) were equally potent in attenuating the behavior of body grooming and scratching, but only 10 mg/kg 6β-naltrexol significantly increased the number of jumps, wet dog shakes, and paw tremors ($P<0.05$). There was no significant difference between the effects of 0.1 mg/kg naltrexone and 10 mg/kg 6β-naltrexol measured at these behavioral endpoints.

Table 1
In vivo affinity values (pA_2 or pK_B) for naltrexone and 6β-naltrexol measured by antagonism of morphine-induced antinociception in the mouse

Antagonist	Hot-plate	Tail-withdrawal
Naltrexone s.c	5.4±0.1[a]	5.8±0.06[b]
6β-naltrexol s.c	5.6±0.1[a]	5.9±0.1[b]
Naltrexone i.p.	NT	5.7±0.1[a]

Affinity values were obtained as [a]pK_B values using single 10 mg/kg doses of antagonist or as [b]pA_2 values using three doses (1, 3 and 10 mg/kg) of antagonist, as described in the Methods section. pA_2 values when not constrained to unity were 6.1 for naltrexone and 5.8 for 6β-naltrexol in the tail-withdrawal test. Values represent means±S.E.M. derived from individual plots for five-six mice at each dose of antagonist. NT=not tested.

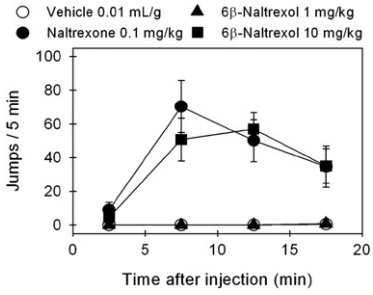

Fig. 2. Withdrawal jumping behavior in morphine-dependent mice. Mice were implanted with a 75 mg morphine pellet. After 72 h withdrawal was precipitated with s.c. injection of vehicle, 6β-naltrexol (1 or 10 mg/kg), or naltrexone (0.1 mg/kg). Jumping behavior was measured in 5 min periods for 20 min immediately following antagonist injection. Data are expressed as the number of jumps in each 5 min period and represent mean±S.E.M. for eight-nine mice in each group.

The effects of naltrexone and 6β-naltrexol in eliciting behavioral withdrawal signs were also compared in mice given repeated injections of morphine (Fig. 4). Naltrexone (0.1 mg/kg, s.c.) significantly increased the number of jumps, wet dog shakes, and paw tremors, and decreased the behaviors of body grooming and scratching as compared with vehicle ($P<0.05$). Both doses of 6β-naltrexol (i.e., 1 and 10 mg/kg, s.c.) were equally potent in

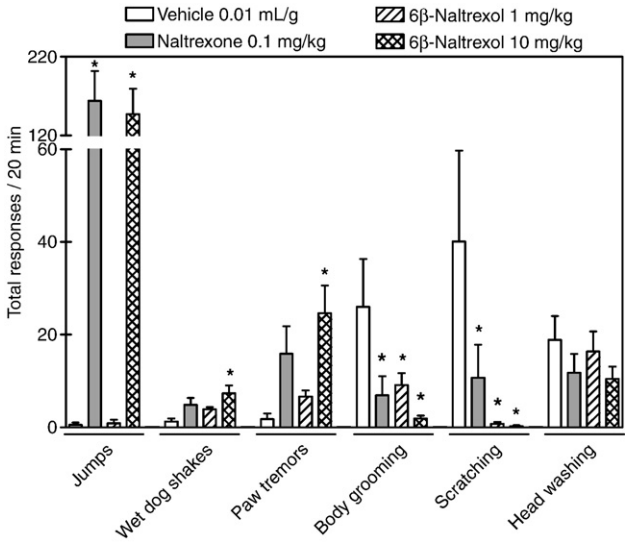

Fig. 3. Behavioral responses in morphine pellet-implanted dependent mice. Mice were implanted with a 75 mg morphine pellet and 72 h later withdrawal was precipitated by s.c. injection of vehicle, 6β-naltrexol (1 or 10 mg/kg), or naltrexone (0.1 mg/kg). Total jumping, wet dog shakes, paw tremors, grooming, scratching, and head washing behaviors were measured for 20 min immediately following antagonist injection. Data are expressed as the total number of responses in a 20 min period and represent mean±S.E.M. for eight-nine mice in each group. *$P<0.05$ compared to vehicle injection.

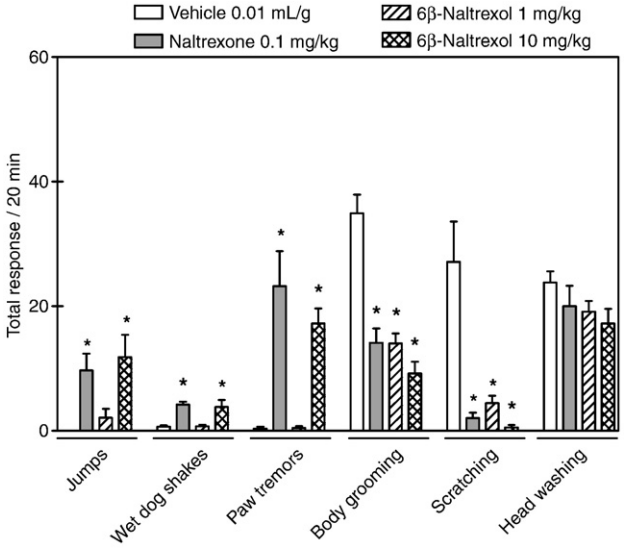

Fig. 4. Behavioral responses in morphine-dependent mice that received five s.c. morphine 30 mg/kg injections 12 h apart as described in Methods. Withdrawal was precipitated by s.c. injection of vehicle, 6β-naltrexol (1 or 10 mg/kg), or naltrexone (0.1 mg/kg). Total jumping, wet dog shakes, paw tremors, grooming, scratching, and head washing behaviors were measured for 20 min immediately following antagonist injection. Data are expressed as the total number of responses in a 20 min period and represent mean±S.E.M. for eight-nine mice in each group.*$P<0.05$ compared to vehicle injection.

attenuating the behavior of body grooming and scratching, but only 10 mg/kg 6β-naltrexol significantly increased the number of jumps, wet dog shakes, and paw tremors ($P<0.05$). In addition, there was no significant difference between effects of 0.1 mg/kg naltrexone and 10 mg/kg 6β-naltrexol measured by these behavioral responses.

Previous studies have suggested that the differences in the ability of naltrexone and 6β-naltrexol to precipitate withdrawal are due to efficacy differences (Wang et al., 2004; Raehal et al., 2005). If naltrexone and 6β-naltrexol have similar affinities but differing efficacies at the μ-opioid receptor the putative neutral antagonist (6β-naltrexol) should inhibit the ability of the putative inverse agonist (naltrexone) to precipitate withdrawal if reversal of constitutive activity is an important component of withdrawal. On the other hand, if they have the same degree of efficacy but different potencies there should be no effect of 6β-naltrexol on naltrexone-induced withdrawal at inactive doses of 6β-naltrexol. To examine for interaction between the two compounds we used a dose of naltrexone (0.1 mg/kg, s.c.) that causes a marked withdrawal response in morphine pellet-implanted dependent mice. We co-administered this dose of naltrexone with a 10-fold higher dose (1 mg/kg, s.c.) of 6β-naltrexol that does not cause withdrawal but, since the compounds have similar receptor affinities, should readily compete with naltrexone and displace this compound from the μ-opioid receptor. If 6β-naltrexol is a neutral antagonist it should reduce the withdrawal signs induced by naltrexone. A higher dose of 6β-naltrexol precipitates withdrawal alone. However, 1 mg/kg 6β-naltrexol did not alter the withdrawal jumping precipitated by 0.1 mg/kg naltrexone (Fig. 5). When the dose of 1 mg/kg 6β-naltrexol was given as a 90 min pretreatment to allow equilibrium to be reached there was still no reversal of the effect of 0.1 mg/kg naltrexone. There was an indication of an enhanced withdrawal response (Fig. 5), but this was not significant [$F(2,14)=1.7$; $P>0.05$].

3.3. Antagonism of the long-lasting μ-opioid receptor agonist BU72

Since naltrexone and 6β-naltrexol have similar *in vivo* receptor affinities measured under equilibrium conditions it is possible that differences in the ability to precipitate withdrawal may be due to a slower access to μ-opioid receptors which would be evident before equilibrium conditions are achieved. To address this, the ability of the compounds to reverse a pre-existing antinociceptive action of BU72 was determined using the mouse 50 °C warm-water tail-withdrawal assay. BU72 is a highly efficacious μ-opioid receptor agonist which displays antinociceptive properties in the mouse that last for at least 8 h at its maximally effective dose (0.32 mg/kg) (Neilan et al., 2004). At equal doses (1 mg/kg, s.c.), both 6β-

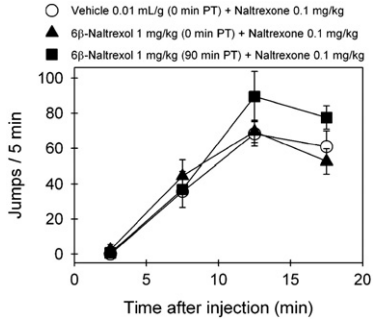

Fig. 5. Withdrawal jumping behavior in morphine-dependent mice implanted with a 75 mg morphine pellet for 72 h. Withdrawal was precipitated by s.c. naltrexone injection (0.1 mg/kg) co-administered with vehicle, co-administered with 6β-naltrexol (1 mg/kg), or following a 90 min pretreatment (PT) with 6β-naltrexol (1 mg/kg). Jumping behavior was measured in 5 min periods for 20 min immediately following naltrexone injection. Data are expressed as the number of jumps per 5 min and represent mean±S.E.M. for eight-nine mice in each group.

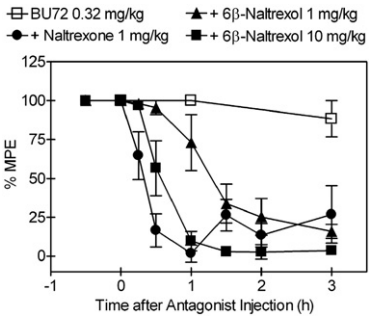

Fig. 6. Time course of s.c. 6β-naltrexol (1 or 10 mg/kg) or s.c. naltrexone (1 mg/kg) to antagonize 0.32 mg/kg BU72-induced antinociception in the warm-water tail-withdrawal assay at 50 °C in NIH Swiss mice. Mice received BU72 s.c. 1 h prior to injection of vehicle, 6β-naltrexol, or naltrexone (time 0). The ability of 6β-naltrexol or naltrexone to antagonize BU72-induced antinociception in the warm-water tail-withdrawal assay was measured at various time points following receptor antagonist injection. Data are expressed as % MPE and represent mean±S.E.M. for five mice in each group.

naltrexol and naltrexone reversed BU72-induced antinociception, but antagonism by naltrexone was more rapid in onset (Fig. 6). When the data were fit to a one-phase exponential decay, 1 mg/kg naltrexone antagonized BU72-induced antinociception with a half-life of 11.3±2.6 min, while 6β-naltrexol antagonized BU72-induced antinociception with a half-life of 72.5±15.7 min. At a higher dose (10 mg/kg) 6β-naltrexol had a faster rate of reversing BU72-induced antinociception with a half-life of 27.3±3.2 min, approaching the rate seen with 1 mg/kg naltrexone.

4. Discussion

An accurate description of the ability of opioid receptor antagonists to precipitate withdrawal is important because this can lead to improved therapies for the treatment of opioid addiction and overdose with reduced adverse withdrawal consequences. Previous work in the mouse led to the proposal that naltrexone precipitates a severe opioid withdrawal response because it is an inverse agonist and reverses signaling of a constitutively active form of the μ-opioid receptor that develops during dependence (Wang et al., 2004; Raehal et al., 2005; Sadee et al., 2005). In contrast, 6β-naltrexol as a neutral antagonist does not precipitate the same degree of withdrawal, unless much higher doses are employed. The present findings show that at appropriate doses naltrexone and 6β-naltrexol do give the same severity of morphine withdrawal in the mouse, but that naltrexone is more potent than 6β-naltrexol. On the other hand, both compounds have similar *in vivo* affinity for the μ-opioid receptor as determined by their apparent pK_B values in the hot plate assay and their apparent pA_2 values in the warm-water tail-withdrawal assay, in agreement with *in vitro* binding assay results (Wang et al., 2001) and confirming the differences in antagonist activity between the two compounds are not due to differences in receptor affinity. Consequently, the differential potency of the two antagonists to induce morphine withdrawal can be explained by the rate at which they reach μ-opioid receptors *in vivo* following s.c. administration and therefore the rate at which they displace μ-opioid receptor agonist from the receptor *in vivo*.

Naltrexone and 6β-naltrexol cause very similar parallel shifts in the morphine dose–response curve for antinociception in both the warm-water tail-withdrawal and the hot plate assays in the mouse when measured at least 30 min after antagonist administration. This result indicates a similar *in vivo* affinity of naltrexone and 6β-naltrexol for the μ-opioid receptor as measured by pharmacological assay (pK_B or pA_2) and in agreement with ligand binding studies in cloned cells (Wang et al., 2001), guinea-pig brain (Nelson et al., 1994) and monkey brain tissue (Ko et al., 2006). Although the pA_2 values for naltrexone and 6β-naltrexol were the same, they are approximately 10-fold greater than the pA_2 for naltrexone reported in male ND4 Swiss–Webster mice (Garner et al., 1997). The difference is not due to the route of administration but is likely due to the strain of mice, since we obtained much higher affinity in ICR mice, although the reason for this strain difference is unclear.

Despite this equivalent *in vivo* receptor affinity, studies using single doses of morphine show a 5- to 10-fold weaker effect of 6β-naltrexol to block morphine-induced antinociception (Wang et al., 2001; Raehal et al., 2005). Likewise, in the present study a dose of 6β-naltrexol between 10 and 100-times greater than naltrexone was required to precipitate the same degree of withdrawal across a variety of measures following dependence induced by two paradigms of morphine administration, morphine pellets or repeated morphine injections. The two methods of inducing dependence produced a similar degree of antagonist-precipitated withdrawal as measured by wet dog shakes, paw tremors, body grooming, scratching, and head washing. In contrast, jumping was much more marked, approximately 10-fold higher, in the morphine-pelleted animals compared to the animals that received repeated morphine injections. Since the level of jumping is associated with the severity of dependence (Blasig et al., 1973; Maldonado et al., 1991) this indicates the pelleted animals are more profoundly dependent. Moreover, the degree of jumping is related to the dose of precipitating antagonist (Blasig et al., 1973). Comparison of 0.1 mg/kg naltrexone with 10 mg/kg 6β-naltrexol showed no significant differences in the precipitation of any measure of withdrawal, including jumping. Consistent with these results, potency differences ranging from 53- to 100-fold have been previously reported for the ability of naltrexone and 6β-naltrexol to precipitate withdrawal (Chatterjie et al., 1975; Fujimoto et al., 1975; Raehal et al., 2005) following systemic administration.

These potency differences have been interpreted as support for two mechanisms by which opioid withdrawal may be induced, namely displacement of agonist with the putative neutral antagonist 6β-naltrexol, or displacement of agonist combined with reversal of a constitutively active μ-opioid receptor by the putative inverse agonist naltrexone. If the compounds have differential efficacies yet similar *in vivo* μ-opioid receptor affinities, then the neutral antagonist 6β-naltrexol should be able to reverse the action of the inverse agonist naltrexone. However, 6β-naltrexol was unable to prevent the withdrawal response induced by naltrexone even when 6β-naltrexol was co-administered at a 10-fold higher dose and given as a 90 min pretreatment to allow for sufficient access to μ-opioid receptors, nor were additive effects seen. Since displacement of naltrexone by 6β-naltrexol must happen over this longer time period, as indicated by the antagonism of BU72-mediated antinociception, then the compounds must be indistinguishable to the receptor. We have also observed similar results in the monkey (Ko et al., 2006). These findings suggest that under the relatively short temporal conditions of the opioid withdrawal assay the two compounds have differential abilities to bind to the μ-opioid receptor following systemic administration rather than different actions at the receptor (Raehal et al., 2005). In contrast to our results in the monkey and mouse, it has been shown that 6β-naltrexol (10–30 mg/kg) can reverse the withdrawal induced by 10 mg/kg naloxone (Raehal et al., 2005), following acute dependence to a single 100 mg/kg dose of morphine. The reason for this discrepancy is not readily apparent but may suggest differences between acute versus chronic dependence, and/or differences between naltrexone and the weaker naloxone.

In support of a differential *in vivo* binding of naltrexone and 6β-naltrexol under the non-equilibrium conditions of the withdrawal assay, there was a marked disparity in the abilities of systemically administered naltrexone and 6β-naltrexol to reverse antinociception produced by the long-lasting highly efficacious μ-opioid receptor agonist BU72, presumably by displacing BU72 from the

μ-opioid receptor. At equal doses (1 mg/kg, s.c.), both compounds were able to reverse BU72-induced antinociception in the warm-water tail-withdrawal assay, yet the effects of naltrexone were significantly more rapid in onset. At the higher dose of 10 mg/kg, the effects of 6β-naltrexol approached the rate seen with 1 mg/kg naltrexone. Since withdrawal is measured on a short time scale following antagonist administration these results of more rapid ability of naltrexone compared to 6β-naltrexol to displace BU72 provide an explanation for the finding that 6β-naltrexol at higher doses can precipitate a severe withdrawal (present results and Chatterjie et al., 1975; Fujimoto et al., 1975; Raehal et al., 2005). In defense of this conclusion, 6β-naltrexol at 10 mg/kg, but not at 1 mg/kg, was able to inhibit morphine-induced antinociception in the hot plate assay measured 30 min post-antagonist injection (Porter et al., 2002). Similarly, 6β-naltrexol rapidly reverses morphine's locomotor effects at a dose 10-times greater than that of naltrexone (Wang et al., 2004; Raehal et al., 2005). These pharmacological studies are supported by the assay of brain levels of the two compounds following systemic (i.p.) administration. When measured 10 min after injection a dose of 1 mg/kg naltrexone and 10 mg/kg 6β-naltrexol provide equal amounts of drug in mouse brain; yet after a dose of 1 mg/kg 6β-naltrexol only limited quantities of the compound are found in brain (Wang et al., 2004). Thus, the results suggest the difference in the *in vivo* ability of the two antagonists to precipitate withdrawal may be explained by relative rates of access to central μ-opioid receptors as assessed by their ability to displace BU72.

The present results do confirm that in the mouse (Wang et al., 2004; Raehal et al., 2005), as with previous work in the monkey (Ko et al., 2006), naltrexone is a more potent inducer of withdrawal than 6β-naltrexol in morphine dependence, but shows that both compounds induce qualitatively similar withdrawal symptoms. However, the present findings suggest the difference between naltrexone and 6β-naltrexol in precipitating morphine withdrawal in the mouse is due to different abilities to reach the site of action and the rapidity of access needed to precipitate a robust withdrawal response. This differential effect is also observed in antagonism of a pre-existing antinociceptive action, yet differences are not seen in conventional antagonism studies when longer antagonist pretreatment times are used to provide equilibrium conditions. Under these latter conditions the properties of the two compounds at the μ-opioid receptor are equivalent. If extrapolated to man the results would agree with the suggestion that 6β-naltrexol may provide a more clinically acceptable method of treating opioid overdose (Sadee et al., 2005). 6β-naltrexol is a metabolite of naltrexone in man and might be expected to contribute to the observed pharmacology of naltrexone. The metabolite is maximally found at 2 to 3-fold higher concentrations than naltrexone in plasma following oral administration (Ferrari et al., 1998). Given the large acute potency difference, 6β-naltrexol may not contribute after naltrexone administration, although it may be responsible for the long duration of naltrexone action (Bullingham et al., 1983). Consequently differential pharmacokinetic profiles of the two compounds may explain why 6β-naltrexol produces a less severe withdrawal than naltrexone and thus be a preferable antagonist for the treatment of overdose.

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References

Aceto, M.D., 1984. Characterization of prototypical opioid antagonists, agonist-antagonists, and agonists in the morphine-dependent rhesus monkey. Neuropeptides 5, 15–18.

Arunlakshana, O., Schild, H.O., 1959. Some quantitative uses of drug antagonists. Br. J. Pharmacol. Chemother. 14, 48–58.

Blasig, J., Herz, A., Reinhold, K., Zieglgansberger, S., 1973. Development of physical dependence on morphine in respect to time and dosage and quantification of the precipitated withdrawal syndrome in rats. Psychopharmacologia (Berl) 33, 19–38.

Blumberg, H., Ikeda, C., 1976. Comparison of naltrexone and 6β-naltrexol in rats and mice. Fed. Proc. 35, 469.

Bullingham, R.E., McQuay, H.J., Moore, R.A., 1983. Clinical pharmacokinetics of narcotic agonist–antagonist drugs. Clin. Pharmacokinet. 8, 332–343.

Chatterjie, N., Inturrisi, C.E., Dayton, H.B., Blumberg, H., 1975. Stereospecific synthesis of the 6β-hydroxy metabolites of naltrexone and naloxone. J. Med. Chem. 18, 490–492.

Ferrari, A., Bertolotti, M., Dell'Utri, A., Avico, U., Sternieri, E., 1998. Serum time course of naltrexone and 6β-naltrexol levels during long term treatment in drug addicts. Drug Alcohol Depend. 52, 211–220.

Fujimoto, J.M., Roerig, S., Wang, R.I.H., Chatterjie, N., Inturrisi, C.E., 1975. Narcotic antagonist activity of several metabolites of naloxone and naltrexone tested in morphine dependent mice. Proc. Soc. Exp. Biol. Med. 148, 443–448.

Garner, H.R., Burke, T.F., Lawhorn, C.D., Stoner, J.M., Wessinger, W.D., 1997. Butorphanol-mediated antinociception in mice: partial agonist effects and mu receptor involvement. J. Pharmacol. Exp. Ther. 282, 1253–1261.

Ko, M.C., Butelman, E.R., Traynor, J.R., Woods, J.H., 1998. Differentiation of kappa opioid agonist-induced antinociception by naltrexone apparent pA_2 analysis in rhesus monkeys. J. Pharmacol. Exp. Ther. 285, 518–526.

Ko, M.C., Divin, M.F., Lee, H., Woods, J.H., Traynor, J.R., 2006. Differential in vivo potencies of naltrexone and 6β-naltrexol in the monkey. J. Pharmacol. Exp. Ther. 316, 772–779.

Maldonado, R., Mico, J.A., Valverde, O., Saavedra, M.C., Leonsegui, I., Gibert-Rahola, J., 1991. Influence of different benzodiazepines on the experimental morphine abstinence syndrome. Psychopharmacology (Berl) 105, 197–203.

Malspeis, L., Bathala, M.S., Ludden, T.M., Bhat, H.B., Frank, S.G., Sokoloski, T.D., Morrison, B., Reuning, R.H., 1975. Metabolic reduction of naltrexone.1. Synthesis, separation and characterization of naloxone and naltrexone reduction products and qualitative assay of urine and bile following administration of naltrexone, alpha-naltrexol or beta-naltrexol. Res. Commum. Chem. Path. Pharmacol. 12, 43–65.

Nelson, T.D., Davis, R.D., Nelson, W.L., 1994. Synthesis and opioid receptor affinity of a series of aralkyl ethers of 6α- and 6β-naltrexol. J. Med. Chem. 37, 4270–4277.

Neilan, C.L., Husbands, S.M., Breeden, S., Ko, M.C., Aceto, M.D., Lewis, J.W., Woods, J.H., Traynor, J.R., 2004. Characterization of the complex morphinan derivative BU72 as a high efficacy, long-lasting mu-opioid receptor agonist. Eur. J. Pharmacol. 499, 107–116.

Porter, S.J., Somogyi, A.A., White, J.M., 2002. In vivo and in vitro potency studies of 6β-naltrexol, the major human metabolite of naltrexone. Addict. Biol. 7, 219–225.

Raehal, K.M., Lowery, J.J., Bhamidipati, C.M., Paoline, R.M., Blair, J.R., Wang, D., Sadee, W., Bilsky, E.J., 2005. In vivo characterization of 6β-naltrexol, an opioid ligand with less inverse agonist activity compared with naltrexone and naloxone in opioid-dependent mice. J. Pharmacol. Exp. Ther. 313, 1150–1162.

Sadee, W., Wang, D., Bilsky, E.J., 2005. Basal opioid receptor activity, neutral antagonists, and therapeutic opportunities. Life Sci. 76, 1427–1437.

Valentino, R.J., Katz, J.L., Medzihradsky, F., Woods, J.H., 1983. Receptor binding, antagonist, and withdrawal precipitating properties of opiate antagonists. Life Sci. 32, 2887–2896.

Van Wimersma Greidanus, T.J., Donker, D.K., Walhof, R., Van Grafhorst, J.C., De Vries, N., Van Schaik, S.J., Maigret, C., Spruijt, B.M., Colbern, D.L., 1985. The effects of neurotensin, naloxone and haloperidol on elements of excessive grooming behavior induced by bombesin. Peptides 6, 1179–1183.

Walker, E.A., Sterious, S.N., 2005. Opioid antagonists differ according to negative intrinsic efficacy in a mouse model of acute dependence. Br. J. Pharmacol. 145, 975–983.

Wang, Z., Bilsky, E.J., Porreca, F., Sadee, W., 1994. Constitutive mu-opioid receptor activation as a regulatory mechanism underlying narcotic tolerance and dependence. Life Sci. 54, PL339–PL350.

Wang, D., Raehal, K.M., Bilsky, E.J., Sadee, W., 2001. Inverse agonists and neutral antagonists at μ opioid receptor (MOR): possible role of basal receptor signaling in narcotic dependence. J. Neurochem. 77, 1590–1600.

Wang, D., Raehal, K.M., Lin, E.T., Lowery, J.J., Kieffer, B.L., Bilsky, E.J., Sadee, W., 2004. Basal signaling activity of μ opioid receptor in mouse brain: role in narcotic dependence. J. Pharmacol. Exp. Ther. 308, 512–520.

Woods, J.H., Gmerek, D.E., 1985. Substitution and primary dependence studies in animals. Drug Alcohol Depend. 14, 233–247.