

Chronic Opioid Antagonist Treatment Selectively Regulates Trafficking and Signaling Proteins in Mouse Spinal Cord

CHINTAN N. PATEL, VIKRAM RAJASHEKARA, KAUSHAL PATEL,
VISHAL PUROHIT, AND BYRON C. YOBURN*

Department of Pharmaceutical Sciences, College of Pharmacy and Allied Health Professions, St. John's University,
Queens, New York 11439

KEY WORDS μ -opioid receptors; GRK; dynamin; G-proteins; constitutive-internalization; upregulation; in vivo

ABSTRACT Chronic opioid antagonist treatment produces functional supersensitivity and μ -opioid receptor (μ OR) upregulation. Studies suggest a role for G-protein receptor kinases (GRKs) and dynamin (DYN), but not signaling proteins (e.g., $G_{i\alpha 2}$), in regulation of μ OR density following opioid treatment. Therefore, this study examined μ OR density, agonist potency, and the abundance and gene expression of GRK-2, DYN-2, and $G_{i\alpha 2}$ in mouse spinal cord after opioid antagonist treatment. Mice were implanted with a 15 mg naltrexone (NTX) or placebo pellet and 8 days later pellets were removed. At 24 and 192 h following NTX treatment, mice were tested for spinal DAMGO analgesia. Other mice were sacrificed at 0 or 192 h following NTX treatment and $G_{i\alpha 2}$, GRK-2, and DYN-2 protein and mRNA levels determined. [3 H] DAMGO binding studies were also conducted. Immediately following NTX treatment (0 h), μ OR density was increased (+ \approx 135%), while 192 h following NTX treatment μ OR density was unchanged. NTX increased DAMGO analgesic potency (3.1-fold) 24 h following NTX treatment, while there was no effect at 192 h. NTX decreased protein and mRNA abundance of GRK-2 (–32%; –48%) and DYN-2 (–25%; –29%) in spinal cord at 0 h. At 192 h following 8-day NTX treatment, GRK-2 protein and mRNA were at control levels, while DYN-2 protein remained decreased (–31%) even though DYN-2 mRNA had returned to control levels. $G_{i\alpha 2}$ was unaffected by NTX treatment. These data suggest that opioid antagonist-induced μ -receptor upregulation is mediated by changes in abundance and gene expression of proteins implicated in receptor trafficking, which may decrease constitutive receptor cycling. **Synapse 50:67–76, 2003.** © 2003 Wiley-Liss, Inc.

INTRODUCTION

The density of opioid receptors can be regulated in a ligand-dependent manner (Law et al., 2000; Yoburn et al., 1995; Zaki et al., 2001). Specifically, opioid antagonists increase opioid receptor density (upregulation), while some opioid agonists decrease opioid receptor density (downregulation) (e.g., Patel et al., 2002a; Stafford et al., 2001). Recent studies have implicated a broad group of proteins implicated in receptor trafficking, including the G-protein receptor kinases (GRKs), dynamin (DYN), and β arrestins in ligand-dependent regulation of opioid receptor density (e.g., Patel et al., 2002b; Zhang et al., 1998; Tsao and von Zastrow, 2001). Studies indicate that agonist-dependent opioid receptor downregulation requires functional trafficking proteins and that receptor internalization by agonists that do not support internalization can be rescued by overexpression of trafficking proteins (Zhang et al., 1998; Whistler and von Zastrow, 1998).

While opioid agonist-induced receptor downregulation is only observed with high intrinsic efficacy agonists, opioid antagonist-induced receptor upregulation has been reported following commonly utilized opioid antagonists (e.g., naloxone and NTX). Studies have demonstrated increased μ , δ , and κ receptor density following chronic opioid antagonist treatment in both cell culture and in vivo systems, although upregulation of μ -opioid receptor (μ OR) appears to be of the largest magnitude (e.g., Yoburn et al., 1995; Zadina et al.,

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*Correspondence to: Byron C. Yoburn, Department of Pharmaceutical Sciences, College of Pharmacy and Allied Health Professions, St. John's University, 8000 Utopia Parkway, Queens, NY 11439. E-mail: Yoburnb@stjohns.edu

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1994). The relevance of the new receptors is evident by the parallel increase in the potency (e.g., analgesic) of μ -opioid agonists (functional supersensitivity) (Tempel et al., 1986; Yoburn et al., 1986, 1995). Unfortunately, the mechanisms that mediate receptor upregulation remain to be fully elucidated.

While antagonist-induced increases in protein synthesis is an attractive explanation for receptor upregulation *in vivo*, an increase in gene expression does not appear to account for upregulation, since changes in mRNA *in vivo* have not been observed (Duttaroy et al., 1999; Castelli et al., 1997; Unterwald et al., 1995). A preliminary study raised the possibility that changes in constitutive internalization mediates opioid antagonist-induced upregulation (Evans et al., 1997). Studies of μ OR splice variants and mutants also suggest that opioid antagonist treatment produces upregulation by stabilizing membrane opioid receptors and interfering with constitutive internalization (Koch et al., 2001; Li et al., 2001). Furthermore, constitutive internalization of μ OR mutants appears to depend on various trafficking proteins, including GRKs, arrestins, and DYNs (Li et al., 2001).

Given the potential role for trafficking proteins in opioid receptor regulation (Patel et al., 2002a; Whistler and von Zastrow, 1998; Zhang et al., 1998), it seemed likely that opioid antagonist treatment might regulate the abundance of these proteins. In a recent study, Patel et al. (2002a) reported that chronic naltrexone treatment increased μ OR density in mouse spinal cord, while at the same time decreasing spinal abundance of two trafficking proteins (GRK-2, DYN-2). We interpreted these results to suggest that chronic antagonist treatment reduced constitutive cycling of μ ORs mediated by GRK-2 and DYN-2. In the present study, we determined if the changes in these trafficking proteins is based on regulation of gene expression and if the decrease in abundance is time-dependent. Furthermore, to determine if opioid antagonist-induced changes observed in trafficking proteins are selective, antagonist-induced changes in a protein directly activated by μ OR ($G_{i\alpha 2}$) were studied. We anticipated that chronic antagonist treatment would have no effect on signaling proteins such as $G_{i\alpha 2}$ since studies do not support a role for functional G-proteins in ligand-mediated regulation of μ OR density (Gomes et al., 2002; Chang et al., 1991; Yoburn et al., 2003).

MATERIALS AND METHODS

Subjects

Male, Swiss-Webster mice (22–24 g) (Taconic Farms, Germantown, NY) were used in all experiments. Animals were housed 10 per cage with free access to food and water. Each mouse was used only once.

General procedure

Mice were implanted subcutaneously with a 15 mg NTX pellet. Controls were implanted with a placebo pellet. Pellets were implanted at the nape of the neck while mice were lightly anesthetized with halothane: oxygen (4:96). Eight days following NTX or placebo treatment, pellets were removed under halothane anesthesia. Immediately and 192 h following 8-day NTX treatment, mice were sacrificed, spinal cords collected for receptor binding, Western blotting, and RT-PCR assays. At 24 and 192 h following termination of 8-day NTX treatment, mice were tested for spinal [D-Ala², N-MePhe⁴, Gly⁵-ol] enkephalin (DAMGO) analgesia. (DAMGO potency was not determined immediately following NTX treatment in order to allow elimination of antagonist.)

μ -Opioid receptor binding

Binding was performed as described previously (Shah et al., 1997). Briefly, mice (n = 12/group) were sacrificed and spinal cords removed, pooled, and homogenized in 80 volumes of ice-cold 50 mM Tris buffer (pH 7.4). Homogenates were centrifuged at 15,000 rpm (2°C) for 15 min, supernatants were discarded, and pellets were resuspended and incubated for 30 min at 25°C in 50 mM Tris buffer (pH 7.4). Homogenates were centrifuged again and the pellets were finally resuspended in 20–80 volumes of 50 mM phosphate buffer (PB; pH 7.2). An aliquot (200 μ l) of homogenate was assayed in triplicate in tubes containing 0.03–5 nM [³H]DAMGO (μ ligand, New England Nuclear, Boston, MA). Nonspecific binding was determined in the presence of 1,000 nM levorphanol. Tubes were incubated for 90 min at 25°C and the incubation was terminated by filtration of samples over GF/B glass fiber filters (Brandel, Gaithersburg, MD). Filters were washed three times with cold PB and transferred to vials containing scintillation cocktail and then counted. Counts per minute (CPMs) were converted to disintegration per minute (DPMs) using the external standard method. Protein was assayed by the Bradford (1976) method using reagent purchased from Bio-Rad (Richmond, CA).

Analgesia assay

DAMGO-induced analgesia (antinociception) was evaluated 24 and 192 h following termination of NTX

Abbreviations:

AP	alkaline phosphatase
DAMGO	[D-Ala ² , N-MePhe ⁴ , Gly ⁵ -ol]-enkephalin
dNTPs	deoxynucleotide triphosphates
DYN	dynamain
GPCR	G-protein coupled receptor
GRK	G-protein coupled receptor kinase
μ OR	μ -opioid receptor
NTX	naltrexone
PVDF	polyvinylidene difluoride.

or placebo treatment. Analgesia was determined using the tail-flick assay in which a beam of light was focused on the dorsal tail surface ~2 cm from the tip of the tail. The intensity of the light was adjusted so that baseline tail flick latencies were 2–4 sec. Mice ($n = 6/\text{dose}/\text{treatment}$) were then briefly anesthetized using halothane and injected intrathecally (IT) (see Yoburn et al., 1989) with DAMGO (0.4–23 ng/4 μl /mouse for placebo; 0.2–15 ng/4 μl /mouse for NTX) and tested for analgesia 30 min later. If a mouse failed to flick by 10 sec following DAMGO administration, the test was terminated and a latency of 10 sec was recorded. Mice that had a latency of 10 sec were defined as analgesic. All antinociception testing was conducted in a blind manner.

Western blotting assay

GRK-2 and DYN-2 Western blots

Individual spinal cords ($n = 5/\text{treatment}$) were rapidly removed on ice and homogenized (Brinkman Polytron Homogenizer, 20,000 rpm 30 sec) in 500 μl lysis buffer (2% SDS, 1 mM sodium orthovanadate, 12.5 mM Tris; pH 7.4), 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 (Bradford, 1976). Spinal cord samples were not pooled. Samples for Western analysis were diluted using a mixture of equal volumes of lysis and sample buffer (4% SDS, 1% β -mercaptoethanol, 20% glycerol, 125 mM Tris base, loading dye). Aliquots (8 μl , 0.3–3 μg protein) from individual cords were loaded side by side so that 10 individual spinal cords (5 cords/treatment; 1 cord/lane) were loaded onto each polyacrylamide gel (Pager Gels 10% Tris-Glycine, BioWhittaker Molecular Applications, Rockland, ME). Samples were separated by electrophoresis (150 V for 60 min). Proteins were transferred to Immobilon-P PVDF membranes (Millipore, Bedford, MA) using the Miniprotean II (Bio-Rad) at 100 V for 85 min. Nonspecific binding sites on the membrane were blocked by incubation (2 h at 24°C, or overnight at 4°C) in blocking buffer (0.2% Aurora™ Blocking Reagent; 1 \times PBS; 0.058 M Na_2HPO_4 , 0.017 M NaH_2PO_4 , 0.068 M NaCl; 0.05% Tween-20 from ICN Biomedicals, Costa Mesa, CA) followed by incubation (1 h, 24°C) with primary antibody in blocking buffer (rabbit polyclonal IgG for GRK-2 (1:200); goat polyclonal IgG for DYN-2 (1:300), Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed twice with blocking buffer and then incubated (1 h at 24°C) with secondary antibody in blocking buffer (anti-rabbit IgG-AP for GRK-2 (1:5,000); anti-goat IgG-AP for DYN-2 (1:5,000), Santa Cruz Biotechnology). Membranes were then washed three times with blocking buffer, followed by two quick rinses with assay buffer (20 mM Tris-HCl, pH 9.8, 1 mM MgCl_2). Bands were visualized using a chemiluminescence assay (CDP Star Substrate, Novagen, Madison, WI). A standard curve (minimum four points) using increas-

ing amounts of spinal cord protein from controls (0.3–3 $\mu\text{g}/\text{lane}$) was included on every gel. All data are reported as standard curve equivalents. Each experiment was repeated three times.

$G_{i\alpha 2}$ Western blots

Spinal cords ($n = 6/\text{treatment}$) were removed and individually homogenized in 500 μl Tris buffer. Samples were not pooled. Homogenates were centrifuged at 20,000 rpm for 15 min, the pellet resuspended in 500 μl Tris buffer, and protein concentration determined. Pellets were obtained by centrifugation at 4°C (20,000 rpm for 15 min), resuspended, and boiled in 10% SDS sample buffer for 5 min. Aliquots (8 μl , 14 μg protein) from individual cords were loaded side-by-side so that 12 individual spinal cords (6 cords/treatment; 1 cord/lane) were loaded onto each polyacrylamide gel and subjected to electrophoresis (0.02 Amp, 85 min). Proteins were transferred to Immobilon-P PVDF membranes at 15 V for 25 min using Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA) using the Aurora Chemiluminescence assay (ICN Biomedicals). Nonspecific binding sites on the membrane were blocked by incubation in blocking buffer overnight. Blocked membranes were incubated at room temperature for 1 h in primary mouse $G_{i\alpha 2}$ antibody (Chemicon International, Temecula, CA) diluted in blocking buffer (1:750). After washing twice in blocking buffer, membranes were incubated for 1 h with goat antimouse AP-conjugated secondary antibody (Santa Cruz Biotechnology) diluted in blocking buffer (1:5,000). The blot was washed three times in blocking buffer, then twice in assay buffer and visualized using the Aurora Chemiluminescence assay (ICN Biomedicals). A standard curve using recombinant $G_{i\alpha 2}$ protein (10–20 ng/lane; Santa Cruz Biotechnology) was included on every gel assay and data are reported as standard curve equivalents. Each experiment was repeated three times.

Reverse-transcriptase polymerase chain reaction

Mice ($n = 6/\text{treatment}$) were sacrificed and total RNA was extracted from individual spinal cords using TRIzol reagent (Invitrogen, Carlsbad, CA) and precipitated in 100% isopropyl alcohol. Samples were not pooled. Yeast tRNA (12.5 μg) was added as a carrier to facilitate the precipitation of RNA prepared from a single spinal cord. The pellet was washed in 75% ethanol, dried, and suspended in 100 μl of RNase-free water for 10 min at 55–60°C. The tubes were then treated with 2 units of RNase free DNase (MAXI script Kit, Ambion, Austin, TX) at 37°C for 30 min. RNA was stored at –80°C until analysis. The yield of RNA was determined using UV spectrometry (260 nm).

The RT-PCR assay was based on the thermostable rTth RNA PCR kit (Perkin Elmer Applied Biosystems,

Foster City, CA). An aliquot of RNA from each individual spinal cord was subjected to RT-PCR in separate tubes yielding an amplification product for each mouse spinal cord. The primers for mouse GRK-2 were AGA AGT ATC TGG AGG ACC GAGG (upstream) and TCT TTG AAA AGG GAT GTG AGC AGGC (downstream) corresponding to bases 131–152 and 355–379 (249 bp product). The primers for mouse DYN-2 were CAG TTT GGA GTG GAC TTT GAG AAGC (upstream) and AGA CAG GGC TCT TTC AGC TTG ACC (downstream) corresponding to bases 1131–1155 and 1379–1402 (272 bp product). The primers for mouse $G_{i\alpha 2}$ were TGA GGA TGA GGA GAT GAA CCGC (upstream) and AAC ACA AAC TGC ACG TTC TTGG (downstream) corresponding to bases 805–826 and 1086–1107 (303 bp product). The reverse transcription reaction used 0.2 μ g of spinal RNA. The initial reaction mixture contained downstream primers (0.75 μ M for GRK-2, 1 μ M for DYN-2 and $G_{i\alpha 2}$), 16 μ M dNTPs, 1 mM $MgCl_2$, 2.5 units of rTth DNA polymerase in rTth reverse transcriptase buffer (10 mM Tris-HCl, pH 8.3, 90 mM KCl) brought to 10 μ l with DEPC water. Tubes were then placed in a Techne Progene Thermal Cycler (Techne, Princeton, NJ) at 70°C for 10 min. Next, PCR mix containing chelating buffer (50% v/v glycerol; 10 mM Tris-HCl, pH 8.3; 0.1 M KCl, 0.5% w/v Tween-20; 0.75 mM EGTA), 1.875 mM $MgCl_2$, and upstream primers (0.75 μ M for GRK-2, 1 μ M for DYN-2 or $G_{i\alpha 2}$) brought to 40 μ l with DEPC water was added to the reaction tubes. PCR was then carried out in the thermocycler under the following conditions: a 60-sec incubation at 95°C, followed by amplification for 35 cycles at 95°C for 10 sec and 60°C for 15 sec, followed by a final extension at 60°C for 7 min. Tubes were brought to 4°C, 1 μ l loading dye (Ambion) was added to each tube and then a 5- μ l aliquot containing amplified product from individual control and treated spinal cord was loaded on 1.8% agarose gels and electrophoresed (100 V, 60 min). Amplified product from a single spinal cord was loaded in each lane so that 12 individual cords (6/treatment) were examined on each gel. The RT-PCR assay yielded the predicted size amplification products for GRK-2, DYN-2, and $G_{i\alpha 2}$.

A standard curve for GRK-2, DYN-2, and $G_{i\alpha 2}$ mRNA was constructed using RNA extracted from control spinal cord. Each gel included 0.05, 0.1, 0.2, and 0.4 μ g of control RNA in the first four lanes. The standard curves were used to convert optical densities to arbitrary RNA units for GRK-2, DYN-2, and $G_{i\alpha 2}$ amplified products. Each experiment was repeated three times.

Drugs

DAMGO, NTX (30 mg), and placebo pellets were obtained from Research Triangle Institute (Research Triangle Park, NC) through the Research Technology Branch of the National Institute on Drug Abuse. The NTX pellets were cut in half (yielding 15-mg implants),

weighed, and then implanted. The pellets were wrapped in nylon mesh before subcutaneous implantation. DAMGO was dissolved in sterile saline for IT injection.

Data analysis

Gel images (RT-PCR, Westerns) were captured using a FluorChem v. 2.0 Imaging System (Alpha Innotech, San Leandro, CA). The images were digitized and analyzed for integrated optical density (IOD) using GelPro image analysis software (v. 3.0 and 3.1, Media Cybernetics, Silver Spring, MD). Optical densities from Western blot and RT-PCR gels were converted to protein or RNA equivalents using the standard curves (Patel et al., 2002b; Yoburn et al., 2003), expressed as percent change from control and evaluated using Student's *t*-test ($P < 0.05$). Quantal dose-response data were analyzed by Probit Analysis (Finney, 1973) using a computerized program (BLISS 21, Department of Statistics, University of Edinburgh, Edinburgh, Scotland) that estimated ED_{50} s and relative potencies. Statistical differences between ED_{50} values were determined using the *t*-test ($P < 0.05$). B_{max} and K_D values were estimated from saturation studies using nonlinear regression (Prism v. 3.02, Graphpad Software, San Diego, CA). Binding data was best fit by a one-site model. Differences between B_{max} and K_D values were evaluated using the Z-test ($P < 0.05$) based on nonlinear regression parameters.

RESULTS

Effect of NTX on spinal μ OR density and IT DAMGO potency

In saturation binding studies (Fig. 1A), chronic NTX treatment produced significant μ OR upregulation immediately following treatment (NTX 0 h) in two independent studies (+136%, +131%). In separate groups of mice, the NTX pellet was removed following 8-day treatment and binding determined in spinal cord 192 h later (NTX 192 h). In two independent experiments, μ -receptor density had returned to control levels 192 h following the 8-day NTX treatment (Fig. 1). No significant effects of NTX treatment on DAMGO K_D were observed at either time point (K_D range = 0.8–1.5 nM).

Mice were tested for IT DAMGO potency (tail flick) 24 and 192 h following 8-day NTX treatment. The potency of DAMGO was significantly increased (ED_{50} shift = 3.1 ± 0.2 fold; mean \pm SEM from three independent experiments) 24 h following 8-day NTX treatment (Fig. 1B). However, 192 h following 8-day NTX treatment, DAMGO potency did not differ significantly from control (ED_{50} shift = 1.3 ± 0.2 from three independent experiments). Prior to DAMGO injection there were no significant differences in baseline tail-flick latencies among the groups at either test time (data not shown). Similarly, there was no difference between ED_{50} s among placebo groups at either time point.

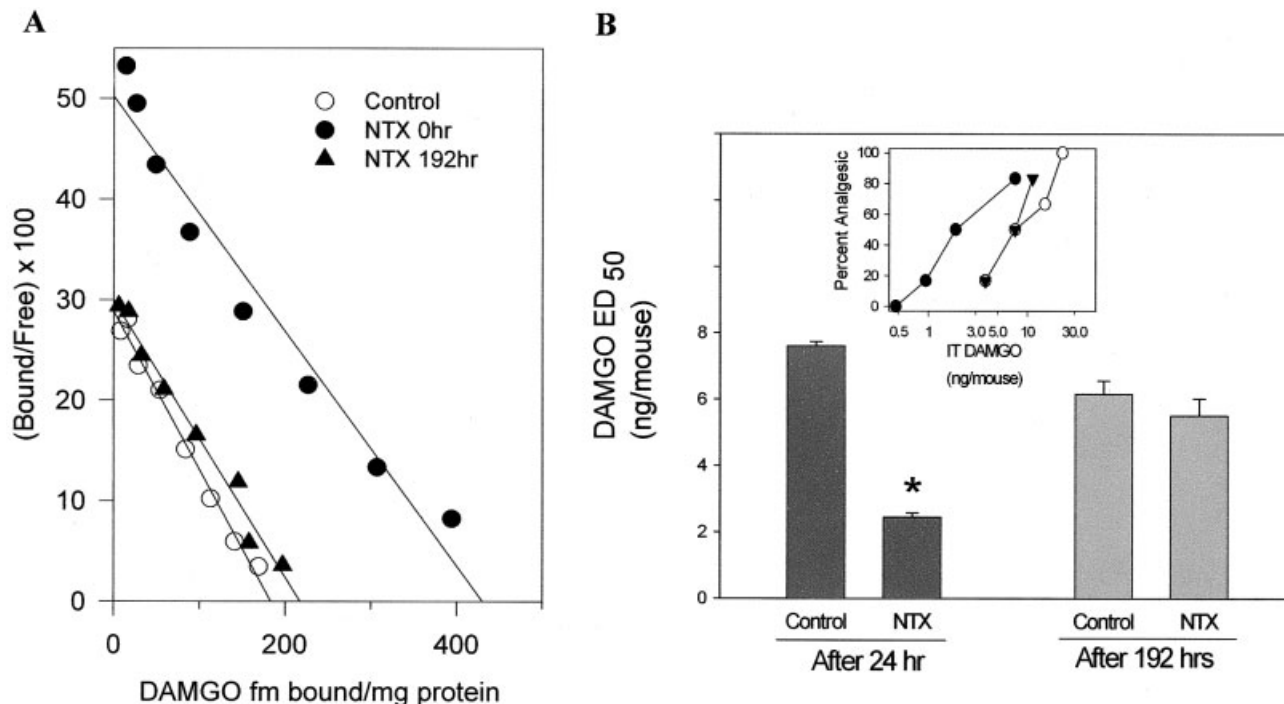


Fig. 1. Effect of naltrexone treatment on μ -opioid receptor density in spinal cord and spinal potency of DAMGO. **A:** Scatchard plot of specific [3 H] DAMGO binding in spinal cord homogenate from mice ($n = 12$ /group) chronically treated with NTX or control. The closed circles (\bullet , NTX 0 h) are data from spinal cord assayed immediately following 8-day NTX treatment. Closed triangles (\blacktriangle , NTX 192hr) are data from spinal cord assayed 192 h following termination of the 8-day NTX treatment. $B_{max} + SEM = 191 + 4; 451 + 6, 220 + 9$ fmol/mg protein for control, NTX 0 h, and NTX 192 h, respectively. Virtually identical results were observed in a second independent study (not

shown). **B:** Effect of NTX on IT DAMGO potency in the tail flick. Mice ($n = 6$ /dose/treatment) were treated with NTX or control pellets for 8 days and the pellets removed and 24 h (NTX 24 h) or 192 h (NTX 192 h) later, IT DAMGO dose-response studies were conducted. Data are presented as a bar graph depicting mean (\pm SEM) ED_{50s} from three experiments with three-four doses/treatment for each time point. The inset shows results from a single representative experiment. (\circ = control, \bullet = NTX 24 h, \blacktriangledown = NTX 192 h). *Significantly different from control $P < 0.05$.

Effect of NTX on GRK-2, DYN-2, and $G_{i\alpha 2}$ protein abundance

Standard curves (see Materials and Methods) for GRK-2, DYN-2, and $G_{i\alpha 2}$ protein were linear or log-linear (mean \pm SEM 3–6 assays $r^2 = 0.92 \pm 0.02; 0.96 \pm 0.01, 0.88 \pm 0.01$, respectively) and included the range of optical densities employed for unknowns (Fig. 2A,C,E). Immediately following 8-day chronic NTX treatment, the abundance of GRK-2 (-32%) and DYN-2 (-25%) were significantly decreased (Figs. 3A, 4A, respectively). GRK-2 protein levels returned to control by 192 h following the 8-day NTX treatment, whereas DYN-2 remained significantly decreased (-31%). $G_{i\alpha 2}$ protein abundance was unchanged immediately following 8-day NTX treatment (Fig. 5A), and consequently was not evaluated again at 192 h.

Effect of NTX on spinal GRK-2, DYN-2, and $G_{i\alpha 2}$ mRNA levels

Standard curves (see Materials and Methods) for GRK-2, DYN-2, and $G_{i\alpha 2}$ were linear or log-linear from 0.05–0.4 μ g of total starting RNA (mean \pm SEM for 3–6 assays $r^2 = 0.91 \pm 0.07; 0.88 \pm 0.05; 0.86 \pm 0.03$, respectively) and included the range of optical densi-

ties employed for unknowns (Fig. 2B,D,F). Immediately following 8-day NTX treatment, GRK-2 (-48%) (Fig. 3B) and DYN-2 mRNA abundance (-29%) (Fig. 4B) were significantly reduced in mouse spinal cord. GRK-2 and DYN-2 mRNA levels returned to control by 192 h following the 8-day NTX treatment. There was no change in $G_{i\alpha 2}$ mRNA immediately following NTX treatment (Fig. 5B).

DISCUSSION

Opioid antagonist-induced μ OR upregulation is a robust phenomenon in cell culture and in vivo (Patel et al., 2002a; Yoburn et al., 1986; Unterwald et al., 1998; Zukin and Tempel, 1986; Zadina et al., 1994). Chronic exposure to opioid antagonists increases μ , δ , and κ opioid receptor density, with the increases in μ OR density typically greatest (Yoburn et al., 1995). As such, upregulation of μ OR density by opioid antagonists has been the focus of many studies (e.g., Diaz et al., 2002; Moudy et al., 1985; Unterwald et al., 1998). However, the underlying mechanisms of μ OR upregulation remain unclear.

Potential mechanisms of antagonist-induced μ OR upregulation include: synthesis of new receptor mol-

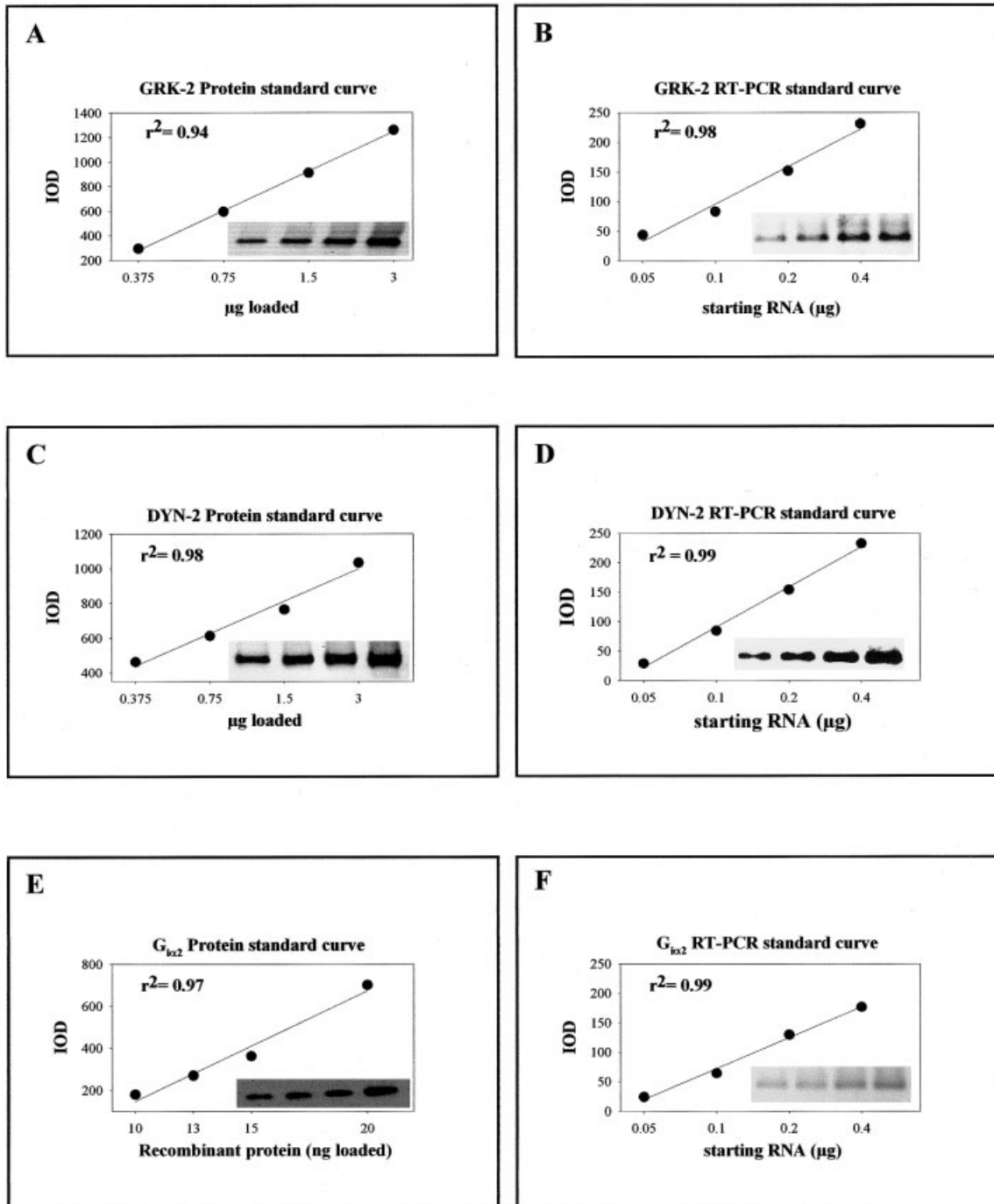


Fig. 2. Representative standard curves for GRK-2, DYN-2, and G₁₀₂ protein (A,C,E, respectively) and mRNA (B,D,F, respectively). See Materials and Methods.

ecules (e.g., Unterwald et al., 1995), activation of latent (cryptic) receptors (e.g., Chan et al., 1995; Moudy et al., 1985; Unterwald et al., 1998), and inhibition of receptor internalization and degradation (Evans et al., 1997; Koch et al., 2001). Among the least likely explanations of upregulation is an increase in the synthesis of new receptors. This conclusion is based on studies that demonstrate little or

no evidence for an increase in μ OR mRNA in association with receptor upregulation (e.g., Duttaroy et al., 1999, Unterwald et al., 1995). Furthermore, protein synthesis inhibition does not block upregulation in mouse spinal cord-ganglion explants (Tempel et al., 1986) and antisense directed at μ OR mRNA does not block antagonist-induced μ OR upregulation or supersensitivity in vivo (Shah et al., 1997).

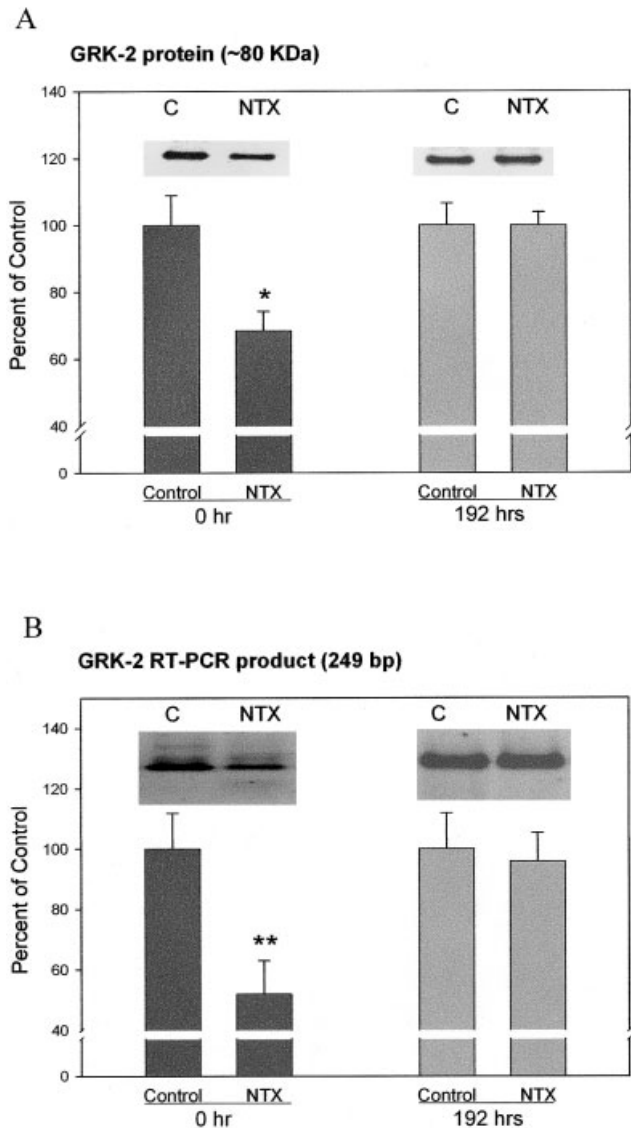


Fig. 3. The effect of naltrexone treatment on GRK-2 in spinal cord. **A:** The effect of chronic NTX on GRK-2 protein abundance in spinal cord. NTX (15 mg) and control pellets were implanted subcutaneously for 8 days. Immediately at the end of treatment (0 h) and 8 days (192 h) following NTX treatment, spinal cords were removed and levels of GRK-2 protein were determined using Western blotting. Data are the mean (+SEM) from three independent experiments for each time point comprising five individual spinal cords per treatment in each assay. Insets are representative blots for GRK-2 (C = control; NTX = naltrexone) for each time point. *Significantly different from control ($P < 0.05$). **B:** The effect of chronic NTX on GRK-2 mRNA abundance in spinal cord. NTX (15 mg) and control pellets were implanted subcutaneously for 8 days. Immediately at the end of treatment (0 h) and 8 days (192 h) following NTX treatment, spinal cords were removed and levels of GRK-2 mRNA were determined using RT-PCR. Data are mean (+SEM) percent of control from three independent experiments for each time point comprising six individual spinal cords per treatment in each assay. Insets are images of representative RT-PCR products for GRK-2 (C = control; NTX = naltrexone) for each time point. **Significantly different from control ($P < 0.01$).

An early proposed mechanism for opioid antagonist-induced upregulation suggested that receptor density increases were mediated by recruitment of latent (cryp-

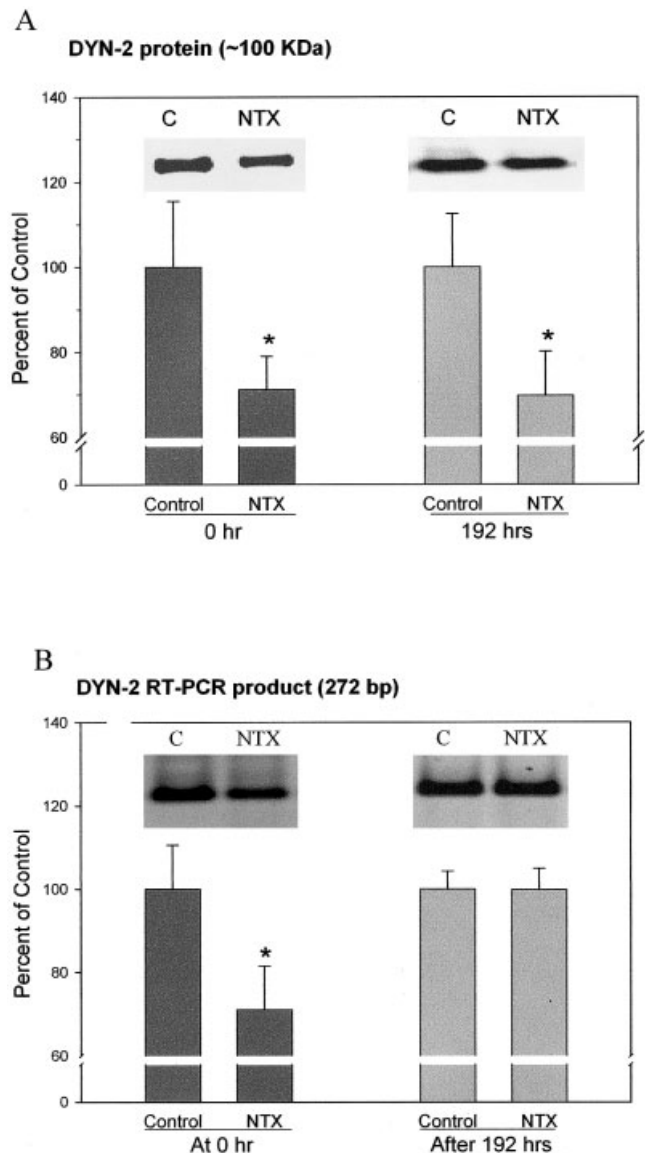


Fig. 4. The effect of naltrexone on DYN-2 in spinal cord. **A:** The effect of chronic NTX on DYN-2 protein abundance in spinal cord. NTX (15 mg) and control pellets were implanted subcutaneously for 8 days. Immediately at the end of treatment (0 h) and 8 days (192 h) following NTX treatment, spinal cords were removed and levels of DYN-2 protein were determined using Western blotting. Data are the mean (+SEM) from three independent experiments for each time point comprising five individual spinal cords per treatment in each assay. Insets are representative blots for DYN-2 (C = control; NTX = naltrexone) for each time point. *Significantly different from control ($P < 0.05$). **B:** The effect of chronic NTX on DYN-2 mRNA abundance in spinal cord. NTX (15 mg) and control pellets were implanted subcutaneously for 8 days. Immediately at the end of treatment (0 h) and 8 days (192 h) following NTX treatment, spinal cords were removed and levels of DYN-2 mRNA were determined using RT-PCR. Data are mean (+SEM) percent of control from three independent experiments for each time point comprising six individual spinal cords per treatment in each assay. Insets are images of representative RT-PCR products for DYN-2 (C = control; NTX = naltrexone) for each time point. *Significantly different from control ($P < 0.05$).

tic) molecules that required final processing and subsequent insertion into the cell membrane (e.g., Zukin and Tempel, 1986). Failure to observe changes in μ OR

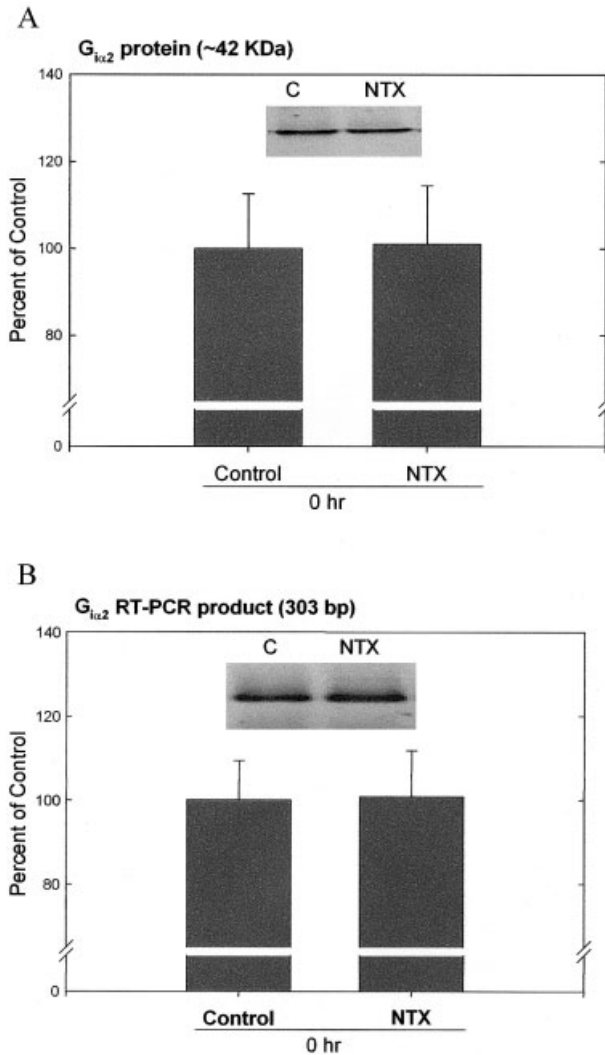


Fig. 5. The effect of naltrexone on $G_{i\alpha 2}$ in mouse spinal cord. **A:** The effect of chronic NTX on $G_{i\alpha 2}$ protein abundance in spinal cord. NTX (15 mg) and control pellets were implanted subcutaneously for 8 days. Immediately at the end of NTX treatment (0 h) spinal cords were removed and levels of $G_{i\alpha 2}$ protein were determined using Western blotting. Data are the mean (+SEM) from three independent experiments comprising six individual spinal cords per treatment in each assay. Inset is a representative blot for $G_{i\alpha 2}$ (C = control; NTX = naltrexone). **B:** The effect of chronic NTX on $G_{i\alpha 2}$ mRNA abundance in spinal cord. NTX (15 mg) and placebo pellets were implanted subcutaneously for 8 days. Immediately at the end of NTX treatment (0 h) spinal cords were removed and levels of $G_{i\alpha 2}$ mRNA were determined using RT-PCR. Data are the mean (+SEM) from three independent experiments comprising six individual spinal cords per treatment in each assay. Inset is an image from a representative RT-PCR gel for $G_{i\alpha 2}$ RT-PCR products (C = control; NTX = naltrexone).

mRNA following antagonist treatment were consistent with this suggestion. A direct examination of this proposal by Unterwald et al. (1998) using a combined immunohistochemical and radioligand approach suggested that activation of immunoreactive cryptic receptors could account for antagonist-induced upregulation in some brain regions. However, activation of cryptic receptors did not fully explain increases in receptor density detected by the radiolabeled ligands. In short,

activation of cryptic receptors may contribute to upregulation, but the magnitude of this effect and the mechanisms involved are not currently known.

Many GPCRs, including μ OR, undergo constitutive (ligand-independent) cycling (Chavkin et al., 2001; Leurs et al., 1998; Wang et al., 2001; Liu et al., 2001). Based on a preliminary study reporting that opioid antagonists increased cell surface density of a promoterless μ OR mutant expressed in HEK-293 cells (Evans et al., 1997), it was proposed that changes in constitutive cycling might mediate antagonist-induced upregulation. More recently, it has been reported that opioid antagonist treatment stabilized membrane μ OR splice variants and mutants and interfered with constitutive internalization (Koch et al., 2001, Li et al., 2001). Constitutive internalization of some μ OR mutants appears to depend on various trafficking proteins, including GRKs, arrestins, and dynamins (Li et al., 2001).

Several proteins involved in receptor trafficking are implicated in opioid tolerance and ligand-dependent regulation of GPCRs, including μ ORs (Bohn et al., 2000; Li et al., 2001; Terwilliger et al., 1994; Whistler and von Zastrow, 1998; Zhang et al., 1998). For example, opioid agonist-induced μ OR downregulation in cell culture (Whistler and von Zastrow, 1998; Zhang et al., 1998) and in vivo (Patel et al., 2002b; Hurle, 2001) appears to depend on GRKs, β -arrestins, and DYNs. Similarly, chronic opioid antagonist treatment decreases GRK-2 and DYN-2 protein abundance in mouse spinal cord (Patel et al., 2002a). While these data support a role for trafficking proteins in opioid antagonist-induced μ OR upregulation, it was not known if the regulation of these proteins was mediated by changes in gene expression or extended to proteins that are directly activated by agonist occupation of the receptor. Furthermore, the reversibility of the decreases in GRK-2 and DYN-2 had not been evaluated.

The present results confirm and extend data that implicate trafficking proteins in opioid antagonist-induced μ OR upregulation. Immediately following chronic NTX treatment, GRK-2 and DYN-2 were decreased (see Patel et al., 2002a), while the signaling protein $G_{i\alpha 2}$ was unchanged. The failure of NTX to regulate $G_{i\alpha 2}$ supports previous studies (Chang et al., 1991; Zaki et al., 2001) that suggest that G-proteins are not critical in mediating μ OR upregulation. Determination of mRNA indicated that changes in trafficking proteins were associated with a decrease in GRK-2 and DYN-2 gene expression. No change in $G_{i\alpha 2}$ mRNA was observed. At 192 h following NTX treatment, GRK-2 protein and mRNA had returned to control levels, while DYN-2 protein remained decreased even though DYN-2 mRNA had returned to control levels. Increases in DAMGO potency and spinal μ ORs paralleled the changes in GRK-2. Although μ OR binding studies were conducted immediately following NTX treatment, dose-response studies required an interval between treatment and analgesia testing to allow

elimination of NTX, which would antagonize DAMGO. Nevertheless, opioid antagonist-induced receptor upregulation and functional supersensitivity were maximal soon after NTX treatment and both declined to control levels by 192 h following treatment termination.

The decline in supersensitivity and receptor upregulation following NTX treatment was paralleled by changes in GRK-2 protein and mRNA abundance. These results suggest that GRK-2 may play a pivotal role in opioid antagonist-induced μ -opioid receptor upregulation. Specifically, the reduction in this trafficking protein may reduce constitutive cycling and downregulation of μ ORs in mouse spinal cord. The $\approx 30\%$ loss in GRK-2 protein may be sufficient to allow receptors to accumulate on the cell surface and produce a net increase in receptor density that mediates functional supersensitivity. The fact that GRK-2 mRNA was decreased concurrently with protein raises the possibility that cellular adaptations to chronic opioid antagonist treatment are mediated at the gene expression level. The return of spinal μ OR density, DAMGO potency, and GRK-2 protein and mRNA to control levels further supports the suggestion that this trafficking protein may play a role in upregulation.

On the other hand, DYN-2 protein was decreased $\approx 25\text{--}30\%$ at both time points, although DYN-2 mRNA was decreased only immediately following NTX treatment. These data raise the possibility that decreases in DYN-2 are not sufficient to mediate opioid antagonist-induced upregulation. However, it is possible that a reduction in DYN-2 is necessary for upregulation and supports antagonist-induced μ -receptor increases mediated by changes in GRK-2. The fact that DYN-2 protein abundance recovery from NTX is delayed may be related to changes in degradative mechanisms. In any case, the recovery of DYN-2 mRNA suggests that DYN-2 protein levels will eventually recover.

A recent report demonstrated an increase in GRKs (2,3,6) and β -arrestin-2 in select rat brain regions following chronic treatment with opioid antagonist (Diaz et al., 2002). Several possibilities may explain the differences in our results. Our studies were conducted in mouse spinal cords, while the Diaz et al. experiments were conducted in rat brain. In addition, the increases in the Diaz et al. (2002) study were reported only at 24 h following chronic antagonist treatment. Since receptor upregulation declines following treatment termination (Tempel et al., 1982; Yoburn and Inturrisi, 1988), it may be the case that the increase in trafficking proteins observed in rat brain are related to processes that are promptly activated to return receptor density to control levels.

$G_{i\alpha 2}$ is a signaling protein that is important in many opioid receptor-mediated actions (Gomes et al., 2002; Parolaro et al., 1990; Raffa et al., 1994; Sanchez-Blazquez et al., 1995; Shen et al., 1998; Standifer et al., 1996). For example, antisense to $G_{i\alpha 2}$ reduces opioid agonist potency and inhibits the development of tolerance to many opioid agonists (Raffa et al., 1994; Stan-

difer et al., 1996; Yoburn et al., 2003). Furthermore, opioid tolerance is associated with increases in $G_{i\alpha 2}$ protein and message in mouse spinal cord (Yoburn et al., 2003). However, PTX-sensitive G-proteins and $G_{i\alpha 2}$, in particular, play little or no role in agonist and antagonist-induced regulation of μ OR density (Gomes et al., 2002; Chang et al., 1991; Zaki et al., 2001). Therefore, it was anticipated that chronic opioid antagonist treatment would not regulate G-proteins. In this study, we found no change in $G_{i\alpha 2}$ protein or mRNA abundance immediately following chronic NTX treatment. These data are consistent with suggestions that opioid receptor density upregulation does not involve G-protein coupled signaling pathways and may depend on changes in receptor trafficking (decreased constitutive cycling).

In all RT-PCR and Western studies, aliquots from the same individual protein and RNA samples were loaded on individual gels for each target of interest (GRK-2, DYN-2, $G_{i\alpha 2}$). The failure to find a change in $G_{i\alpha 2}$ protein or mRNA serves as a control for the changes noted for GRK-2 and DYN-2. These results confirm the selective effect of NTX on GRK-2 and DYN-2 that were reported previously with actin as the control (Patel et al., 2002a). Furthermore, we analyzed the protein and mRNA from individual mouse spinal cords. Samples were not pooled. Thus, variations in loading would be expected to average out over the three replicate experiments with 5–6 individual cords assayed per treatment.

Taken together, these results indicate that there is a time-dependent decline in supersensitivity and receptor upregulation following NTX treatment that is paralleled by changes in GRK-2 protein and mRNA. These results suggest a role for GRK-2 in opioid antagonist-induced μ OR upregulation in vivo. While changes in DYN-2 protein abundance do not correspond to the time-dependent changes in binding and functional supersensitivity, it is possible that decreases in DYN-2 constitute a permissive effect that is required for expression of chronic opioid antagonist-induced effects. The failure to note a change in $G_{i\alpha 2}$ mRNA and protein abundance confirms reports that pertussis toxin-sensitive G-proteins, and $G_{i\alpha 2}$ in particular, do not play an important role in opioid antagonist-induced μ OR upregulation. Thus, chronic treatment with opioid antagonists differentially regulates trafficking and signaling proteins. The regulation of trafficking proteins by chronic opioid antagonist treatment may be related to antagonism of tonic influences of endogenous opioids (see Brady et al., 1999). Finally, given the decreases in GRK-2 observed in the present report, future studies may focus on chronic antagonist-induced changes in basal μ OR phosphorylation, which may be a necessary antecedent event for constitutive internalization.

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