

Opioid agonist and antagonist treatment differentially regulates immunoreactive μ -opioid receptors and dynamin-2 in vivo

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Abstract

Opioid agonists and antagonists can regulate the density of μ -opioid receptors in whole animal and in cell culture. High intrinsic efficacy agonists (e.g., etorphine), but not lower intrinsic efficacy agonists (e.g., morphine), produce μ -opioid receptor down-regulation and can alter the abundance of μ -opioid receptor mRNA. Conversely, opioid antagonists substantially increase the density of μ -opioid receptors without changing its mRNA. μ -Opioid receptor up-regulation has been associated with decreases in the trafficking protein dynamin-2, whereas μ -opioid receptor down-regulation produces an increase in dynamin-2 abundance. To probe the differences between opioid agonist and antagonist-induced μ -opioid receptor regulation, the current study determined changes in μ -opioid receptor density using a combined radioligand binding ($[^3\text{H}]$ DAMGO) and quantitative Western blotting approach in mouse spinal cord. Furthermore, the differences between intermittent and continuous dosing protocols were evaluated. Continuous (7–8 days) s.c. infusions of naloxone (5 mg/kg/day) or naltrexone (15 mg s.c. implant pellet) increased μ -opioid receptor density in radioligand binding assays ($\approx +80\%$) in mouse spinal cord and down-regulated dynamin-2 abundance ($\approx -30\%$), but had no effect on the abundance of immunoreactive μ -opioid receptor. Continuous (7 days) s.c. infusion of etorphine (200 $\mu\text{g/kg/day}$) decreased immunoreactive μ -opioid receptor ($\approx -35\%$) and $[^3\text{H}]$ DAMGO binding ($\approx -30\%$), and concurrently increased dynamin-2 abundance ($\approx +40\%$). Continuous (7 days) morphine infusion (40 mg/kg/day plus 25 mg s.c. implant pellet) had no effect on any outcome measure. Delivery of the same daily dose of etorphine or naloxone using intermittent (every 24 h for 7 days) s.c. administration had no effect on immunoreactive μ -opioid receptor, $[^3\text{H}]$ DAMGO binding or dynamin-2 abundance. These data indicate that μ -opioid receptor density, determined in radioligand binding assays, and immunoreactive dynamin-2 abundance are regulated by continuous, but not intermittent, opioid ligand treatment. Furthermore, the differential regulation of μ -opioid receptor abundance by agonists and antagonists in immunoblotting assays contrasts with changes in $[^3\text{H}]$ DAMGO binding. Taken together, these results suggest that etorphine-induced down-regulation may depend upon μ -opioid receptor degradation and changes in dynamin-2-mediated receptor trafficking. Conversely, antagonist-induced up-regulation does not require an increase in μ -opioid receptor synthesis and may entail conversion of receptors to an appropriate conformation to bind ligand, as well as changes in receptor trafficking.

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1. Introduction

Opioid agonists and antagonists have been shown to regulate the density of μ opioid receptors in cell culture and whole animal studies (e.g., Chakrabarti et al., 1997;

Rajashekara et al., 2003; Yoburn et al., 2003; Zaki et al., 2000). In the mouse spinal cord, studies indicate that chronic treatment with high intrinsic efficacy agonists (e.g., etorphine) will down-regulate μ -opioid receptors, while chronic opioid antagonist treatment (e.g., naltrexone, naloxone) can increase the density of μ -opioid receptor (Patel et al., 2002a; Rajashekara et al., 2003). Interestingly, despite robust tolerance induced by morphine, it does not produce μ -opioid receptor down-regulation (Patel et al.,

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2002b). These results have led some investigators to suggest that intrinsic efficacy is a determinant of the regulation of μ -opioid receptor density (Yoburn et al., 1993; Patel et al., 2002b); although alternate factors have been proposed as well (e.g., Alvarez et al., 2002).

The role of trafficking proteins (e.g., β arrestins, dynamins, G-protein receptor kinases (GRKs)) in G-protein coupled receptor (GPCR) desensitization and internalization has been well documented (e.g., Pierce et al., 2002; Ferguson, 2001). Recent findings have raised the possibility that regulation of trafficking proteins may also play a role in up- and down-regulation of μ -opioid receptors in mouse spinal cord. Specifically, continuous opioid antagonist infusion produces a dose-dependent increase in μ -opioid receptor density and a dose-dependent decrease in dynamin-2 and GRK-2 in mouse spinal cord (Patel et al., 2003; Rajashekara et al., 2003). Continuous etorphine infusion has been shown to down-regulate μ -opioid receptors and to increase dynamin-2 abundance in mouse spinal cord (Patel et al., 2002b). Morphine treatment, which does not regulate μ -opioid receptor density, has no effect on either GRK-2 or dynamin-2 (Patel et al., 2002b). While other proteins are likely to contribute to agonist-induced μ -opioid receptor regulation, these data indicate that up-regulation may be partially explained by slowing of constitutive GRK-2 mediated phosphorylation and dynamin-2 associated internalization. Furthermore, down-regulation may depend upon acceleration of dynamin-2-dependent internalization.

Despite the reliability of μ -opioid receptor regulation by opioid antagonists and some agonists, the mechanisms that account for up- and down-regulation remain unclear. In order to gain further insight into the source of μ -opioid receptors following chronic opioid antagonist treatment and the fate of μ -opioid receptors during chronic opioid agonist treatment, we examined the abundance of immunoreactive μ -opioid receptors and dynamin-2 following both agonist and antagonist treatment. In general, this study was designed to determine if opioid antagonist-induced up-regulation and opioid agonist-induced down-regulation are mediated by differential regulation of μ -opioid receptor protein in the mouse spinal cord. The population of receptors identified using immunoblotting would be all μ -opioid receptors that retain the antigenic determinant for the antibody, regardless of whether the receptor is in a conformation to bind ligand. Conversely, saturation radioligand binding studies, by definition, will only measure receptors that are in the appropriate conformation. Given that changes in μ -opioid receptor mRNA are associated with down-regulation measured in radioligand binding studies (Duttaroy and Yoburn, 2000; Sehba et al., 1997), we hypothesized that immunoreactive μ -opioid receptor protein abundance would decline in parallel with changes in radioligand binding. On the other hand, since up-regulation in radioligand binding studies is not associated with changes in μ -opioid receptor mRNA (Duttaroy et al., 1999; Unterwald et al., 1995), it seemed likely that antagonist treatment would not regulate μ -opioid receptor

protein abundance in immunoreactive assays. This study also included continuous and intermittent dosing protocols, since these schedules have been shown to produce different degrees of tolerance (Duttaroy and Yoburn, 1995). We anticipated that μ -opioid receptor density would be differentially regulated by intermittent and continuous dosing.

2. Materials and methods

2.1. Subjects

Male, Swiss–Webster mice (22–30 g) obtained from Taconic Farms (Germantown, NY) were used throughout. The animals were maintained 5–10 per cage with free access to food and water and housed for at least 24 h prior to experimentation. Mice were used only once.

2.2. General procedure

In general, mice were treated chronically with opioid agonists (morphine, etorphine) or antagonists (naloxone, naltrexone) for 7–8 days. Following treatment, mice were sacrificed, spinal cord removed and various assays performed.

For etorphine treatment, mice were implanted subcutaneously with an osmotic minipump (Alzet model 2001: 1 μ l/h; Durect, Cupertino, CA) that infused etorphine (200 μ g/kg/day). Other mice were injected s.c. once per day with etorphine (200 μ g/kg). For morphine treatment, mice were implanted subcutaneously with a minipump that infused morphine (40 mg/kg/day) plus one 25-mg morphine implant pellet. For naloxone treatment, mice were implanted subcutaneously with a minipump that infused naloxone (5 mg/kg/day). Other mice were injected s.c. once per day with naloxone (5 mg/kg). For naltrexone treatment, mice were implanted s.c. with a pellet containing 15 mg of naltrexone. Controls were included for all treatment protocols. Control mice for drug infusions and pellets were implanted with inert placebo pellets. Control mice for intermittent injections were injected daily with saline. All pumps and pellets were implanted at the nape of the neck while mice were lightly anesthetized with halothane/oxygen (4:96%). Following 7 (naloxone, morphine, etorphine) or 8 (naltrexone) days of treatment, mice were sacrificed and the spinal cord collected for immunoblotting and binding assays. For μ OR radioligand binding studies for the continuous etorphine treatment group only, pumps and pellets were removed at the end of treatment, mice sacrificed and cord collected 16 h later. This interval insures that residual etorphine does not interfere with the radioligand binding assay (see Yoburn et al., 1993).

2.3. μ -Opioid receptor binding

Spinal cords ($n=12$ /group) were rapidly removed, weighed and then homogenized (Brinkman Polytron

Homogenizer 20,000 rpm, 20 s) in 15 ml of ice-cold 50 mM Tris buffer (pH 7.4). Homogenates were then centrifuged at 15,000 rpm for 15 min (2–4 °C), the supernatant discarded and the pellet resuspended in buffer and centrifuged again and then frozen at –80 °C, until analysis. The pellets were thawed, resuspended in Tris buffer, incubated (30 min at 25 °C), centrifuged and finally resuspended in 13 ml of 50 mM phosphate buffer (pH 7.2). An aliquot (200 µl) of homogenate was then assayed in triplicate in tubes containing 0.04–5.0 nM [³H] [D-Ala²-MePhe⁴-Gly(ol⁵)] enkephalin (DAMGO). Nonspecific binding was determined in the presence of 1000 nM levorphanol. The homogenates were incubated for 90 min at 25 °C. Incubation was terminated by addition of ice-cold phosphate buffer and the samples were filtered over GF/B glass fiber filters. Filters were washed three times with cold buffer, transferred to vials, scintillation cocktail added, and then counted using liquid scintillation spectroscopy. Counts per minute (CPMs) were converted to disintegrations per minute (DPMs) using the external standard method. Protein concentration was determined using a micro-assay technique based on the method of Bradford (1976) using reagent purchased from Bio-Rad (Hercules, CA). Binding experiments were conducted two to three times for each treatment condition.

2.4. Western blotting assays

2.4.1. μ -Opioid receptors and actin

Individual spinal cords ($n=5$ /group) were rapidly removed on ice and homogenized (Brinkman Polytron Homogenizer 20,000 rpm, 45 s) in 750 µl of ice-cold 50 mM Tris buffer (pH 7.4) and centrifuged at 12,000 rpm (2–4 °C) for 20 min. Supernatant was discarded and the pellet was resuspended in 200 µl of lysis buffer (10% SDS (sodium dodecyl sulfate), 1 mM sodiumorthovanadate, 12.5 mM Tris, pH 7.4). Samples were boiled for 15 min and centrifuged again at 12,000 rpm (2–4 °C) for 60 min. The supernatant was collected for analysis and determination of protein concentration. Samples were diluted with an equal volume of sample buffer (10% SDS, 1% β -mercaptoethanol, 20% glycerol, 12.5 mM Tris base, bromophenol blue dye). An aliquot of diluted sample from one spinal cord was loaded on each gel lane (15–25 µg protein), so that 10 individual spinal cords (5 cords/treatment; 1 cord/lane) and a standard curve (see below) were loaded onto each polyacrylamide gel (Pager Gels 10% Tris–Glycine, Cambrex Bioscience, Rockland, ME). Samples were separated by electrophoresis (0.02 amp for 85 min) and protein transferred to Immobilon-P PVDF (polyvinylidene difluoride) membranes (Millipore, Bedford, MA) using the miniprotean II (Bio-Rad) at 85 V for 100 min. Nonspecific binding sites on the membrane were blocked by overnight incubation (4 °C) in blocking buffer (0.2% Aurora Blocking Reagent (ICN Biomedicals, Aurora, OH), in phosphate buffered saline (0.058M Na₂HPO₄, 0.017M NaH₂PO₄, 0.068M NaCl); 0.05% Tween-20, Sigma, St. Louis, MO).

Membranes were then incubated (4 h, 25 °C) with primary antibody (μ -opioid receptor, 1:500; Neuromics, Northfield, MN; Actin, 1:300; Santa Cruz Biotechnology, Santa Cruz, CA) in blocking buffer. Membranes were washed thrice with blocking buffer and then incubated (1 h, 25 °C) with secondary antibody (for μ -opioid receptor: Goat Antirabbit Immunoglobulin G - alkaline phosphatase (IgG-AP) (1:5000), ICN Biomedicals, Costa Mesa, CA; for Actin: Donkey Antigoat IgG-AP(1:5000) Santa Cruz Biotechnology). Membranes were then washed thrice with blocking buffer, followed by two quick rinses with Assay buffer (20 mM Tris HCl, pH 9.8, 1 mM MgCl₂). Bands were visualized using an alkaline phosphatase chemiluminescence assay (CDP Star Substrate, Novagen, Madison, WI). A standard curve using increasing amounts of spinal cord protein from control mice (10–40 µg/lane) was included on every gel. All data are converted into standard curve equivalents prior to analysis. Each experiment was repeated two to three times.

Finally, in a series of control experiments, the selectivity of the primary antibody for the μ -opioid receptor was assessed. Briefly, tissues in which there was little or no specific binding for [³H] DAMGO (heart, lung, cerebellum, kidney, liver) were negative for immunoreactive μ -opioid receptor. In addition, GH3 cells expressing the opioid receptor or the μ -opioid receptor were analyzed using the Western blot protocol. There were no specific bands in samples from GH3 cells expressing opioid receptors, whereas a single broad band was observed for samples from GH3 cells expressing μ -opioid receptors. The GH3 cells were a generous gift of Dr. Paul Prather (University of Arkansas Medical Center). Finally, preincubation of the primary antibody with blocking peptide significantly reduced band density.

2.4.2. *Dynamin-2*

Briefly, spinal cords ($n=5$ /group) were removed on ice and homogenized (20,000 rpm 30 s) in 500-µl lysis buffer, boiled for 5 min, and centrifuged at 10,000 rpm (15 °C) for 10 min. The supernatant was removed for analysis and protein concentration was determined. Samples were diluted using a mixture of equal volumes of lysis and sample buffer. Aliquots (0.5 µg protein/lane) of diluted sample were loaded on each lane of polyacrylamide gels and separated by electrophoresis (150 V for 60 min). Ten individual spinal cords (5 cords/treatment; 1 cord/lane) and a standard curve were included in each gel. Proteins were transferred to Immobilon-P PVDF membranes using the Miniprotean II at 100 V for 85 min. Nonspecific binding sites on the membrane were blocked by overnight incubation (4 °C) in blocking buffer, followed by incubation (1 h, 24 °C) with primary antibody directed at dynamin-2 (Goat Polyclonal IgG (1:300) #SC6400, Santa Cruz Biotechnology) in blocking buffer. Membranes were washed twice with blocking buffer and then incubated (1 h, 25 °C) with secondary antibody (Donkey Antigoat IgG-AP (1:5000),

Santa Cruz Biotechnology). The rest of the procedure was as described above for μ -opioid receptor protein.

A standard curve using increasing amounts of spinal cord protein from control mice (0.25–2 μ g/lane) was included on every gel. All data are converted into standard curve equivalents prior to analysis. Each experiment was repeated two to three times.

2.5. Drugs and reagents

Etorphine HCl, morphine sulfate, and morphine and naltrexone pellets, as well as inert placebo pellets, were obtained from the Research Triangle Institute (Research Triangle Park, NC). Naloxone HCl was obtained from Dupont Pharmaceuticals (Willington, DE). All compounds were dissolved in normal saline (0.9%). Doses were calculated as the free base. [3 H] DAMGO was obtained from NEN Life Sciences (Boston, MA).

2.6. Data analysis

Gel images were captured using a FluorChem ver 2.0 Imaging System (Alpha Innotech, San Leandro, CA). The images were digitized and analyzed for optical density using GelPro image analysis software (ver 3.1, Media Cybernetics, Silver Spring, MD). Optical densities from Western blot data were converted to protein equivalents using the standard curves and evaluated using two-tailed Student *t*-tests ($P < 0.05$). B_{\max} and K_D values were estimated from saturation studies using nonlinear regression (Prism ver 3.02, Graphpad Software, San Diego, CA). Binding data

were best fit by a one-site model. Differences between B_{\max} and K_D values were evaluated using analysis of variance ($P < 0.05$) with appropriate post hoc comparisons or using the Z-test ($p < 0.05$) based on nonlinear regression parameters.

3. Results

3.1. μ -Opioid receptor radioligand binding

Continuous treatment with naloxone significantly increased μ -opioid receptor density in saturation binding assays. Naloxone increased the density of μ -opioid receptors in mouse spinal cord by 84% without changing affinity (Fig. 1). Similar results were obtained in another independent study (+120%). We have previously reported that μ -opioid receptor up-regulation in mouse spinal cord by continuous naloxone infusion is dose-dependent (Rajashekara et al., 2003). However, unlike continuous naloxone treatment, intermittent administration of the same daily dose (5 mg/kg) did not significantly alter μ -opioid receptor density or affinity in two independent experiments (Fig. 1). Although not presented here, naltrexone pellet treatment is well documented to increase μ -opioid receptor density (e.g., Patel et al., 2003).

Continuous treatment with etorphine significantly down-regulated μ -opioid receptor density in spinal cord (–30%) with no significant change in affinity (Fig. 2), as reported previously (Patel et al; 2002b). On the other hand, the same dose of etorphine administered once every 24 h did not alter μ -opioid receptor density or affinity in three independent

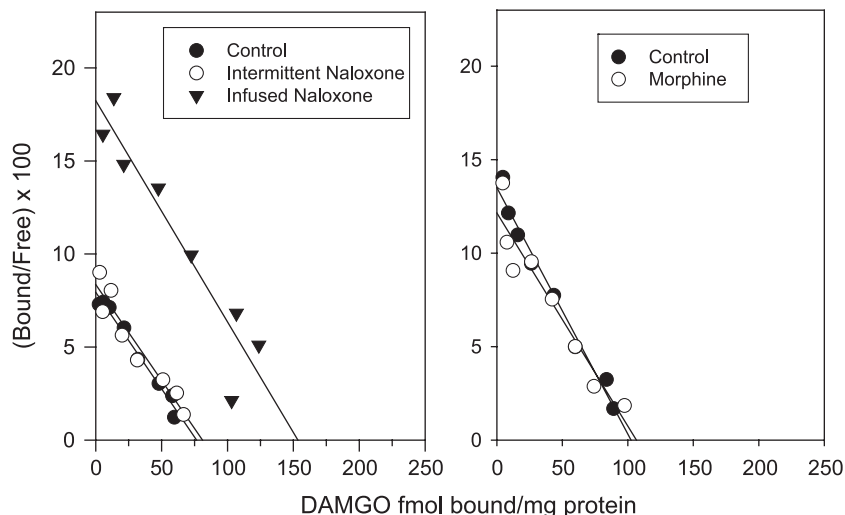


Fig. 1. The effect of naloxone and morphine on the density of μ OR in mouse spinal cord. Mice were injected s.c. with naloxone (5 mg/kg) every 24 h (intermittent) or were continuously infused s.c. using an osmotic pump with naloxone (5 mg/kg/day) for 7 days then sacrificed. An additional group of mice was treated with morphine (40 mg/kg/day by osmotic pump plus s.c. implantation of a 25-mg morphine pellet) for 7 days then sacrificed. Controls were either implanted with an inert placebo pellet or injected s.c. with saline. Following sacrifice, spinal cord was removed and prepared for [3 H] DAMGO binding assays. The results are representative data from two to three independent studies ($N=12$ /group). The binding parameters for the left panel are: B_{\max} (fmol/mg protein) = 73 ± 3 , 84 ± 3 , $134 \pm 12^*$ (mean \pm S.E.M.) for control, intermittent and continuous naloxone treated mice, respectively. The binding parameters for the right panel are B_{\max} = 106 ± 3 , 112 ± 5 (mean \pm S.E.M.) for control and morphine treated mice, respectively. *Significantly different from control ($P < 0.05$). No significant differences were observed for K_D 's (range = 0.8–1.0 nM).

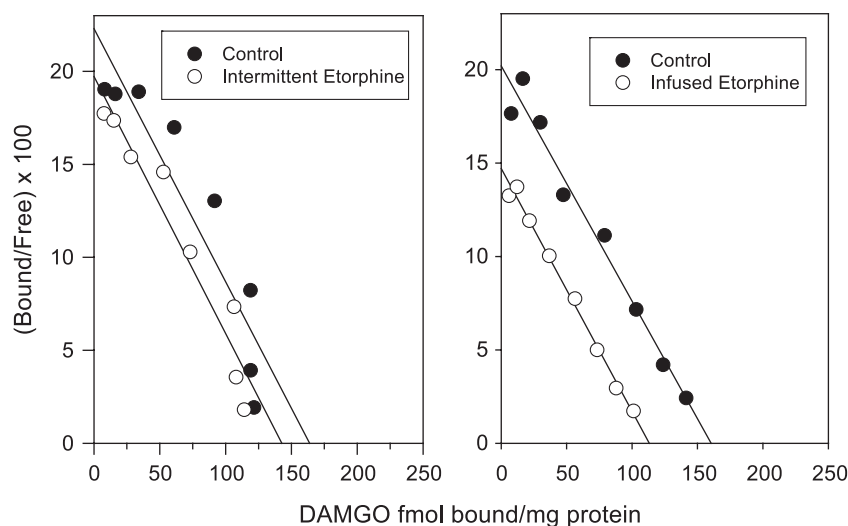


Fig. 2. The effect of intermittent (left panel) and continuous (Infused, right panel) etorphine treatment on spinal cord μ opioid receptors. Mice were continuously infused via an osmotic minipump with etorphine (200 $\mu\text{g/kg/day}$) or injected s.c. once per day (intermittent) with etorphine (200 $\mu\text{g/kg/day}$). After 7 days, the pumps were removed and 16 h later mice ($N=12$) sacrificed, spinal cord removed and prepared for radioligand binding studies. For mice injected once per day, mice ($N=12$) were sacrificed 24 h after the 7th injection and spinal cord removed and prepared for radioligand binding studies. The data are representative of one of three independent studies with similar results for intermittently treated mice (left panel). The data for continuous etorphine infusion are from a single study (right panel), but we have reported similar results for identical treatment (see Patel et al., 2002b; Yoburn et al., 1993). The binding parameters for the above data are: B_{max} (fmol/mg protein) = 139 ± 7 , 129 ± 6 (S.E.M.) for control and intermittently treated mice, respectively. B_{max} = 161 ± 2 , 113 ± 1 (S.E.M.) for control and infused mice, respectively. *Significantly different from control ($P < 0.05$). No significant differences were observed for K_D 's (range = 0.4–0.8 nM).

experiments. In agreement with prior observations (e.g., Gomes et al., 2002), morphine treatment had no effect on μ -opioid receptor density or affinity (Fig. 1).

3.2. Immunoreactive dynamin-2

We have suggested that opioid ligand-induced regulation of μ -opioid receptor density in mouse spinal cord may depend upon changes in abundance in receptor trafficking proteins such as dynamin-2. In support of that suggestion, both continuous etorphine and naloxone treatment regulated dynamin-2 abundance in mouse spinal cord (Fig. 3). Etorphine increased dynamin-2 abundance, whereas naloxone decreased abundance. Previous reports have shown that morphine treatment identical to that used in the present study did not change spinal levels of dynamin-2 and that naltrexone pellet treatment significantly decreased dynamin-2 (Patel et al., 2002a,b, 2003). If regulation of dynamin-2 contributes to changes in μ -opioid receptor density, then intermittent dosing protocols should not alter dynamin-2 abundance. Neither intermittent etorphine nor naloxone significantly altered dynamin-2 abundance (Fig. 3).

3.3. Immunoreactive μ -opioid receptor

Continuous infusion of etorphine significantly reduced immunoreactive levels of μ -opioid receptor in mouse spinal cord, whereas intermittent administration of the same dose was without effect (Fig. 4). Etorphine infusions had no effect on actin abundance (Fig. 4). Although both contin-

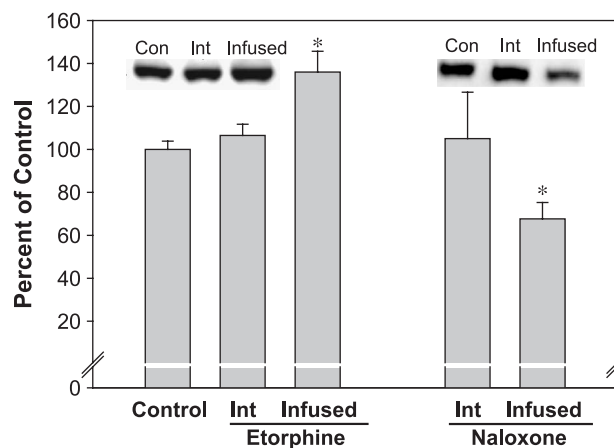


Fig. 3. The effect of continuous (Infused) and intermittent opioid treatment on Dynamin-2 abundance in mouse spinal cord. Mice were infused with naloxone (5 mg/kg/day) or etorphine (200 $\mu\text{g/kg/day}$) for 7 days. Other mice were injected s.c. with naloxone (5 mg/kg) or etorphine (200 $\mu\text{g/kg}$) once per day for 7 days. Controls (Con) were injected once per day with saline. Mice were sacrificed 7 days following the start of treatment (at the end of 192-h infusion, or 24 h following the 7th s.c. injection), spinal cord removed and prepared for Western blot analysis. Individual cords from a single mouse were loaded in each lane in multiple gels. In addition, each gel included a standard curve so that accurate percent changes in protein abundance could be quantitated (not shown; however, see Patel et al., 2002b). The continuous and intermittent etorphine results are based on three independent experiments ($N=5/\text{group}$). The continuous and intermittent naloxone results are based on one experiment ($N=5/\text{group}$). The decrease in Dynamin-2 abundance following continuous naloxone and the increase in Dynamin-2 abundance following continuous etorphine have been previously reported (Patel et al., 2002b, 2003). The insets show representative Western blot data. *Significantly different from control ($p < 0.05$).

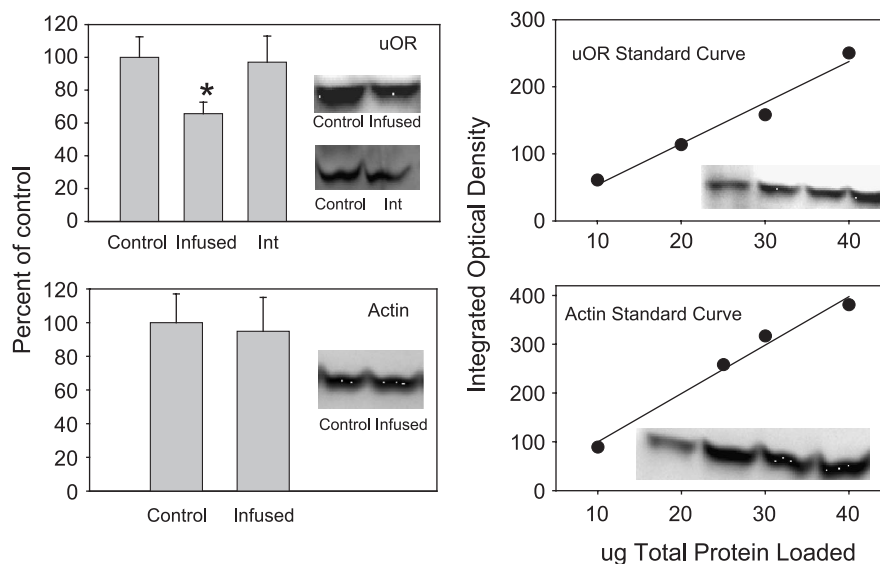


Fig. 4. The effect of etorphine treatment on immunoreactive μ OR abundance in mouse spinal cord. Mice were infused via an osmotic minipump with etorphine (200 μ g/kg/day) or intermittently (Int) injected s.c. once per day with etorphine (200 μ g/kg/day). At the end of 7 days of treatment, mice ($N=5$ /group) were sacrificed, spinal cord removed and prepared for Western blotting studies for μ OR or actin. Individual cords from a single mouse were loaded in each lane in multiple gels. In addition, each gel included a standard curve so that accurate percent changes in protein abundance could be quantitated (representative standard curves are presented in the right panels). The actin and μ OR data are the mean (\pm S.E.M.) results from three independent studies. The insets show representative Western blot data. *Significantly different from control, $p<0.05$.

uous infusion of naloxone and naltrexone pellet treatment dramatically increase μ -opioid receptor density determined by radioligand binding studies, neither treatment significantly changed immunoreactive μ -opioid receptor abun-

dance in spinal cord (Fig. 5). Similarly, intermittent naloxone treatment had no effect on immunoreactive μ -opioid receptor abundance. Representative standard curves for actin and μ -opioid receptor are presented in Fig. 4.

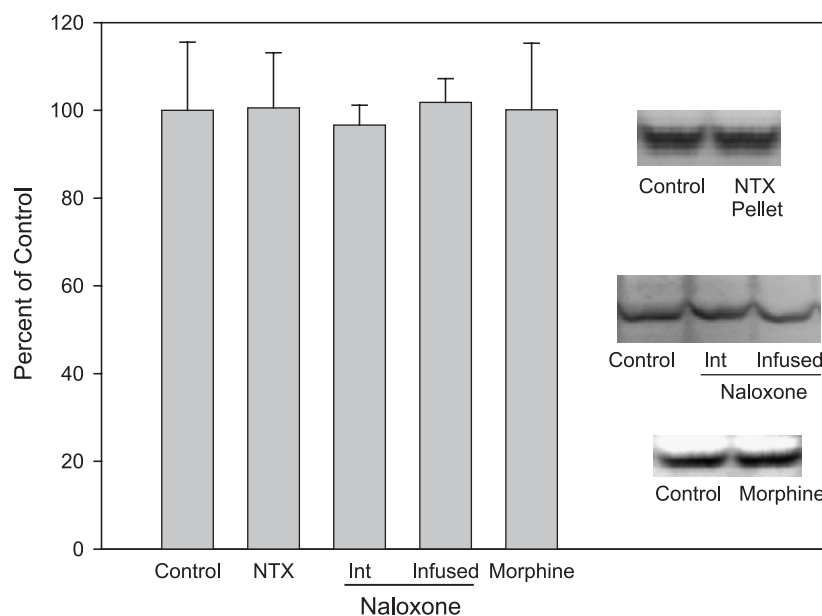


Fig. 5. The effect of naltrexone (NTX), naloxone and morphine on immunoreactive μ OR abundance in mouse spinal cord. Mice were implanted s.c. with a 15-mg naltrexone pellet and at the end of 8 days were sacrificed. Other groups of mice were injected every 24 h (Int) s.c. with naloxone (5 mg/kg) or were infused s.c. using an osmotic pump with naloxone (5 mg/kg/day) for 7 days then sacrificed. An additional group of mice was treated with morphine (40 mg/kg/day by osmotic pump plus s.c. implantation of a 25-mg morphine pellet) for 7 days then sacrificed. Controls were either implanted with an inert placebo pellet or injected s.c. with saline. Following sacrifice, spinal cord was removed and prepared for Western blot analysis. Individual cords from a single mouse were loaded in each lane in multiple gels. In addition, each gel included a standard curve so that accurate percent changes in protein abundance could be quantitated (see Fig. 4). The results are from two to three independent studies ($N=5$ /group). The insets show representative Western blot data.

4. Discussion

Opioid receptor density can be regulated by chronic treatment with opioid agonist and antagonists. Whole animal and cell culture studies have shown that μ -opioid receptor density is increased following treatment with opioid antagonists such as naloxone or naltrexone (e.g., [Rajashekara et al., 2003](#)), while μ -opioid receptor density is down-regulated following treatment with high intrinsic efficacy agonists such as etorphine or DAMGO (e.g., [Patel et al., 2002b](#); [Whistler et al., 1999](#)). Although agonists and antagonists regulate μ -opioid receptor density in vivo, studies suggest these effects rely on different mechanisms. For example, antagonist-induced up-regulation does not alter μ -opioid receptor mRNA abundance in vivo. In contrast, chronic etorphine treatment biphasically regulates μ -opioid receptor mRNA abundance (e.g., [Sehba et al., 1997](#); [Duttaory et al. 1999](#), [Duttaory and Yoburn, 2000](#)). Furthermore, antagonist-induced up-regulation decreases the abundance of dynamin-2 and GRK-2 in mouse spinal cord ([Patel et al., 2002a](#); [Rajashekara et al., 2003](#)), while etorphine treatment increases dynamin-2 protein abundance and has no effect on GRK-2 ([Patel et al., 2002b](#)). Despite these differences, neither opioid antagonist-induced up-regulation nor etorphine-induced down-regulation requires functional pertussis toxin-sensitive G-proteins ([Chang et al 1991](#); [Gomes et al., 2002](#); [Yoburn, et al., 2003](#); [Zaki et al 2000](#)). Since neither up-regulation nor down-regulation requires a full complement of functional $G_{i/o}$ proteins, it is possible that receptor occupancy by some opioid ligands shifts the receptor into a conformation that engages the mechanisms of density regulation.

While studies suggest that the mechanisms that mediate opioid ligand-induced up- and down-regulation of μ OR may be different, the fate of down-regulated receptors and the source of up-regulated receptors remain unclear. It is possible that agonist-induced down-regulation may be related to μ -opioid receptor degradation, whereas antagonist-induced up-regulation may depend upon increased availability of μ -opioid receptor. To address this possibility, we examined changes in μ -opioid receptor protein following treatment using both radioligand binding assays and Western blotting. Continuous naloxone and etorphine treatment regulated μ -opioid receptor density determined in radioligand binding assays in mouse spinal cord. Furthermore, continuous etorphine treatment decreased immunoreactive levels of μ -opioid receptor, which is consistent with degradation of receptor protein. Consequently, etorphine-induced μ -opioid receptor down-regulation may require proteolysis of the receptor. On the other hand, continuous naloxone treatment had no effect on immunoreactive μ -opioid receptor abundance, which suggests that changes in protein abundance may not be required for up-regulation. Morphine treatment did not regulate [3 H] DAMGO binding or immunoreactive μ -opioid receptor. The lack of effect of morphine treatment on immunoreactive μ -opioid receptor or

[3 H] DAMGO binding in mouse spinal cord confirms previous findings ([Cichewicz et al., 2001](#); [Patel et al., 2002b](#)).

The failure to find an increase in immunoreactive μ -opioid receptor following continuous antagonist treatment in mouse spinal cord suggests that increases in [3 H] DAMGO binding are unlikely to require de novo synthesis of μ -opioid receptor. These results might have been anticipated since there are no antagonist-induced changes in μ -opioid receptor mRNA and protein synthesis inhibition and antisense directed at μ -opioid receptor mRNA does not block up-regulation (e.g., [Duttaory et al., 1999](#); [Shah et al., 1997](#); [Tempel et al., 1986](#); [Unterwald et al., 1995](#)). Up-regulation of radioligand binding sites may depend upon several mechanisms. For example, up-regulation may be due to activation of cryptic receptors that are detected by immunoreactive assays, but not by radioligand techniques. Antagonist treatment may shift this pool of cryptic receptors into an active conformation that binds radioligand. Thus, antagonist treatment might recruit latent receptor molecules that require final processing and subsequent insertion into the cell membrane (e.g., [Zukin and Tempel, 1986](#)). This explanation would account for the increase in [3 H] DAMGO binding with no change in immunoreactive μ OR and agrees with recent studies that propose that opioid antagonists can act as pharmacological chaperones for opioid receptors expressed in HEK 293 cells ([Petaja-Repo et al., 2002](#)). These authors propose that pharmacological chaperones such as naltrexone will accelerate maturation of opioid receptors and cell surface targeting. In addition to recruitment of latent or cryptic receptors, correlated decreases in GRK-2 and dynamin-2 ([Patel et al, 2002a, 2003](#)) might slow constitutive internalization and increase μ -opioid receptor density in radioligand binding assays. It should be noted that the failure to find regulation of immunoreactive μ -opioid receptor by continuous naloxone or naltrexone pellet treatment was not due to the fact that this treatment had no effect, since dynamin-2 abundance determined in immunoblotting assays was significantly decreased. Taken together, we speculate that the increase in [3 H] DAMGO binding sites may be due to accelerated processing of cryptic receptors and/or slowing of constitutive internalization.

In a previous study, [Unterwald et al., \(1998\)](#) examined changes in rat brain μ -opioid receptor following continuous naltrexone using radioligand based autoradiography and an immunohistological approach. They found increases in immunoreactive μ -opioid receptor in four brain areas (mean \pm S.E.M. increase = $64.8 \pm 20.4\%$), whereas significant increases in radioligand binding were observed in nine brain regions (mean \pm S.E.M. increase = $106.3 \pm 20.6\%$). Furthermore, the increases observed using immunohistochemistry were less than 50% for all but one of the four areas, while the increases in radioligand binding were greater than 100%

for five of the nine areas. Thus, the increase in μ -opioid receptor detected by radioligand was greater and more pervasive than that observed using the immunohistological technique. Overall, these data are not inconsistent with the present findings from mouse spinal cord. Finally, antagonism of endogenous opioid peptide-induced down-regulation may contribute to up-regulation in vivo, since enkephalin knockout mice have increased opioid receptor density (Brady et al., 1999). However, the fact that up-regulation occurs in cell culture (e.g., Zadina et al., 1994; Li et al., 2001; Zaki et al., 2000) in the absence of endogenous opioids indicates that antagonism of these peptides is not required and suggests that other mechanisms play critical roles.

Intermittent naloxone and etorphine treatment did not regulate μ -opioid receptor or dynamin-2, despite the fact that the total amount of drug administered was the same as continuous treatment. These results suggest that changes in μ -opioid receptor density in both immunoblotting and radioligand assays require the continuous presence of ligand and not simply phasic occupation of the receptor. In addition, the fact that neither μ -opioid receptor density nor dynamin-2 abundance was changed following intermittent treatment supports the suggestion that opioid ligand regulation of μ -opioid receptor may require up- or down-regulation of trafficking proteins such as dynamin-2.

Although down-regulation of immunoreactive μ -opioid receptor and [3 H] DAMGO binding was observed following etorphine treatment, tolerance does not require down-regulation. For example, using the identical morphine and etorphine dosing as described in the present study, Patel et al. (2002b) found that intrathecal DAMGO potency was reduced. Furthermore, the intermittent etorphine dosing protocol described in this report, which does not regulate μ -opioid receptor density, produces tolerance to s.c. morphine (ED_{50} (95% CL)=4.8 mg/kg (3.3–6.7); 22.2 mg/kg (17.2–28.5) for control and intermittent etorphine, respectively; Purohit and Yoburn, unpublished). Lastly, lower-dose continuous infusions of etorphine that do not produce down-regulation induce tolerance (Stafford et al., 2001). In short, as reported earlier, receptor down-regulation is not required for tolerance. However, μ -opioid receptor down-regulation is not benign. In cases where μ -opioid receptor down-regulation is observed, the magnitude of tolerance is increased (see Shen et al., 2000; Stafford et al., 2001).

The present results support a role for intrinsic efficacy in μ -opioid receptor regulation. The combined results of several studies indicate that the ordinal relationship of intrinsic efficacy (or efficacy) for the ligands used in the present study is etorphine>morphine>naloxone=naltrexone (e.g., Barrett et al., 2003; Walker et al., 1998; Emmerson et al., 1996; Yu et al., 1997). Specifically, naltrexone and naloxone (negative or zero intrinsic efficacy) increase μ -opioid receptor density, while the low intrinsic efficacy ligand (morphine) had no effect on density, and the high

intrinsic efficacy agonist (etorphine) resulted in down-regulation. These results are similar to studies that have focused on μ -opioid receptor internalization (e.g., Keith et al., 1996, 1998; Zaki et al., 2000). Despite these observations, some investigators have suggested that efficacy is not necessarily a predictor of endocytosis (Alvarez et al., 2002; Borgland et al., 2003). However, exceptions to the relationship between efficacy and internalization are less common than the overall correspondence between these two parameters. For example, Borgland et al. (2003) reported that agonist relative efficacy and μ -opioid receptor internalization have the same ordinal relationship (DAMGO>methadone>morphine>pentazocine). Taking a broad view, most data, including recent reports (Clever et al., 2004; Cox and Crowder, 2004), support a correlation between efficacy and internalization. Since there is likely a sequential relationship between internalization and down-regulation (e.g., Li et al., 2000), the association between intrinsic efficacy and μ -opioid receptor down-regulation in in vivo studies is not surprising (Yoburn et al., 1993; Keith et al., 1996). Nevertheless, studies that specifically estimate intrinsic efficacies of μ agonists and the induction of μ -opioid receptor endocytosis and down-regulation would be useful. Furthermore, clarification of the differences between efficacy (often used synonymously with E_{max} ; e.g., Bourne and von Zastrow, 2004), efficacy as defined by Stephenson (1956) and intrinsic efficacy (see Kenakin, 1997) and the predictive accuracy of each parameter for internalization/down-regulation is needed.

In summary, these data demonstrate differential regulation of μ -opioid receptor detected by radioligand binding studies and immunoreactive assays. These data suggest that opioid antagonist-induced μ -opioid receptor up-regulation does not require changes in protein synthesis or degradation. The mechanisms that account for the increase in radioligand binding may include a change in constitutive internalization or the recruitment of a pool of cryptic μ -opioid receptor, or both. The concurrent down-regulation of μ -opioid receptor density in both Western and radioligand binding assays raises the possibility that some agonists can accelerate μ -opioid receptor degradation, or slow synthesis, or both in mouse spinal cord in vivo. Taken together, these results indicate that significantly different mechanisms account for μ -opioid receptor up-regulation and down-regulation in the mouse spinal cord.

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