

# $\mu$ -Opioid Receptor Up-Regulation and Functional Supersensitivity Are Independent of Antagonist Efficacy

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## ABSTRACT

Chronic opioid antagonist treatment up-regulates opioid receptors and produces functional supersensitivity. Although opioid antagonists vary from neutral to inverse, the role of antagonist efficacy in mediating the chronic effects of opioid antagonists is not known. In this study, the effects of two putative inverse agonists (naltrexone, naloxone) and a putative neutral antagonist ( $6\beta$ -naltrexol) were examined. Initially, peak effect (40 min, naltrexone and naloxone; 70 min,  $6\beta$ -naltrexol) and relative potency to antagonize morphine analgesia were determined (relative potencies = 1, 2, and 16,  $6\beta$ -naltrexol, naloxone, and naltrexone, respectively). Next, mice were infused for 7 days with naloxone (0.1–10 mg/kg/day), naltrexone (10 or 15 mg s.c. pellet), or  $6\beta$ -naltrexol (0.2–20 mg/kg/day), and spinal  $\mu$ -opioid receptor density was examined, or morphine analgesia dose-response studies were conducted. All antagonists up-regulated

$\mu$ -opioid receptors (60–122%) and induced supersensitivity (1.8–2.0-fold increase in morphine potency). There were no differences in antagonist potency to produce up-regulation or supersensitivity. These data suggest that opioid antagonist-induced  $\mu$ -opioid receptor up-regulation and supersensitivity require occupancy of the receptor and that antagonist efficacy is not critical. Finally, the ED<sub>50</sub> to precipitate withdrawal jumping was examined in morphine-dependent mice. Naltrexone, naloxone, and  $6\beta$ -naltrexol produced withdrawal jumping, although potencies relative to  $6\beta$ -naltrexol were 211, 96, and 1, respectively. Thus, antagonist potency to precipitate opioid withdrawal was related to inverse agonist efficacy. Overall, the estimated relative potency of the opioid antagonists was a function of the outcome measured, and inverse agonist activity was not required for  $\mu$ -opioid receptor up-regulation and supersensitivity.

Receptor density regulation is a well documented phenomenon in the opioid system (e.g., Yoburn et al., 1986; Keith et al., 1996, 1998; Patel et al., 2002). Both opioid agonists and antagonists have been shown to regulate  $\mu$ -opioid receptor density (Chakrabarti et al., 1997; Zaki et al., 2000; Rajashekara et al., 2003), and these changes can have an impact on the potency of opioid agonists (e.g., Stafford et al., 2001; Patel et al., 2003). The efficacy of an opioid agonist has been proposed to play an important role in  $\mu$ -opioid receptor regulation (Patel et al., 2002; Pawar et al., 2007). For example, chronic treatment with high-efficacy agonists (e.g., etorphine, DAMGO) can induce internalization and down-regulation of  $\mu$ -opioid receptors in vitro and in vivo (Duttaroy and Yoburn, 1995; Keith et al., 1998; Whistler and von Zastrow, 1998). Conversely, low-efficacy agonists (e.g., morphine, oxy-

codone) are less likely to produce either internalization or down-regulation (Keith et al., 1998; Pawar et al., 2007; however, see Haberstock-Debic et al., 2005). Chronic treatment with opioid antagonists (e.g., naltrexone, naloxone) has been shown to up-regulate  $\mu$ -,  $\delta$ -, and  $\kappa$ -opioid receptors (Yoburn et al., 1995; Lesscher et al., 2003; Patel et al., 2003) and increase the potency of opioid agonists (functional supersensitivity) (Yoburn and Inturrisi, 1988; Yoburn et al., 1989, 1995).

The concept that unliganded receptors can display constitutive activity has gained broad acceptance (Bond and Ijzerman, 2006). Studies have shown that all three opioid receptors can display basal activity in the unliganded state (e.g., Costa and Herz, 1989; Becker et al., 1999; Burford et al., 2000). Ligands that suppress constitutive signaling activity are termed inverse agonists or negative antagonists, whereas those that only block agonist-induced effects without altering constitutive signaling are termed neutral antagonists (Kenakin, 2001). Antagonists, like agonists, will display pro-tean behavior, such that an inverse opioid agonist will appear to be neutral in a system that lacks constitutively active receptors (Kenakin, 2001; Prather, 2004). Under appropriate

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**ABBREVIATIONS:** DAMGO, [D-Ala<sup>2</sup>, N-MePhe<sup>4</sup>, Gly-O<sup>5</sup>]-enkephalin; CL, confidence limit.

experimental conditions, studies suggest that antagonists such as naltrexone and naloxone are inverse agonists, whereas  $6\beta$ -naltrexol is a putative neutral antagonist (Wang et al., 2004; Raehal et al., 2005).

Recently, it has been proposed that the population of constitutively active opioid receptors is increased by chronic treatment with opioid agonists and the development of dependence (Wang et al., 2001; Sadée et al., 2005; Walker and Sterious, 2005). Furthermore, it has been demonstrated that antagonists that inhibit constitutive signaling (i.e., inverse agonists) are highly effective in precipitating withdrawal (e.g., Walker and Sterious, 2005; Wang et al., 2007). Conversely, neutral antagonists are significantly less potent in precipitating opioid withdrawal signs and can antagonize the withdrawal produced by inverse agonists such as naloxone (Wang et al., 2001; Raehal et al., 2005; Walker and Sterious, 2005). In brain homogenates from morphine-dependent rats, naloxone and naltrexone, but not  $6\beta$ -naltrexol, decrease guanosine 5'-O-(3-[ $^{35}$ S]thio)triphosphate binding (Wang et al., 2004), and  $6\beta$ -naltrexol pretreatment can reduce the effect of naloxone (Raehal et al., 2005). Taken together, precipitated withdrawal appears to be related to antagonist efficacy (e.g., Sadée et al., 2005), and there is strong support for classifying  $6\beta$ -naltrexol as a neutral antagonist or, at minimum, as an antagonist with substantially reduced inverse agonist activity.

Although studies have documented the effect of inverse agonists in suppressing constitutive opioid receptor activity and precipitating withdrawal (Wang et al., 2004; Walker and Sterious, 2005), little is known about the role of antagonist efficacy in  $\mu$ -opioid receptor up-regulation and functional supersensitivity. It seemed likely that antagonist efficacy might determine up-regulation and supersensitivity given that previous studies indicate that agonist efficacy plays a role in  $\mu$ -opioid receptor down-regulation and tolerance (e.g., Pawar et al., 2007). Although data indicate that antagonist-induced up-regulation and agonist-induced down-regulation are mechanistically distinct (e.g., Yoburn et al., 2004; Pawar et al., 2007), the evidence linking agonist efficacy, tolerance, and receptor regulation is compelling (Patel et al., 2002; Pawar et al., 2007), and some studies have suggested that antagonist efficacy may be important in up-regulation and supersensitivity (Morris and Millan, 1991; Milligan and Bond, 1997). Therefore, it seemed plausible that antagonist efficacy might have an impact on  $\mu$ -opioid receptor up-regulation and functional supersensitivity.

In the present study, two putative inverse agonists (naltrexone and naloxone) and a neutral antagonist ( $6\beta$ -naltrexol) were compared in terms of  $\mu$ -opioid receptor regulation, functional supersensitivity, and precipitated withdrawal. Surprisingly, antagonist efficacy was not correlated with  $\mu$ -opioid receptor up-regulation and functional supersensitivity, despite the fact that the neutral antagonist was far less potent in precipitating withdrawal in the morphine-dependent state.

## Materials and Methods

**Subjects.** Male Swiss-Webster mice, weighing 22 to 30 g, obtained from Taconic Farms (Germantown, NY), were used throughout the study. Animals were housed 10 per cage for at least 24 h after arrival with food and water available ad libitum. All protocols were ap-

proved by the St. John's University Institutional Animal Care and Use Committee.

**Drugs.** Naltrexone HCl, naloxone HCl,  $6\beta$ -naltrexol HCl, and morphine sulfate were obtained from the Research Triangle Institute (Research Triangle Park, NC) through the Research Technology Branch of the National Institute on Drug Abuse (Bethesda, MD). Subcutaneous implant pellets containing naltrexone (30- and 10-mg naltrexone base) or morphine (25-mg morphine base) and placebo pellets were also obtained from the Research Triangle Institute. The 30-mg naltrexone pellets were cut in half (yields 15-mg pellet), and all implant pellets were wrapped in nylon mesh before s.c. implantation. Drugs for injection or infusion were dissolved in 0.9% saline, and doses are expressed as the free base.

**General Procedure.** Initially, the time of peak effect to antagonize morphine (5 mg/kg s.c.) analgesia for naltrexone, naloxone, and  $6\beta$ -naltrexol was determined. This dose of morphine produces analgesia (see below) in all mice at 30 min following administration. Subsequently, the ED<sub>50</sub>s for antagonism of morphine analgesia (5 mg/kg s.c.) were determined for naltrexone, naloxone, and  $6\beta$ -naltrexol. Mice were then treated for 7 days (infusion with osmotic minipumps, Alzet model 2001; DURECT Corporation, Cupertino, CA; or s.c. drug pellet implantation) with each antagonist, and changes in  $\mu$ -opioid receptor density were examined in the spinal cord. Other mice were treated for 7 days with each antagonist, treatment was terminated, and 24 h later, morphine analgesia dose-response studies were conducted. To examine the potency of each antagonist to precipitate withdrawal, mice were implanted s.c. with a single morphine pellet (25 mg) and 72 h later injected with antagonist and the ED<sub>50</sub> for withdrawal jumping (pellet implanted) estimated. Pumps and pellets were implanted and removed while mice were lightly anesthetized with halothane/oxygen (4:96).

**Peak Effect Estimation.** Mice (five to six mice/group) were injected s.c. with naltrexone (0.25 mg/kg), naloxone (0.2 mg/kg), or  $6\beta$ -naltrexol (0.45 mg/kg). At various times following antagonist treatment (1, 10, 30, 60, 90, 120, 150, and 210 min) mice were injected s.c. with morphine (5 mg/kg) and then tested for analgesia (tail-flick, see below) 30 min following morphine. This procedure resulted in antagonism of morphine being determined at 31, 40, 60, 90, 120, 150, 180, and 240 min following antagonist treatment.

**ED<sub>50</sub> Studies.** Mice (six/group) were injected s.c. with naltrexone (0.001–0.25 mg/kg), naloxone (0.01–5.0 mg/kg), or  $6\beta$ -naltrexol (0.05–0.45 mg/kg). Morphine (5 mg/kg) was injected 10 min following naltrexone and naloxone and 40 min following  $6\beta$ -naltrexol. Mice were tested for analgesia 30 min following morphine administration at the time of peak effect for morphine and each antagonist.

**Analgesia Assay.** Antinociception was assayed using the tail-flick assay (Model TF6; Emdie Instrument Co., Maidens, VA), in which a beam of light was focused on the dorsal surface of the tail of the mouse, approximately 2 cm from the tip of the tail. The intensity of the light was adjusted so that baseline tail-flick latency was 2 to 4 s. If a mouse did not remove its tail from the heat source by 10 s, the test was terminated, a latency of 10 s was recorded, and the mouse was defined as analgesic. All testing was conducted by an experimenter who was unaware of the treatment of an individual mouse.

**Functional Supersensitivity Studies.** Mice (8–10/group) were infused with naloxone (10.0 mg/kg/day) or  $6\beta$ -naltrexol (20.0 mg/kg/day) for 7 days. Other mice (8–10/group) were implanted with naltrexone pellets s.c. (10 or 15 mg) for 7 days. For all treatment groups, controls were implanted with a placebo pellet for 7 days. At the end of treatment, the pellets and pumps were removed, and 24 h later, the analgesic potency of morphine was determined using a cumulative dose-response protocol (Duttaroy et al., 1997). For the cumulative dose-response protocol, mice were injected s.c. with a starting dose of morphine and tested for analgesia 30 min later. If the mouse had a tail-flick latency of 10 s, it was defined as analgesic and not tested further. Otherwise, the mouse was immediately injected s.c. with a second dose of morphine and retested. This procedure was continued until all mice were analgesic. The individual dose se-

quence used for cumulative dosing for morphine following naltrexone treatment was 1.5, 1.5, 2.0, 3.0, and 4.0 mg/kg, which yields cumulative doses of 1.5, 3.0, 5.0, 8.0, and 12.0 mg/kg. The individual dose sequence used for cumulative dosing for morphine following naloxone or 6 $\beta$ -naltrexol was 0.5, 1.0, 1.5, 2.0, and 3.0 mg/kg, which yields cumulative doses of 0.5, 1.5, 3.0, 5.0, and 8.0 mg/kg. Cumulative dosing was used to reduce the number of mice and the cost of supplies (e.g., osmotic pumps).

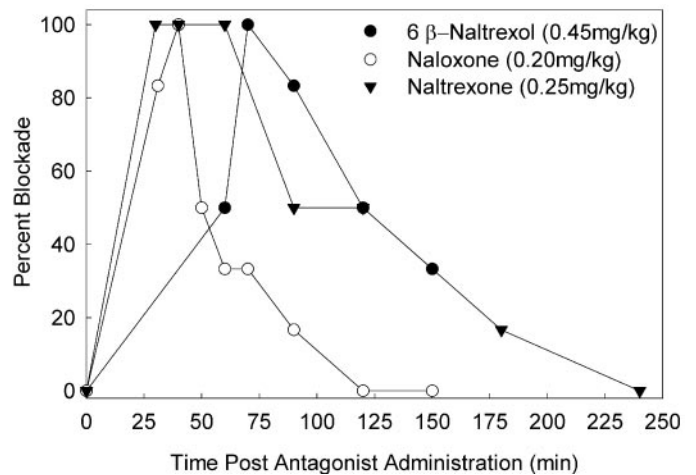
**Radioligand Binding Assay.** Mice (10/dose) were infused with naloxone (0.1, 1.0, and 10.0 mg/kg/day) or 6 $\beta$ -naltrexol (0.2, 2.0, and 20.0 mg/kg/day) for 7 days. Other mice (10/dose) were implanted with naltrexone pellets (10 or 15 mg). For all treatment groups, controls were implanted with placebo pellets for 7 days. At the end of naloxone and 6 $\beta$ -naltrexol treatments, mice were sacrificed. At the end of naltrexone pellet treatment, the pellets were removed, and 24 h later, mice were sacrificed. For all groups, spinal cords were rapidly removed and pooled in tubes containing 15 ml of ice-cold 50 mM Tris buffer, pH 7.4. Samples were homogenized (Brinkmann Polytron Homogenizer, Westbury, NY) at 20,000 rpm on ice for 40 s. Homogenates were centrifuged at 15,000 rpm for 15 min (3–9°C). The supernatant was discarded, and pellets were stored (–80°C) until analysis. On the day of assay, the pellet was thawed on ice, resuspended in 15 ml of Tris buffer, centrifuged at 15,000 rpm for 15 min (3–9°C), and the supernatant was discarded. The pellet was resuspended in 35 ml of Tris buffer and incubated for 30 min in a shaking water bath (25°C). Samples were centrifuged again for 15 min, the supernatant was discarded, and pellets were suspended in 18 ml of ice-cold 50 mM potassium phosphate buffer, pH 7.2. An aliquot of homogenate was assayed in triplicate in saturation binding assays using [<sup>3</sup>H]DAMGO (range, 0.02–10 nM) ( $\mu$ -opioid receptor ligand; PerkinElmer Life Sciences, Boston, MA). Nonspecific binding was determined in triplicate in the presence of levorphanol (1000 nM). Tubes were incubated for 90 min at 25°C. Incubation was terminated by the addition of ice-cold phosphate buffer and filtering the samples over GF/B filters (Brandel, Gaithersburg, MD). Tubes were washed three times with phosphate buffer, and filters were placed in vials containing scintillation cocktail and counted. Counts per minute were converted into disintegrations per minute using the external standard method. Protein was assayed by the Bradford method (Bradford, 1976) using reagent from Bio-Rad (Richmond, CA). Binding studies were conducted one to two times for each treatment.

**Withdrawal Jumping Assay.** Mice (five/group) were implanted s.c. with a placebo pellet or a single morphine pellet (25 mg) for 72 h and at the end of treatment injected with naltrexone (0.003–0.1 mg/kg), naloxone (0.01–1.0 mg/kg), or 6 $\beta$ -naltrexol (1.0–20.0 mg/kg). Immediately following antagonist treatment, mice were placed in a clear plastic container (5 liters) and observed for 15 min for jumping. Jumping was defined as all four paws leaving the bottom of the plastic container. All jumping was observed by an experimenter who was unaware of the treatment of an individual mouse. For the purpose of quantal dose-response analysis, mice that jumped 50 or more times in the 15-min observation period were defined as positive for withdrawal jumping. The ED<sub>50</sub> for each antagonist to precipitate withdrawal jumping was estimated.

**Data Analysis.** Dose-response data were analyzed using the BLISS-21 computer program (Department of Statistics, University of Edinburgh, Edinburgh, UK). This program uses Probit analysis (Finney, 1973) to calculate ED<sub>50</sub> values, S.E.s, 95% confidence intervals, and potency estimates. Binding data from saturation studies were analyzed by Prism version 4.03 (Graph Pad Software, San Diego, CA) using nonlinear regression. All binding data were best fit by a one-site model.

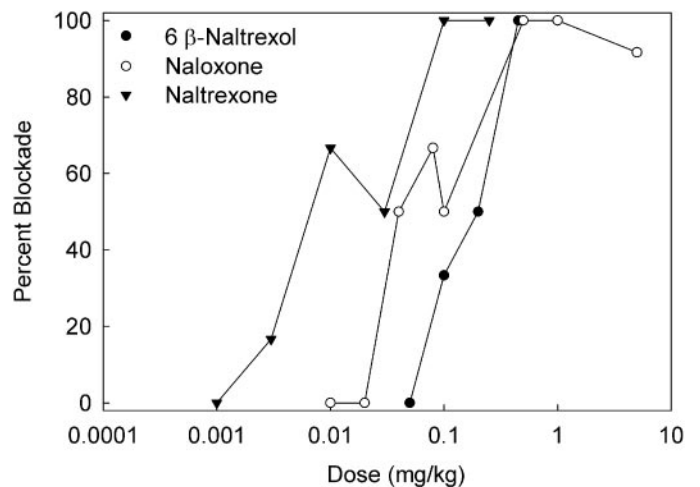
## Results

Time action profile studies (Fig. 1) indicated that the peak antagonist effect for blockade of morphine analgesia (5 mg/kg) was approximately 40 min for naltrexone and nal-



**Fig. 1.** Time course of antagonism of morphine-induced analgesia by naltrexone, naloxone, and 6 $\beta$ -naltrexol. Individual groups of mice (five to six/drug/time point) were injected with naltrexone (0.25 mg/kg), naloxone (0.20 mg/kg), and 6 $\beta$ -naltrexol (0.45 mg/kg). At various times (1–210 min) following antagonist treatment, mice were injected with morphine (5 mg/kg) and tested for analgesia 30 min later. Each mouse was tested only once. This procedure resulted in antagonism of morphine being determined at 31, 40, 60, 90, 120, 150, and 240 min following antagonist treatment. Tail-flick latency of less than 10 s was defined as blockade of morphine-induced analgesia. The percentage of mice demonstrating blockade is plotted versus the time following antagonist administration. Time of maximal blockade of analgesia was estimated as 40 min for naltrexone and naloxone and 70 min for 6 $\beta$ -naltrexol.

oxone and 70 min for 6 $\beta$ -naltrexol. In subsequent studies, these peak effect estimates were used to determine relative antagonist potency. The estimated ED<sub>50</sub>s (95% CL) for naltrexone, naloxone, and 6 $\beta$ -naltrexol to block morphine analgesia (5 mg/kg) were 0.01 (0.005–0.027), 0.08 (0.04–0.20), and 0.16 (0.10–0.28) mg/kg, respectively (Fig. 2). The order



**Fig. 2.** Dose-response functions for antagonism of morphine-induced analgesia by naltrexone, naloxone, and 6 $\beta$ -naltrexol. Mice (6/dose/drug) were injected s.c. with naltrexone (0.001–0.25 mg/kg), naloxone (0.01–5.0 mg/kg), or 6 $\beta$ -naltrexol (0.05–0.45 mg/kg). Ten minutes following naltrexone and naloxone and 40 min following 6 $\beta$ -naltrexol administration, all mice were injected s.c. with morphine (5 mg/kg) and tested for analgesia 30 min later at the time of peak effect for each antagonist (see Fig. 1). Each mouse was tested only once. Tail-flick latency of less than 10 s was defined as blockade of morphine-induced analgesia. The percentage of mice demonstrating blockade is plotted versus the dose of antagonist. The ED<sub>50</sub>s (95% CL) for naltrexone, naloxone, and 6 $\beta$ -naltrexol were estimated as 0.01 (0.01–0.03), 0.08 (0.04–0.20), and 0.16 (0.10–0.28) mg/kg, respectively.

of potency relative to 6 $\beta$ -naltrexol was naltrexone (16) > naloxone (2) > 6 $\beta$ -naltrexol (1).

Next, we determined the effect of chronic antagonist treatment on  $\mu$ -opioid receptors in mouse spinal cord. All three drugs significantly increased the density of  $\mu$ -opioid receptors in mouse spinal cord (Fig. 3). The effect of naloxone and 6 $\beta$ -naltrexol on  $\mu$ -opioid receptors was examined in dose-response studies (Fig. 3A). Both drugs produced a dose-dependent increase in  $\mu$ -opioid receptor density, with no significant change in  $K_d$ . The highest infusion dose for naloxone and 6 $\beta$ -naltrexol increased the density of  $\mu$ -opioid receptors by 85 to 122%. There was no potency difference for receptor up-regulation between naloxone and 6 $\beta$ -naltrexol. Typical results for 6 $\beta$ -naltrexol and naloxone are shown in Fig. 3, B and C. Naltrexone pellet (10 or 15 mg) treatment also increased the density of spinal  $\mu$ -opioid receptors (+60–77%) without altering affinity (Fig. 3D). There was no significant difference in the magnitude of up-regulation between the groups treated with the 10- or 15-mg naltrexone pellet.

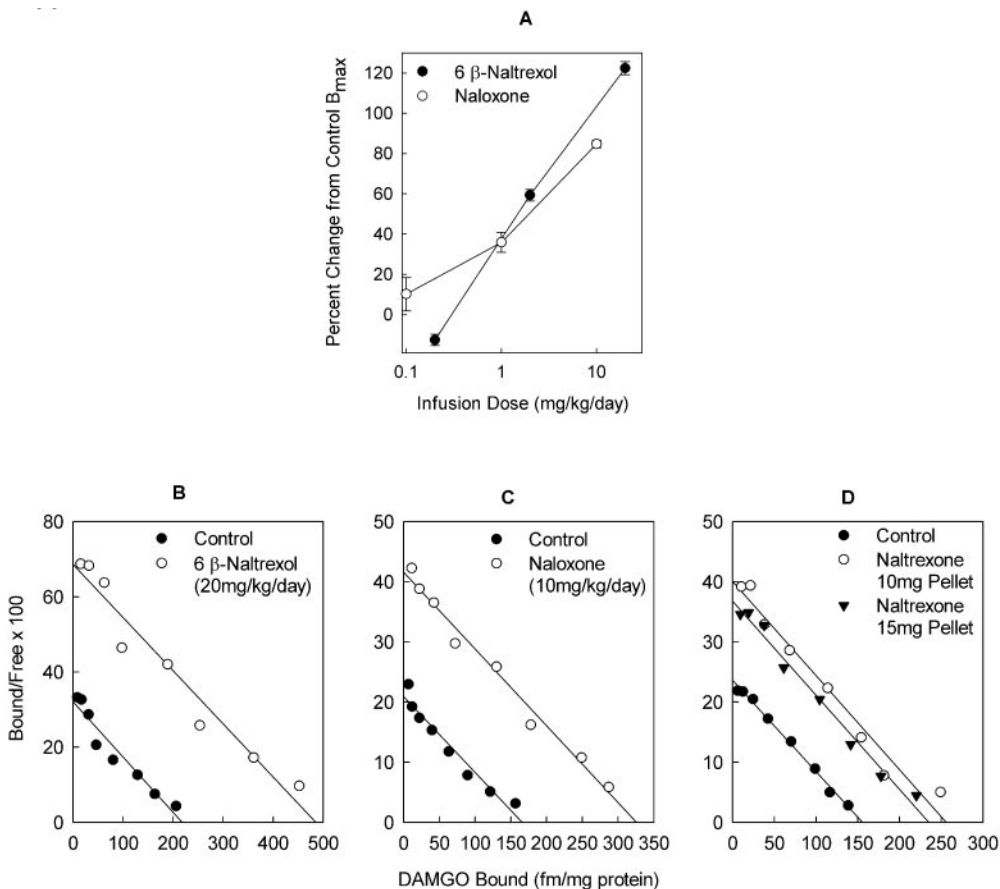
Chronic treatment with the three antagonists similarly increased the analgesic potency of morphine (Fig. 4). Table 1 presents the ED<sub>50</sub>s and relative potencies for morphine in control and treated groups. Naltrexone pellet treatment (10 or 15 mg) (Fig. 4, left) increased morphine analgesic potency by  $\approx$ 2-fold, whereas naloxone and 6 $\beta$ -naltrexol (Fig. 4, right) increased morphine potency by  $\approx$ 1.8- to 1.9-fold.

Finally, the potency of the three antagonists to precipitate withdrawal was determined. Mice were treated for 72 h with a single morphine pellet (25 mg) and then injected with antagonist and observed (see *Materials and Methods*). Nal-

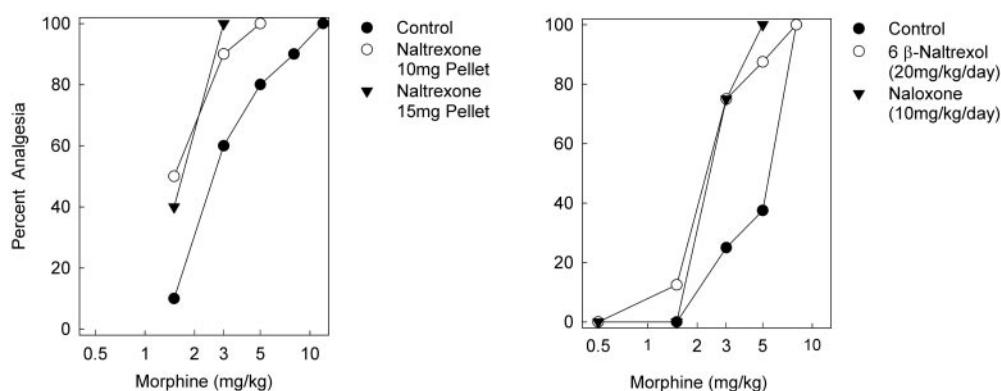
trexone, naloxone, and 6 $\beta$ -naltrexol all produced withdrawal jumping in mice made dependent on morphine (Fig. 5). Placebo-treated mice exhibited no significant jumping following 0.1 mg/kg naloxone or 20.0 mg/kg 6 $\beta$ -naltrexol or 0.1 mg/kg naltrexone. Table 2 presents the ED<sub>50</sub>s and relative potency of naltrexone, naloxone, and 6 $\beta$ -naltrexol to induce withdrawal jumping. Based on this analysis, the relative potencies for the antagonists to precipitate withdrawal relative to 6 $\beta$ -naltrexol were naltrexone (211) > naloxone (96)  $\gg$  6 $\beta$ -naltrexol (1). The ED<sub>50</sub> for 6 $\beta$ -naltrexol to precipitate withdrawal was  $\approx$ 100- to 200-fold greater than that for naltrexone and naloxone.

## Discussion

Previous studies have shown that opioid ligand efficacy can be an important determinant of the regulation of opioid receptor trafficking and density. In both cell culture and in *in vivo* studies, opioid agonists with higher efficacy have been shown to induce  $\mu$ -opioid receptor internalization and down-regulation (Keith et al., 1996, 1998; Stafford et al., 2001; Patel et al., 2002; Yoburn et al., 2004; Pawar et al., 2007). A classic example of the role of agonist efficacy in receptor regulation is the failure of morphine, a lower efficacy agonist, to induce internalization and down-regulation, whereas etorphine and DAMGO, higher efficacy agonists, readily produce both effects (e.g., Keith et al., 1996, 1998). Nevertheless, it should be noted that in some cases, morphine can produce internalization, although this may be limited to particular brain regions and structural elements of the cell and may not



**Fig. 3.** The effect of chronic naltrexone, naloxone, and 6 $\beta$ -naltrexol treatment on  $\mu$ -opioid receptor density in mouse spinal cord. Mice (10/group) were implanted s.c. with a naltrexone pellet (10 or 15 mg) or an osmotic pump that infused naloxone (0.1, 1.0, or 10 mg/kg/day) or 6 $\beta$ -naltrexol (0.2, 2.0, or 20 mg/kg/day) for 7 days. Controls were implanted with placebo pellets. At the end of treatment, naloxone and 6 $\beta$ -naltrexol treated mice were sacrificed. For naltrexone-treated mice, at the end of treatment, the pellets were removed, and 24 h later, mice were sacrificed. After sacrifice, spinal cords were removed and assayed in [<sup>3</sup>H]DAMGO saturation binding studies (see *Materials and Methods*). A, combined results for naloxone and 6 $\beta$ -naltrexol treatment as a function of antagonist infusion dose. Data are from one experiment for each dose ( $n = 10$  spinal cords per dose) and are plotted as the percent change in  $B_{max}$  ( $\pm$ S.E.M.) from the control group. B, C, and D, typical results from each experiment. In these studies the calculated  $B_{max}$  ( $\pm$ S.E.) for control and 6 $\beta$ -naltrexol was 245.6 ( $\pm$  6.2) and 546.3 ( $\pm$ 17.9), and the  $K_d$  ( $\pm$ S.E.) was 1.0 ( $\pm$ 0.1) and 1.1 ( $\pm$ 0.1), respectively. The calculated  $B_{max}$  ( $\pm$ S.E.) for control and naloxone was 187.3 ( $\pm$  7.3) and 345.9 ( $\pm$ 7.7), and  $K_d$  ( $\pm$ S.E.) was 1.2 ( $\pm$ 0.1) and 1.0 ( $\pm$ 0.1), respectively. The calculated  $B_{max}$  ( $\pm$ S.E.) for control and 10- and 15-mg naltrexone pellets was 155.2 ( $\pm$ 2.6), 274.6 ( $\pm$ 16.0), and 248.1 ( $\pm$ 6.9), and  $K_d$  was 0.7 ( $\pm$ 0.1), 0.9 ( $\pm$ 0.1), and 0.8 ( $\pm$ 0.1), respectively.



**Fig. 4.** The effect of chronic antagonist treatment on morphine potency. Mice (8–10/treatment) were implanted s.c. with naltrexone pellets (10 or 15 mg) for 7 days (left). Other mice (8–10/treatment) were infused s.c. with naloxone (10.0 mg/kg/day) or 6 $\beta$ -naltrexol (20.0 mg/kg/day) for 7 days (right). For all treatment groups, controls were implanted with placebo pellets. At the end of treatment, the pellets and pumps were removed, and 24 h later, the analgesic potency of morphine was determined using a cumulative dose-response protocol (see *Materials and Methods*). The ED<sub>50</sub> (95% CL) for morphine was estimated as 3.03 (2.22–3.94), 1.48 (0.96–2.05), and 1.47 (0.96–2.07) mg/kg for control and 10- and 15-mg naltrexone pellets, respectively (left). The ED<sub>50</sub> (95% CL) for morphine was estimated as 4.58 (3.46–6.06), 2.51 (1.91–3.23), and 2.48 (1.79–3.30) mg/kg for control, naloxone, and 6 $\beta$ -naltrexol, respectively.

TABLE 1

Morphine ED<sub>50</sub>s following chronic antagonist treatment

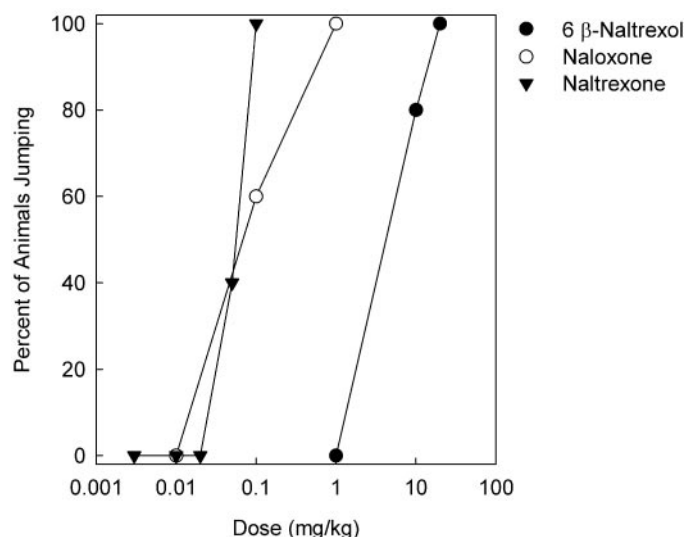
Mice (8–10/treatment) were implanted s.c. with naltrexone pellets (10 or 15 mg) for 7 days. Other mice (8–10/treatment) were infused s.c. with naloxone (10.0 mg/kg/day) or 6 $\beta$ -naltrexol (20.0 mg/kg/day) for 7 days. For all treatment groups, controls were implanted s.c. with placebo pellets. At the end of treatment, pellets and pumps were removed, and 24 h later, the analgesic potency of morphine was determined using a cumulative dose-response protocol (see *Materials and Methods*). The ED<sub>50</sub>s (95% CL) and relative potencies of morphine following antagonist treatment are presented.

Antagonist	ED <sub>50</sub> mg/kg $\pm$ CL	Relative Potency
Control	3.03 (2.22–3.94)	1.00
Naltrexone (10-mg pellet)	1.48 (0.96–2.05)	2.05*
Naltrexone (15-mg pellet)	1.47 (0.96–2.07)	2.06*
Control	4.58 (3.46–6.06)	1.00
Naloxone (10 mg/kg/day)	2.51 (1.91–3.23)	1.82*
6 $\beta$ -Naltrexol (20 mg/kg/day)	2.48 (1.79–3.30)	1.85*

\* Significantly different ( $P < 0.05$ ) from control based on Probit analysis (Finney, 1973).

be associated with subsequent down-regulation (Haberstock-Debic et al., 2003, 2005). An extension of the importance of efficacy in regulating  $\mu$ -opioid receptors is the effect of opioid antagonists on receptor density. Numerous studies have reported that treatment with antagonists, which by definition are neutral or negative in efficacy, will substantially increase the density of  $\mu$ -opioid receptors and produce a shift to the left of the agonist dose-response function (functional supersensitivity, e.g., Patel et al., 2003). Taken together, these data indicate that regulation of  $\mu$ -opioid receptors occurs over a continuum with increases in density observed for antagonists, little change in density for lower efficacy agonists, and internalization and down-regulation reported for higher efficacy agonists.

Although both agonists and antagonists can regulate  $\mu$ -opioid receptor density, the mechanisms by which this is accomplished appear to be different, at least in vivo studies. Higher efficacy agonists, such as etorphine, induce changes in  $\mu$ -opioid receptor mRNA, increases in G<sub>i $\alpha$ 2</sub> and Dynamin-2, but no changes in GRK-2 (Sehba et al., 1997; Patel et al., 2002; Yoburn et al., 2003). In addition, the high-efficacy agonist etorphine induces decreases in  $\mu$ -opioid receptor density detected using both radioligand binding as-



**Fig. 5.** Potency of naltrexone, naloxone, and 6 $\beta$ -naltrexol to induce withdrawal jumping in the mouse. Mice were implanted s.c. with a placebo or morphine pellet (25 mg) for 72 h and at the end of treatment injected s.c. with naltrexone (0.003–0.1 mg/kg;  $n = 5$ /dose), naloxone (0.01–1.0 mg/kg;  $n = 5$ /dose), or 6 $\beta$ -naltrexol (1.0–20.0 mg/kg;  $n = 5$ /dose). Immediately following antagonist treatment, jumping was recorded for 15 min (see *Materials and Methods*). The data are presented as percentage of mice positive for jumping as a function of antagonist dose. The ED<sub>50</sub> (95% CL) for each antagonist for precipitating withdrawal jumping was estimated as 0.05 (0.03–0.09), 0.11 (0.06–0.26), and 10.53 (4.75–16.80) mg/kg for naltrexone, naloxone, and 6 $\beta$ -naltrexol, respectively. Placebo-treated mice exhibited no significant jumping following 0.1 mg/kg naloxone or 20.0 mg/kg 6 $\beta$ -naltrexol or 0.1 mg/kg naltrexone.

says and Western immunoblotting, which suggests proteolysis of receptor protein or slowing of synthesis (Yoburn et al., 2004). These results contrast with the effects of chronic antagonist treatment. In the intact mouse, chronic antagonist treatment induces no change in  $\mu$ -opioid receptor mRNA and decreases in Dynamin-2 and GRK-2 (Duttaroy et al., 1999; Rajashekara et al., 2003). Interestingly, antagonist-induced up-regulation of  $\mu$ -opioid receptors is detected by radioligand binding studies, but not by Western blotting, a finding that suggests that up-regulation may involve recruitment of immature receptors (Yoburn et al., 2004). Taken together, there appears to be substantial differences between the mecha-

TABLE 2

Antagonist ED<sub>50</sub>s for precipitated withdrawal jumping following 72-h morphine treatment

Mice were implanted s.c. with a placebo or morphine pellet (25 mg) for 72 h and at the end of treatment injected s.c. with naltrexone (0.003–0.1 mg/kg; *n* = 5/dose), naloxone (0.01–1.0 mg/kg; *n* = 5/dose), or 6 $\beta$ -naltrexol (1.0–20.0 mg/kg; *n* = 5/dose). Immediately following antagonist treatment, jumping was recorded for 15 min (see *Materials and Methods*). Placebo-treated mice exhibited no significant jumping following 0.1 mg/kg naloxone or 20.0 mg/kg 6 $\beta$ -naltrexol or 0.1 mg/kg naltrexone. The ED<sub>50</sub>s (95% CL) and relative potencies of 6 $\beta$ -naltrexol, naloxone, and naltrexone are presented.

Antagonist	ED <sub>50</sub> mg/kg $\pm$ CL	Relative Potency
6 $\beta$ -Naltrexol	10.53 (4.75–16.80)	1*
Naloxone	0.11 (0.06–0.26)	96
Naltrexone	0.05 (0.03–0.09)	211

\* Significantly different (*P* < 0.05) from naloxone- and naltrexone-treated groups based on Probit analysis (Finney, 1973).

nisms of agonist-induced down-regulation and antagonist-induced up-regulation.

To date, there has been no direct examination of the possible role of antagonist efficacy in opioid receptor up-regulation. Recent data indicate that opioid antagonists can vary over a range in terms of the level of inverse agonist efficacy (Walker and Sterious, 2005). It had been suggested that negative intrinsic efficacy is required to produce opioid receptor up-regulation and functional supersensitivity (Morris and Millan, 1991; Milligan and Bond, 1997). Therefore, in the present study, we compared the effects of chronic treatment with putative inverse agonists (naltrexone, naloxone) and a putative neutral antagonist (6 $\beta$ -naltrexol).

Initially, the time of peak effect and the ED<sub>50</sub> for the three drugs to block the analgesic effect of morphine were determined. Naltrexone and naloxone peak effect was 40 min, whereas the peak effect for 6 $\beta$ -naltrexol was  $\approx$ 70 min, which is generally consistent with earlier findings (Berkowitz et al., 1975; Yan et al., 2003; Raehal et al., 2005) (Fig. 1). Similar to previous reports in the mouse (Wang et al., 2001; Raehal et al., 2005), naltrexone and naloxone were more potent than 6 $\beta$ -naltrexol (16 and 2 times more potent, respectively) in blocking the analgesic effect of morphine (Fig. 2).

To determine the effect of the antagonists on spinal  $\mu$ -opioid receptors, naloxone and 6 $\beta$ -naltrexol were infused for 7 days in mice. The infusion doses were determined to be equipotent using the dose-response data for antagonism of morphine analgesia (Fig. 2). In saturation binding studies, both naloxone and 6 $\beta$ -naltrexol produced significant  $\mu$ -opioid receptor up-regulation and were approximately equipotent (Fig. 3). In a related experiment, naltrexone pellets (10 or 15 mg/pellet) also significantly up-regulated spinal  $\mu$ -opioid receptors (Fig. 3D). These data with naloxone and naltrexone are consistent with earlier studies (e.g., Patel et al., 2003; Rajashekara et al., 2003), whereas up-regulation following 6 $\beta$ -naltrexol is a novel finding. Naloxone and 6 $\beta$ -naltrexol were equipotent in producing  $\approx$ 2-fold increase in morphine potency (Fig. 4, right). Similarly, naltrexone pellets also increased the analgesic potency of morphine  $\approx$ 2-fold (Fig. 4, left). Increases in the functional potency of opioid agonists following antagonist treatment have been reported often (e.g., Patel et al., 2003; Rajashekara et al., 2003). Overall, these findings demonstrate that inverse agonists and a neutral antagonist are equieffective in up-regulating  $\mu$ -opioid receptors and producing functional supersensitivity. These data indicate that negative efficacy is not required for  $\mu$ -opi-

oid receptor regulation and agonist potency shifts. Taken together, we propose that receptor up-regulation and functional supersensitivity require receptor blockade and are not contingent upon negative efficacy.

To determine whether the antagonists differed in relative efficacy, the potency of each ligand to precipitate withdrawal jumping in morphine-dependent mice was estimated. 6 $\beta$ -Naltrexol was  $\approx$ 211 and  $\approx$ 96 times less potent than naltrexone and naloxone, respectively. This difference in potency to precipitate withdrawal is dramatically different from the potency differences observed for antagonism of morphine analgesia and is consistent with earlier findings (Wang et al., 2001; Walker and Sterious, 2005). The potency differences to precipitate withdrawal support proposals that 6 $\beta$ -naltrexol has significantly lower inverse agonist activity than naloxone or naltrexone and that it may function as a neutral antagonist (e.g., Raehal et al., 2005; Wang et al., 2007).

Opioid agonists can be characterized according to efficacy. Opioid agonists with higher efficacy (e.g., etorphine) regulate  $\mu$ -opioid receptor density, whereas lower efficacy agonists (e.g., morphine, oxycodone) are generally ineffective (Patel et al., 2002; Pawar et al., 2007). Conversely, higher efficacy opioid agonists induce less tolerance at equieffective doses than lower efficacy agonists (Duttaroy and Yoburn, 1995; Pawar et al., 2007). In the present study, we found that antagonist efficacy was not a critical determinant of receptor regulation or functional supersensitivity. Both inverse agonists as well as a neutral antagonist were capable of producing comparable  $\mu$ -opioid receptor up-regulation and functional supersensitivity. These data suggest that  $\mu$ -opioid receptor up-regulation and functional supersensitivity require only receptor blockade and are independent of antagonist efficacy. This contrasts with withdrawal jumping, which was more sensitive to inverse agonists; a result that may be related to termination of basal signaling of constitutively active receptors in the opioid-dependent state (e.g., Wang et al., 2007).

Taken together, the current data are consistent with suggestions that opioid antagonist-induced effects and opioid agonist-induced effects depend upon different mechanisms (Yoburn et al., 2004). Although agonist-induced down-regulation appears to involve degradation of receptor protein, changes in  $\mu$ -opioid receptor gene expression, and increases in trafficking proteins, antagonist-induced up-regulation produces a different profile of correlated events. Furthermore,  $\mu$ -opioid receptor down-regulation and tolerance are related to agonist efficacy, whereas up-regulation and supersensitivity are independent of antagonist efficacy. Overall, it is clear that opioid antagonists can display functional selectivity (Urban et al., 2007), with 6 $\beta$ -naltrexol demonstrating relative equipotency for antagonism, up-regulation, and supersensitivity but dramatically reduced potency for precipitation of withdrawal. Thus, antagonist potency is dependent on experimental assay and is not an absolute property of the ligand.

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