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μ-Opioid Receptor Upregulation and Functional Supersensitivity are Independent of Antagonist Efficacy

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DAMGO, [D-Ala^2, N-MePhe^4, Gly-Ol^5] - enkephalin

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Abstract

Chronic opioid antagonist treatment upregulates 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 2 putative inverse agonists (naltrexone, naloxone) and a putative neutral antagonist (6 β-naltrexol) were examined. Initially, peak effect (40 min naltrexone and naloxone; 70 min 6 β-naltrexol) and relative potency to antagonize morphine analgesia were determined (relative potencies = 1, 2 and 16; 6 β-naltrexol, naloxone, naltrexone; respectively). Next, mice were infused for 7 days with naloxone (0.1-10mg/kg/day), naltrexone (10 or 15mg s.c. pellet) or 6 β-naltrexol (0.2-20mg/kg/day) and spinal μ-opioid receptor density examined; or morphine analgesia dose-response studies conducted. All antagonists upregulated μ-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 upregulation or supersensitivity. These data suggest that opioid antagonist-induced μ-opioid receptor upregulation and supersensitivity require occupancy of the receptor and that antagonist efficacy is not critical. Finally, the ED_{50} to precipitate withdrawal jumping was examined in morphine-dependent mice. Naltrexone, naloxone and 6 β-naltrexol produced withdrawal jumping; although potencies relative to 6 β-naltrexol were 211, 96, 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 μ-opioid receptor upregulation and supersensitivity.

Introduction

Receptor density regulation is a well-documented phenomenon in the opioid system (e.g., Keith et al., 1996, 1998; Patel et al., 2002; Yoburn et al., 1986). Both opioid agonists and antagonists have been shown to regulate μ-opioid receptor density (Chakrabarti et al., 1997; Rajashekara et al., 2003; Zaki et al., 2000) 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 μ-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 downregulation of μ- opioid receptors in vitro and in vivo (Keith et. al., 1998; Duttaroy and Yoburn, 1995; Whistler and von Zastrow, 1998). Conversely, low efficacy agonists (e.g., morphine, oxycodone) are less likely to produce either internalization or downregulation (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 upregulate μ, δ and κ -opioid receptors (Lesscher et al., 2003; Yoburn et al., 1995; 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., Becker et al., 1999; Burford et al., 2000; Costa and Herz, 1989). Ligands that suppress constitutive signaling activity are termed *inverse agonists or negative antagonists*, while those that only block

agonist induced effects without altering constitutive signaling are termed *neutral antagonists* (Kenakin, 2001). Antagonists, like agonists, will display protean behavior, such that an inverse opioid agonists will appear to be neutral in a system that lacks constitutively active receptors (Prather, 2004; Kenakin, 2001). Under appropriate experimental conditions, studies suggest that antagonists such as naltrexone and naloxone are inverse agonists, whereas, 6 β-naltrexol is a putative neutral antagonist (Raehal et al., 2005; Wang et al., 2004).

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 (Sadée et al., 2005; Walker and Sterious, 2005; Wang et al., 2001). 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 (Raehal et al., 2005; Walker and Sterious, 2005; Wang et al., 2001). In brain homogenates from morphine dependent rats, naloxone and naltrexone, but not 6 β-naltrexol, decrease [^{35}S]GTPγ S binding (Wang et al., 2004); and 6 β-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 β-naltrexol as a neutral antagonist; or at minimum as an antagonist with substantially reduced inverse agonist activity.

While 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 μ-opioid receptor upregulation and functional supersensitivity. It seemed likely that antagonist efficacy might determine upregulation and supersensitivity given that previous studies indicate that agonist efficacy plays a role in μ-opioid receptor downregulation and tolerance (e.g., Pawar et al., 2007). Although data indicate that antagonist-induced upregulation and agonist-induced downregulation are mechanistically distinct (e.g., Pawar et al., 2007; Yoburn et al., 2004), the evidence linking agonist efficacy, tolerance and receptor regulation are compelling (Pawar et al., 2007; Patel et al., 2002) and some studies have suggested that antagonist efficacy may be important in upregulation and supersensitivity (Morris and Millan, 1991; Milligan and Bond, 1997). Therefore, it seemed plausible that antagonist efficacy might have an impact on μ-opioid receptor upregulation and functional supersensitivity.

In the present study, two putative inverse agonists (naltrexone and naloxone) and a neutral antagonist (6 β-naltrexol) were compared in terms of μ-opioid receptor regulation, functional supersensitivity and precipitated withdrawal. Surprisingly, antagonist efficacy was not correlated with μ-opioid receptor upregulation 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 22-30 gm, obtained from Taconic Farms (Germantown, NY) were used throughout the study. Animals were housed 10 per cage for at least 24 hr after arrival with food and water available *ad-libitum*. All protocols were approved by the St John's University Institutional Animal Care and Use Committee.

Drugs:

Naltrexone HCl, naloxone HCl, 6 β-naltrexol HCl and morphine sulphate were obtained from the Research Triangle Institute (Research Triangle Park, NC) through the Research Technology Branch of NIDA. Subcutaneous implant pellets containing naltrexone (30mg and 10mg naltrexone base) or morphine (25mg morphine base), and placebo pellets were also obtained from the Research Triangle Institute. The 30mg naltrexone pellets were cut in half (yields 15mg 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 (5mg/kg, s.c.) analgesia for naltrexone, naloxone and 6 β-naltrexol was determined. Subsequently, the ED_{50}'s for antagonism of morphine analgesia (5mg/kg, s.c.) were determined for naltrexone, naloxone and 6 β-naltrexol. Mice were then treated for 7 days (infusion with osmotic mini pumps; Alzet Model 2001; DURECT Corporation, CA; or subcutaneous drug pellet implantation) with each antagonist and changes in μ-opioid receptor density examined in

spinal cord. Other mice were treated for 7 days with each antagonist, treatment terminated and 24hr 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 72hr later injected with antagonist and the ED_{50} 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 (5-6 mice/group) were injected s.c. with naltrexone (0.25 mg/kg), naloxone (0.2 mg/kg) or 6 β-naltrexol (0.45 mg/kg). At various times following antagonist treatment (1, 10, 30, 60, 90, 120, 150 and 210min) mice were injected s.c. with morphine (5mg/kg) and then tested for analgesia (tailflick, see below) 30 min following morphine. This procedure resulted in antagonism of morphine being determined at 31, 40, 60, 90, 120, 150, 180 and 240min following antagonist treatment.

ED_{50} Studies:

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

Analgesia Assay:

Antinociception was assayed using the tailflick 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 2cm from the tip of the tail. The intensity of the light was adjusted so that baseline tailflick latency was 2-4sec. If a mouse did not remove its tail from the heat source by 10 sec, the test was terminated, a latency of 10sec was recorded and the mouse was defined as analgesic. All testing was conducted by an experimenter who was unaware of the treatment of individual mouse.

Functional Supersensitivity Studies:

Mice (8-10/group) were infused with naloxone (10.0 mg/kg/day) or 6 β-naltrexol (20.0 mg/kg/day) for 7 days. Other mice (8-10/group) were implanted with naltrexone pellets s.c. (10 or 15mg) 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 24hr 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 30min later. If the mouse had a tailflick latency of 10sec, 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 sequence 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 β-naltrexol was 0.5, 1.0, 1.5, 2.0 and 3.0mg/kg; which yields cumulative doses of 0.5, 1.5, 3.0, 5.0 and 8.0mg/kg. Cumulative dosing was used to reduce the number of mice and the cost of supplies (e.g. osmotic pumps).

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Radioligand Binding Assay:

Mice (10/dose) were infused with naloxone (0.1, 1.0, 10.0 mg/kg/day) or 6 β-naltrexol (0.2, 2.0, 20.0 mg/kg/day) for 7 days. Other mice (10/dose) were implanted with naltrexone pellets (10 or 15mg). For all treatment groups, controls were implanted with placebo pellets for 7 days. At the end of naloxone and 6 β-naltrexol treatments, mice were sacrificed. At the end of naltrexone pellet treatment, the pellets were removed and 24hr later mice were sacrificed. For all groups, spinal cords were rapidly removed and pooled in tubes containing 15ml ice cold 50mM TRIS buffer (pH 7.4). Samples were homogenized (Brinkmann Polytron Homogenizer, Westbury, NY) at 20,000 rpm on ice for 40sec. 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, re-suspended in 15ml TRIS buffer and centrifuged at 15,000 rpm for 15 min (3-9°C) and the supernatant discarded. The pellet was re-suspended in 35ml TRIS buffer and incubated for 30 min in a shaking water bath (25˚C). Samples were centrifuged again for 15min, the supernatant 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 [^{3}H] DAMGO (range: 0.02-10nM) (μ-opioid receptor ligand; PerkinElmer Life Sciences, Boston, MA). Non-specific binding was determined in triplicate in the presence of levorphanol (1000nM). Tubes were incubated for 90min 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

(cpm) were converted into disintegration per minute (dpm) 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 1-2 times for each treatment.

Withdrawal Jumping Assay:

Mice (5/group) were implanted s.c. with a placebo pellet or a single morphine pellet (25 mg) for 72 hr and at the end of treatment injected with naltrexone (0.003-0.1 mg/kg), naloxone (0.01- 1.0 mg/kg) or 6 β-naltrexol (1.0 – 20.0 mg/kg). Immediately following antagonist treatment, mice were placed in a clear plastic container (5L) and observed for 15min for jumping. Jumping was defined as all 4 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 15min observation period, were defined as positive for withdrawal jumping. The ED_{50} 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). This program uses Probit analysis (Finney, 1973) to calculate ED_{50} values, standard errors, 95% confidence intervals and potency estimates. Binding data from saturation studies were analyzed by Prism ver 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 naloxone, and 70 min for 6 β-naltrexol. In subsequent studies, these peak effect estimates were used to determine relative antagonist potency. The estimated ED_{50}'s (95 % CL) for naltrexone, naloxone and 6 β-naltrexol to block morphine analgesia (5 mg/kg) were 0.01 mg/kg (0.005-0.027), 0.08 mg/kg (0.04-0.20) and 0.16 mg/kg (0.10-0.28), respectively. (Fig. 2) The order of potency relative to 6 β-naltrexol was naltrexone (16) > naloxone (2) > 6 β-naltrexol (1).

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

Chronic treatment with the three antagonists similarly increased the analgesic potency of morphine (Fig .4). Table 1 presents the ED_{50}'s and relative potencies for

morphine in control and treated groups. Naltrexone pellet treatment (10 or 15 mg) (Fig. 4, left panel) increased morphine analgesic potency by ≈ 2-fold, while naloxone, and 6 β-naltrexol (Fig. 4, right panel) increased morphine potency by ≈ 1.8-1.9 fold.

Finally, the potency of the 3 antagonists to precipitate withdrawal was determined. Mice were treated for 72hr with a single morphine pellet (25 mg) and then injected with antagonist and observed (see methods). Naltrexone, naloxone and 6 β-naltrexol all produced withdrawal jumping in mice made dependent on morphine (Fig. 5). Placebo treated mice exhibited no significant jumping following 0.1mg/kg naloxone; or 20.0 mg/kg 6 β-naltrexol; or 0.1 mg/kg naltrexone. Table 2 presents the ED_{50}'s and relative potency of naltrexone, naloxone and 6 β-naltrexol to induce withdrawal jumping. Based on this analysis, the relative potencies for the antagonists to precipitate withdrawal relative to 6 β-naltrexol were naltrexone (211) > naloxone (96) >>> 6 β-naltrexol (1). The ED_{50} for 6 β-naltrexol to precipitate withdrawal was ≈ 100-200 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 μ-opioid receptor internalization and downregulation (Keith et al., 1996, 1998; Patel et al., 2002; Pawar et al., 2007; Stafford et al., 2001; Yoburn et al., 2004). 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 downregulation, while 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 be associated with subsequent downregulation (Haberstock-Debic et al., 2003, 2005). An extension of the importance of efficacy in regulating μ-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 μ-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 μ-opioid receptors occurs over a continuum with increases in density observed for antagonists, little change in density for lower efficacy agonists, and internalization and downregulation reported for higher efficacy agonists.

While both agonists and antagonists can regulate μ-opioid receptor density, the mechanisms by which this is accomplished appears to be different; at least in in vivo studies. Higher efficacy agonists, such as etorphine, induce changes in μ-opioid receptor mRNA, increases in $G_{i\alpha 2}$ and Dynamin-2, but no changes in GRK-2 (Patel et al., 2002; Sehba et al., 1997; Yoburn et al., 2003). In addition, the high efficacy agonist etorphine induces decreases in μ-opioid receptor density detected using both radioligand binding assays 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 μ-opioid receptor mRNA, and decreases in Dynamin-2 and GRK-2 (Duttaroy et al., 1999; Rajashekara et al., 2003). Interestingly, antagonist-induced upregulation of μ-opioid receptors is detected by radioligand binding studies, but not by western blotting; a finding that suggests that upregulation may involve recruitment of immature receptors (Yoburn et al., 2004). Taken together, there appears to be substantial differences between the mechanisms of agonist-induced downregulation and antagonist-induced upregulation.

To date, there has been no direct examination of the possible role of antagonist efficacy in opioid receptor upregulation. 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 upregulation 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 β-naltrexol).

Initially, the time of peak effect and the ED_{50} for the three drugs to block the analgesic effect of morphine were determined. Naltrexone and naloxone peak effect was 40min, while the peak effect for 6 β-naltrexol was ≈ 70min; which is generally consistent with earlier findings (Berkowitz et al., 1975; Raehal et al., 2005; Yan et al., 2003) (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 β-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 μ-opioid receptors, naloxone and 6 β-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 β-naltrexol produced significant μ-opioid receptor upregulation and were approximately equipotent (Fig. 3). In a related experiment, naltrexone pellets (10 or 15mg/pellet) also significantly upregulated spinal μ-opioid receptors (Fig. 3, D.). These data with naloxone and naltrexone are consistent with earlier studies (e.g., Patel et al., 2003, Rajashekara et al., 2003) while upregulation following 6 β-naltrexol is a novel finding. Naloxone and 6 β-naltrexol were equipotent in producing ≈ 2-fold increase in morphine potency (Fig 4, right panel). Similarly, naltrexone pellets also increased the analgesic potency of morphine ≈ 2 fold (Fig 4, left panel). 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 equi-effective in upregulating μ-opioid receptors and producing functional supersensitivity. These data indicate that negative efficacy is not required for μ-opioid receptor regulation and agonist potency shifts. Taken together, we propose that receptor upregulation and functional supersensitivity require receptor blockade and are not contingent upon negative efficacy.

To determine if the antagonists differed in relative efficacy, the potency of each ligand to precipitate withdrawal jumping in morphine-dependent mice was estimated. 6 β-naltrexol was ≈ 211 and ≈ 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 β-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 μ-opioid receptor density, while lower efficacy agonists (e.g., morphine, oxycodone) are generally ineffective (Pawar et al., 2007; Patel et al., 2002). 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 a neutral antagonist were capable of producing comparable μ-opioid receptor upregulation and functional supersensitivity. These data suggest that μ-opioid receptor upregulation 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). Whereas, agonist-induced downregulation appears to involve degradation of receptor protein, changes in μ-opioid receptor gene expression and increases in trafficking proteins; antagonist-induced upregulation produces a different profile of correlated events. Furthermore, μ-opioid receptor downregulation and tolerance are related to agonist efficacy, while upregulation and supersensitivity are independent of antagonist efficacy. Overall, it is clear that opioid antagonists can display functional selectivity (Urban et al., 2007) with 6 β-naltrexol demonstrating relative equipotency for antagonism, upregulation 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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Footnotes:

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Legends for Figures:

Figure 1. Time course of antagonism of morphine-induced analgesia by naltrexone, naloxone and 6 β-naltrexol. Individual groups of mice (5-6 /drug/time point) were injected with naltrexone (0.25 mg/kg), naloxone (0.20 mg/kg) and 6 β-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. Failure to tailflick by 10 sec was defined as blockade of morphine-induced analgesia. The percent 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 β-naltrexol.

Figure 2. Dose response functions for antagonism of morphine-induced analgesia by naltrexone, naloxone and 6 β-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 β-naltrexol (0.05-0.45 mg/kg). Ten min following naltrexone and naloxone, and 40 min following 6 β-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. Failure to tailflick by 10 sec was defined as blockade of morphine-induced analgesia. The percent of mice demonstrating blockade is plotted versus the dose of antagonist. The ED_{50}'s (95 % CL) for naltrexone, naloxone and 6 β-naltrexol were estimated as 0.01 mg/kg (0.01-0.03), 0.08 mg/kg (0.04-0.20) and 0.16 mg/kg (0.10-0.28); respectively.

Figure 3. The effect of chronic naltrexone, naloxone and 6 β- naltrexol treatment on μ-opioid receptor density in mouse spinal cord. Mice (10/group) were implanted s.c. with a naltrexone pellet (10 or 15mg), or an osmotic pump that infused naloxone (0.1, 1.0, 10mg/kg/day) or 6 β- naltrexol (0.2, 2.0, 20mg/kg/day) for 7 days. Controls were implanted with placebo pellets. At the end of treatment, naloxone and 6 β- naltrexol treated mice were sacrificed. For naltrexone treated mice, at the end of treatment the pellets were removed and 24hr later mice were sacrificed. Following sacrifice, spinal cords were removed and assayed in [^{3}H] DAMGO saturation binding studies (see methods). **Panel A** presents the combined results for naloxone and 6 β- 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} (± SEM) from the control group. **Panel B, C, D** presents typical results from each experiment. In these studies the calculated B_{MAX} (±SE) for control and 6 β-naltrexol was 245.6 (± 6.2) and 546.3 (±17.9) and the K_D (±SE) was 1.0 (±0.1) and 1.1 (±0.1); respectively. The calculated B_{MAX} (±SE) for control and naloxone was 187.3 (± 7.3) and 345.9 (±7.7) and K_D (±SE) was 1.2 (±0.1) and 1.0 (±0.1); respectively. The calculated B_{MAX} (±SE) for control, naltrexone 10 mg and naltrexone 15 mg pellets was 155.2 (± 2.6), 274.6 (±16.0) and 248.1 (±6.9) and K_D was 0.7 (±0.1), 0.9 (±0.1) and 0.8 (±0.1); respectively.

Figure 4. The effect of chronic antagonist treatment on morphine potency. Mice (8-10/treatment) were implanted s.c. with naltrexone pellets (10 or 15mg) for seven days (left panel). Other mice (8-10/treatment) were infused s.c. with naloxone (10.0

mg/kg/day) or 6 β-naltrexol (20.0 mg/kg/day) for seven days (right panel). For all treatment groups, controls were implanted with placebo pellets. At the end of treatment, the pellets and pumps were removed and 24hr later the analgesic potency of morphine was determined using a cumulative dose-response protocol (see methods). The ED_{50} (95% CL) for morphine was estimated as 3.03 mg/kg (2.22-3.94), 1.48 mg/kg (0.96-2.05) and 1.47 mg/kg (0.96-2.07) for control, naltrexone 10 mg and naltrexone 15 mg pellets; respectively (left panel). The ED_{50} (95% CL) for morphine was estimated as 4.58 mg/kg (3.46-6.06), 2.51 mg/kg (1.91-3.23) and 2.48 (1.79-3.30) for control, naloxone and 6 β-naltrexol respectively (right panel).

Figure 5. Potency of naltrexone, naloxone and 6 β-naltrexol to induce withdrawal jumping in the mouse. Mice were implanted s.c. with a placebo or morphine pellet (25 mg) for 72 hr 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 β-naltrexol (1.0-20.0 mg/kg; N=5/dose). Immediately following antagonist treatment, jumping was recorded for 15min (see methods). The data are presented as percent of mice positive for jumping as a function of antagonist dose. The ED_{50} (95% CL) for each antagonist for precipitating withdrawal jumping was estimated as 0.05 mg/kg (0.03-0.09), 0.11 mg/kg (0.06-0.26) and 10.53 mg/kg (6.06 – 15.27) for naltrexone, naloxone and 6 β-naltrexol; respectively. Placebo treated mice exhibited no significant jumping following 0.1mg/kg naloxone; or 20.0 mg/kg 6 β-naltrexol; or 0.1 mg/kg naltrexone.

Table 1. Morphine ED_{50}'s Following Chronic Antagonist Treatment. Mice (8-10/treatment) were implanted s.c. with naltrexone pellets (10 or 15mg) for 7 days. Other mice (8-10/treatment) were infused s.c. with naloxone (10.0 mg/kg/day) or 6 β-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 24hr later the analgesic potency of morphine was determined using a cumulative dose-response protocol (see methods). The ED_{50}'s (95% CL) and relative potencies of morphine following antagonist treatment are presented. * significantly different ($p<0.05$) from control based on Probit Analysis (Finney, 1973).

Antagonist	ED_{50} (mg/kg ± CL)	Relative Potency
Control	3.03 (2.22-3.94)	1.00
Naltrexone (10mg pellet)	1.48 (0.96-2.05)	2.05*
Naltrexone (15mg pellet)	1.47 (0.96-2.07)	2.06*
Control	4.58 (3.46-6.06)	1.00
Naloxone (10mg/kg/day)	2.51 (1.91-3.23)	1.82*
6β-Naltrexol (20mg/kg/day)	2.48 (1.79-3.30)	1.85*

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Table 2. Antagonist ED_{50}'s for Precipitated Withdrawal Jumping Following 72hr Morphine Treatment. Mice were implanted s.c. with a placebo or morphine pellet (25 mg) for 72 hr 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 β-naltrexol (1.0-20.0 mg/kg; N=5/dose). Immediately following antagonist treatment, jumping was recorded for 15min (see methods). Placebo treated mice exhibited no significant jumping following 0.1mg/kg naloxone; or 20.0 mg/kg 6 β-naltrexol; or 0.1 mg/kg naltrexone. The ED_{50}'s (95% CL) and relative potencies of 6 β-naltrexol, naloxone and naltrexone are presented. * significantly different ($p<0.05$) from naloxone and naltrexone treated groups based on Probit Analysis (Finney, 1973).

Antagonist	ED_{50} (mg/kg ± CL)	Relative Potency
6 β- Naltrexol	10.53 (4.75-16.80)	1*
Naloxone	0.11 (0.06-0.26)	96
Naltrexone	0.05 (0.03-0.09)	211

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Figure 1

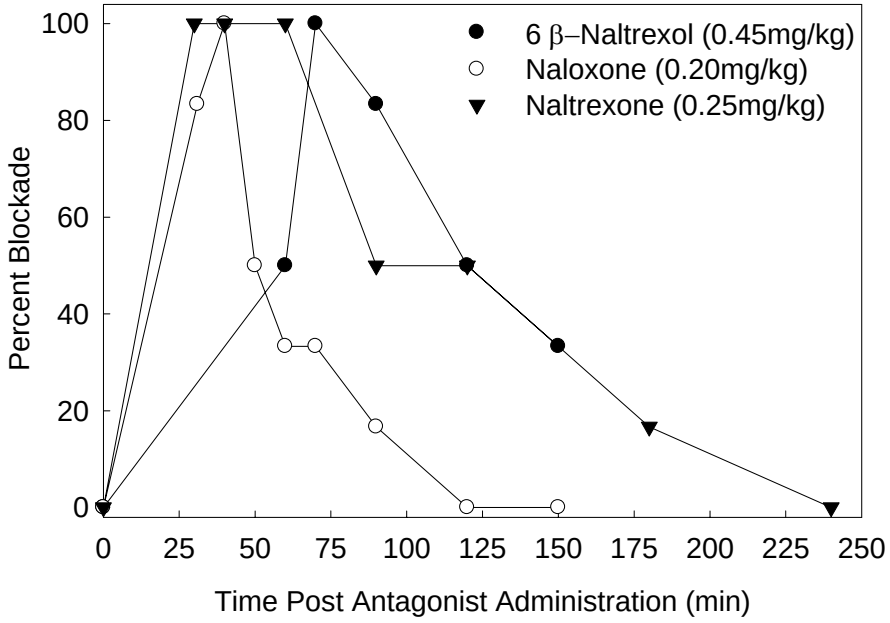

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Figure 2

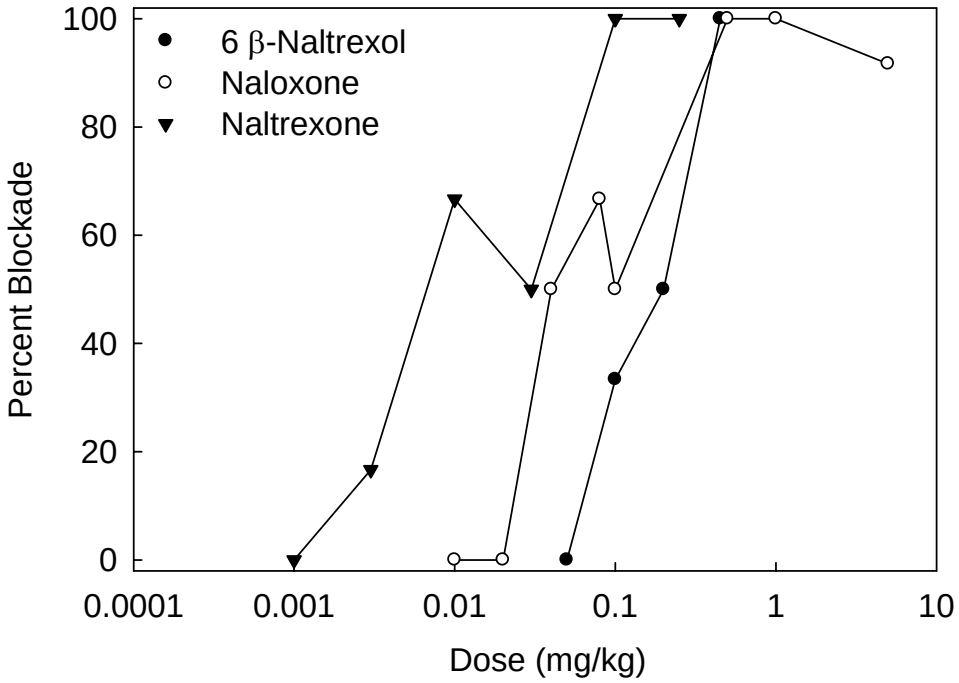

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Figure 3

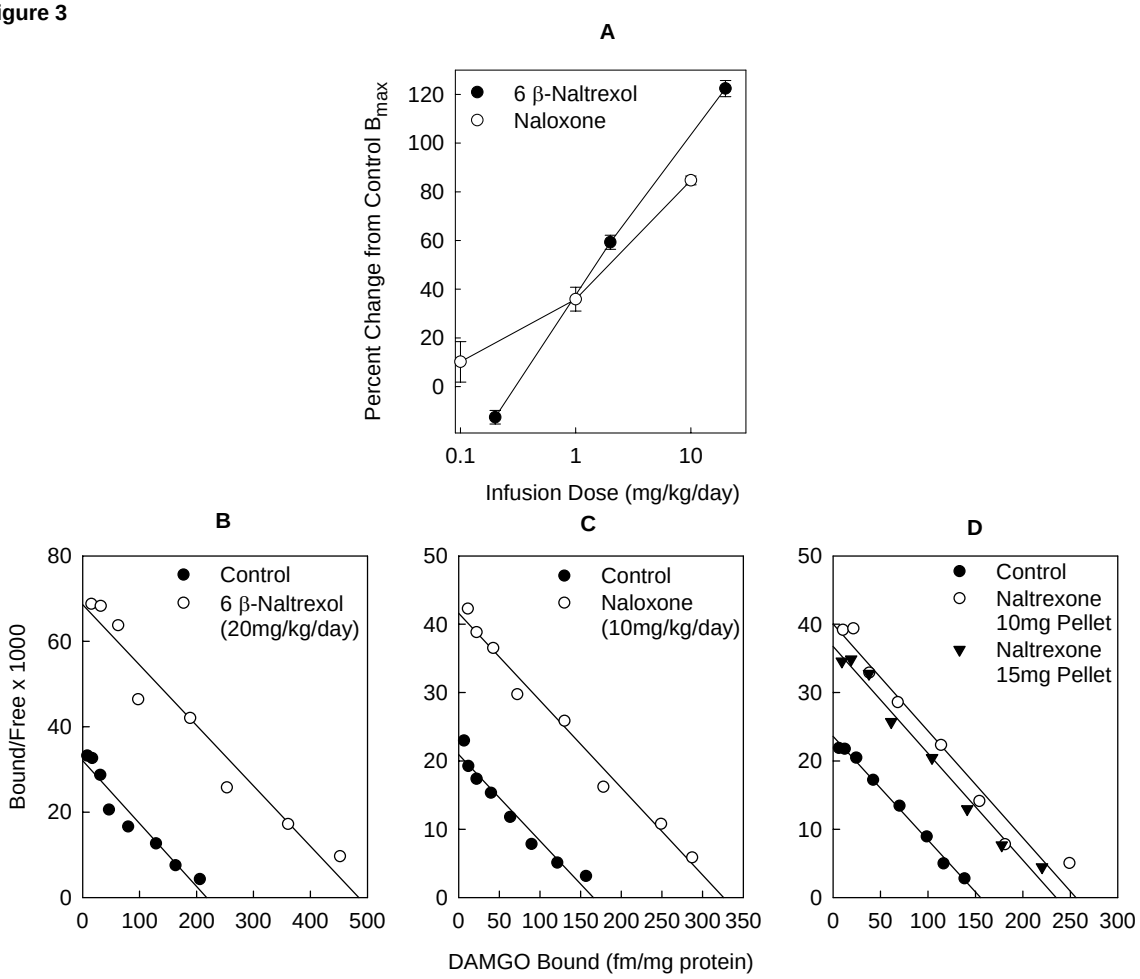

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Figure 4

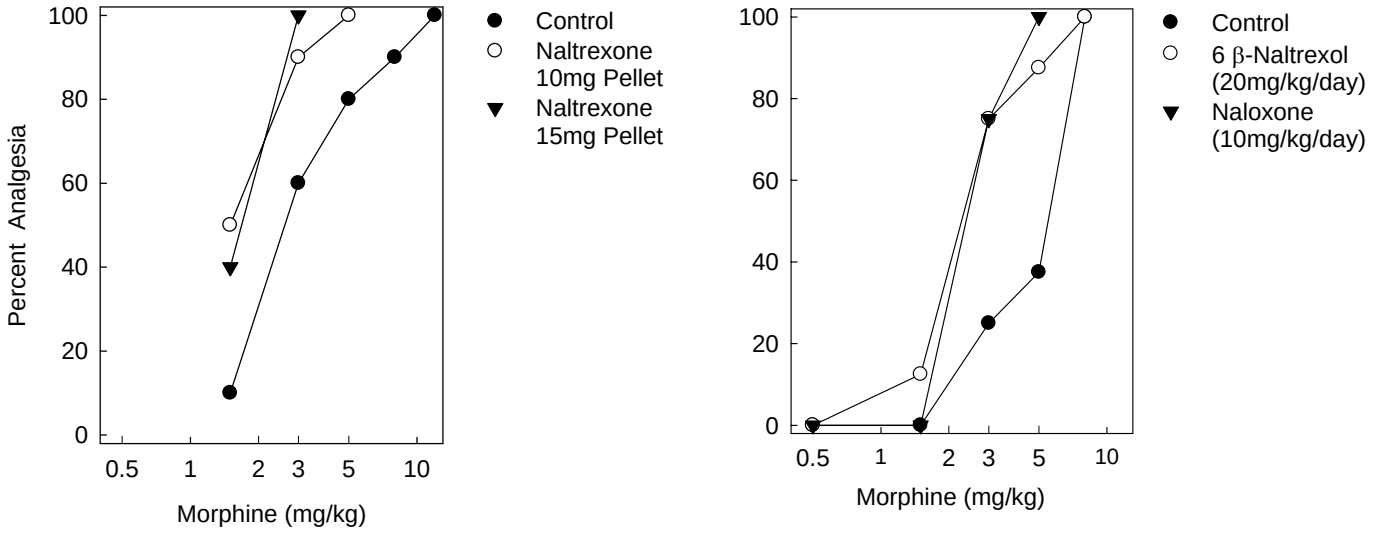

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Figure 5