

Antagonist-induced μ -opioid receptor up-regulation decreases G-protein receptor kinase-2 and dynamin-2 abundance in mouse spinal cord

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Abstract

Chronic treatment with opioid receptor antagonists has been shown to increase the density of μ -, δ - and κ -opioid receptors in cell culture and in the intact animal. Although opioid receptor antagonist-induced up-regulation is a robust phenomenon, the mechanisms responsible for the increase in receptor density remain unclear. In the present study, changes in a kinase and a GTPase that have been implicated in G-protein-coupled receptor regulation were examined following opioid receptor antagonist treatment. Mice were implanted s.c. with a naltrexone pellet or placebo pellet. On the eighth day following implantation, spinal cord was removed and G-protein receptor kinase-2 (GRK-2) and dynamin-2 abundance were determined using a quantitative immunoblot approach. Changes in μ -opioid receptor density were also determined. Naltrexone treatment produced a significant (145%) increase in μ -opioid receptor density. Naltrexone treatment was associated with a significant 36% decrease in GRK-2 and 30% decrease in dynamin-2 abundance in spinal cord. These data raise the possibility that opioid receptor antagonist-induced μ -opioid receptor up-regulation in the intact animal may be due to a reduction in constitutive internalization of opioid receptors. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: μ -Opioid receptor; Naltrexone; Receptor up-regulation; Constitutive internalization; Dynamin-2; G-protein receptor kinase-2

1. Introduction

Treatment with opioid receptor agonists or antagonists can regulate opioid receptor density as well as the potency of opioid agonists. For example, chronic *in vivo* opioid receptor antagonist (e.g., naltrexone) treatment has been shown to produce a robust increase in the density of μ -, δ - and κ -opioid receptors (i.e., up-regulation) and a parallel increase in the potency of opioid agonists (i.e., functional supersensitivity) (Zukin et al., 1982, 1984; Yoburn et al., 1986, 1995). Prolonged exposure (7–8 days) to an opioid receptor antagonist is required to produce receptor up-regulation (Yoburn et al., 1986, 1989a,b), and functional supersensitivity is proportional to the degree of up-regulation (Paronis and Holtzman, 1991; Yoburn et al., 1986, 1989b, 1995).

While the density of opioid receptors reliably increases after chronic opioid receptor antagonist treatment, the origin

of the new receptors remains unclear. A concurrent change in receptor density and gene expression (i.e., steady state mRNA level) has not been reliably observed following antagonist treatment (Duttaroy et al., 1999; Castelli et al., 1997; Jenab et al., 1995; Unterwald et al., 1995; however, see Brodsky et al., 1995). The increase in receptor density without a change in receptor mRNA levels *in vivo* suggests that increases in receptor density may not require the synthesis of the new receptors. These results are consistent with findings that an antisense oligodeoxynucleotide directed at the μ -opioid receptor does not interfere with opioid receptor antagonist-induced μ -opioid receptor up-regulation *in vivo* (Shah et al., 1997). Similarly, an *in vitro* study showed that antagonist-induced up-regulation was not blocked by inhibiting protein synthesis with cycloheximide (Tempel et al., 1986). Taken together, these data suggest that it is possible that the opioid receptor antagonist-induced up-regulation may be related to activation of a pre-existing pool of “cryptic” receptors (Candido et al., 1992; Chan et al., 1995; Moudy et al., 1985; Unterwald et al., 1998) or a decrease in receptor degradation (Belcheva et al., 1991). Early preliminary data also support opioid receptor antago-

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nist-induced changes in constitutive receptor cycling (Evans et al., 1997). More recent studies with μ -opioid receptor splice variants have supported these suggestions that antagonist treatment interferes with constitutive internalization of μ -opioid receptor (Koch et al., 2001).

G-protein receptor kinase-2 (GRK) and dynamin have been shown to play an important role in opioid agonist-induced receptor internalization (Whistler and von Zastrow, 1998; Zhang et al., 1998). These data raise the possibility that opioid receptor antagonist-induced up-regulation may be associated with alterations in the abundance or activity of GRK and dynamin. Therefore, in the present study, we examined GRK-2 and dynamin-2 abundance following chronic opioid receptor antagonist treatment. We focused on the regulation of μ -opioid receptors in spinal cord, since studies demonstrate opioid receptor antagonist-induced μ -opioid receptor up-regulation in spinal cord and there is substantial support for spinal mediation of opioid effects (Shah et al., 1997).

2. Materials and methods

2.1. Subjects

Male, Swiss–Webster mice (24–28 g) from Taconic Farms (Germantown, NY) were used in all experiments. The animals were housed 10 per cage for at least 24 h prior to experimentation with free access to food and water. Mice were used only once.

2.2. Procedure

Mice were implanted subcutaneously at the nape of the neck with an inert placebo pellet or a 15 mg naltrexone pellet for 8 days while mice were lightly anesthetized with halothane/oxygen (4:96). At the end of 8 days, mice were killed and spinal cords were collected for receptor binding or Western blotting studies.

2.3. μ -Opioid receptor binding assay

Binding was performed as described previously (Yoburn et al., 1995). Briefly, mice ($n = 12$ /treatment) were sacrificed, spinal cords were removed and homogenized in 80 volumes of cold 50 mM Tris buffer (pH—7.4). Homogenates were centrifuged at 15,000 rpm for 15 min, supernatants were discarded and pellets were resuspended and incubated (30 min, 25 °C) in Tris buffer. Homogenates were centrifuged again and the pellets were finally resuspended in 50 mM phosphate buffer (pH 7.2). An aliquot of homogenate was assayed in triplicate in tubes containing 0.03–5 nM [3 H] [D-Ala², N-MePhe⁴, Gly⁵-ol] enkephalin (DAMGO) (μ -opioid receptor ligand, New England Nuclear, Boston, MA). Non-specific binding was determined in the presence of 1 μ M levorphanol. Tubes were incubated for 90 min at 25 °C and

then the incubation was terminated by filtration of samples over GF/B glass fiber filters (Brandel, Gaithersburg, MD). Filters were washed three times with cold phosphate buffer, 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 using the Bradford method (Bradford, 1976) with reagent purchased from Bio-Rad (Richmond, CA).

2.4. Western blotting

Mice ($n=12$ /treatment) were sacrificed, spinal cords were removed and homogenized (Brinkman Polytron Homogenizer, 20,000 rpm, 30 s) in 2 ml lysis buffer [2% sodium dodecyl sulfate (SDS), 1 mM sodium orthovanadate, 12.5 mM Tris (pH 7.4)], boiled for 5 min and centrifuged at 10,000 rpm for 10 min. The supernatant was removed for analysis and protein concentration was determined (Bradford, 1976). An aliquot (four spinal cords/tube) of the sample (8 μ l, 0.6–12 μ g protein) was loaded on polyacrylamide gels (Pager Gels 10% Tris–Glycine, BioWhittaker Molecular Applications, Rockland, ME) and subject to electrophoresis at 150 V for 60 min. Transfer of protein to PVDF membrane (Bio-Rad, Hercules, CA) was carried out at 100 V for 75 min. Nonspecific binding sites on the membrane were blocked by incubation (1 h, 24 °C) in blocking buffer (1.5% BSA in Tris-buffered saline with Tween-20 (TBST): (25 mM Tris, 150 mM NaCl, 0.05% Tween-20) for colorimetric assays; or AuroraTM Blocking Buffer (ICN Biomedicals, Costa Mesa, CA) for chemiluminescence assays), followed by incubation (1 h, 24 °C) with primary antibody in appropriate blocking buffer [rabbit polyclonal immunoglobulin G (IgG) for GRK-2 (1:200); goat polyclonal IgG for dynamin-2 (1:300); goat polyclonal IgG for actin (1:300)] (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed twice with blocking buffer and then incubated (1 h, 24 °C) with secondary antibody in TBST or in blocking buffer [anti-rabbit IgG-AP for GRK-2 (1:5000); anti-goat IgG-AP for dynamin-2 (1:5000); anti-goat IgG-AP for actin (1:5000)] (Santa Cruz Biotechnology). Membranes for colorimetric assays were washed twice with TBST buffer followed by two quick rinses with Tris-buffered saline (25 mM Tris, 150 mM NaCl). Membranes for chemiluminescence assays were washed thrice with blocking buffer followed by two quick rinses with assay buffer (200 mM Tris–HCl, pH 9.8; 10 mM MgCl₂). Target proteins were visualized using the Starlight alkaline phosphatase Chemiluminescent Assay (Aurora, ICN Biomedicals) according to the manufacturer's instructions. Target proteins were visualized for the colorimetric assay using 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitroblue tetrazolium color development substrate (Promega, Madison, WI) according to the manufacturer's instructions. Chemiluminescence and colorimetric results were captured using a FluorChem ver. 2.0 Imaging

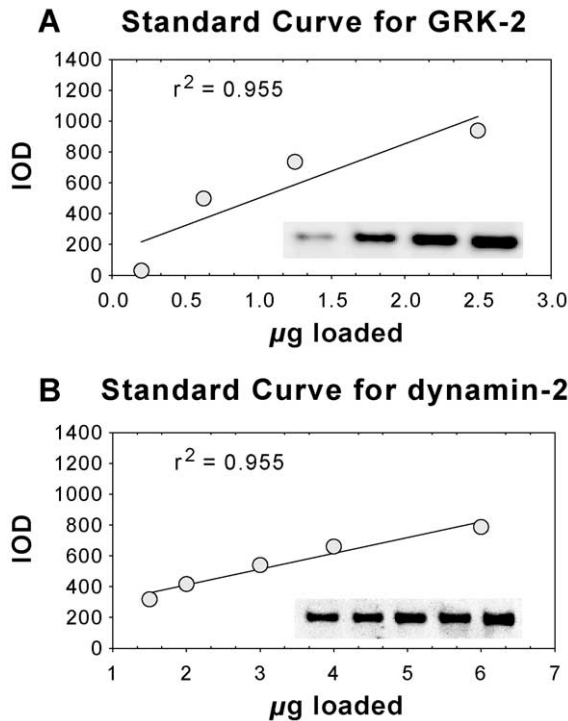


Fig. 1. Typical standard curves for GRK-2 (A) and dynamin-2 (B). Increasing amounts of total protein were loaded on polyacrylamide gels.

System (Alpha Innotech, San Leandro, CA) and images were then quantitated for optical density using GelPro ver. 3.0 (Media Cybernetics, Silver Spring, MD). All assays for GRK-2 and actin used chemiluminescence, while two assays for dynamin-2 employed the colorimetric method.

A standard curve (minimum 4 points) using increasing amounts (0.2–32 µg) of untreated spinal cord sample was included in each gel assay (dynamin-2, GRK-2, actin). This allowed conversion of optical density into valid estimates of percent changes in protein. All data are expressed in these protein equivalents. The standard curves were linear from 0.2 to 32 µg of total protein, which included the range of optical densities employed for the unknowns. Typical chemiluminescent standard curves are shown in Fig. 1A (GRK-2: mean $r^2=0.87\pm0.05$; six assays); Fig. 1B (dynamin-2: mean $r^2=0.89\pm0.05$; five assays) (actin: mean $r^2=0.98\pm0.01$; five assays, data not shown). Results from colorimetric standard curves were similar.

2.5. Drugs

Naltrexone (30 mg) and corresponding placebo pellets were obtained from Research Triangle Institute (Research Triangle Park, North Carolina) through the Research Technology Branch of the National Institute on Drug Abuse. The pellets were cut in half (yielding 15 mg implants), weighed and then implanted. The pellets were wrapped in nylon mesh before subcutaneous implantation.

2.6. Data analysis

Differences between means were assessed using Student's *t*-test ($P<0.05$). B_{MAX} and K_D were estimated from saturation studies using nonlinear regression (Prism ver. 3.02, Graphpad Software, San Diego, CA). Binding data was best fit by a one-site model.

3. Results

3.1. Effects on GRK-2 and dynamin-2 abundance

Changes in the abundance of GRK-2 and dynamin-2 in spinal cord were determined following chronic naltrexone treatment. Western blotting of SDS-solubilized spinal cord

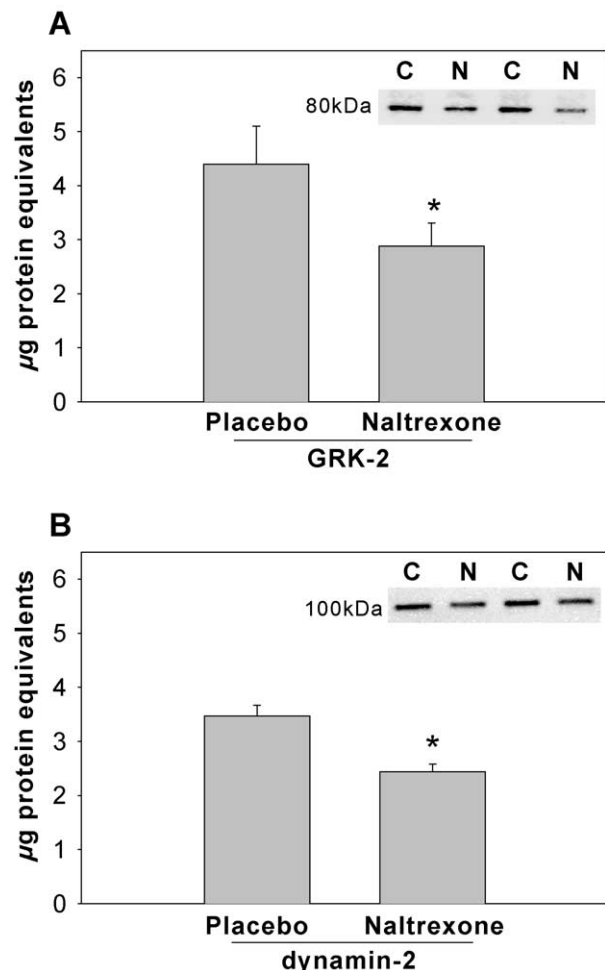


Fig. 2. The effect of chronic naltrexone on GRK-2 and dynamin-2 abundance in spinal cord. Naltrexone (15 mg) and placebo pellets were implanted subcutaneously for 8 days. At the end of the treatment, spinal cords were removed and levels of GRK-2 and dynamin-2 were determined using Western blotting. Insets are representative blots for GRK-2 (A) and dynamin-2 (B) (C=control; N=naltrexone). Data are mean \pm S.E.M. from five or six independent experiments. *Significantly different from placebo ($P<0.05$).

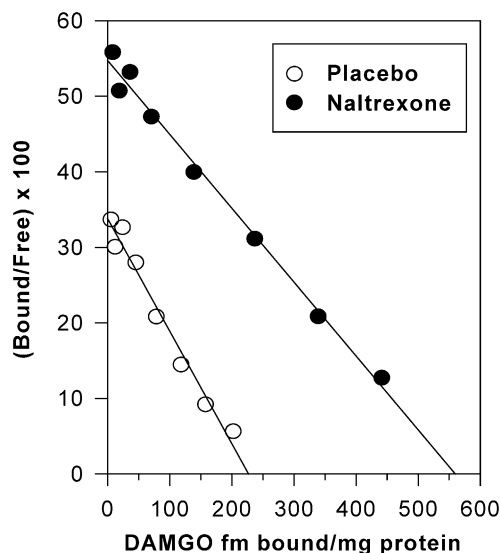


Fig. 3. Scatchard plot of specific [^3H] DAMGO binding in spinal cord homogenate from mice ($n=12$) chronically treated subcutaneously with naltrexone or placebo pellet for 8 days. The K_D and B_{MAX} for placebo: 0.92 nM, 247 ± 7 fmol/mg protein, and for naltrexone: 1.38 nM, 605 ± 4 fmol/mg protein. The results are representative data from a single experiment.

preparation indicated that chronic naltrexone treatment significantly ($P<0.05$) decreased the abundance of GRK-2 (36%) and dynamin-2 (30%) in spinal cord as compared to placebo group (Fig. 2A and B). A representative blot for each GRK-2 and dynamin-2 is shown in the insets in Fig. 2A and B. Immunoblot assay indicated no significant changes in actin abundance (data not shown).

3.2. Saturation binding study

In saturation binding, chronic naltrexone treatment produced significant ($P<0.05$) μ -opioid receptor up-regulation ($B_{\text{MAX}}=247 \pm 7$ and 606 ± 4 , control and naltrexone, respectively) without altering affinity ($K_D=0.92$ and 1.38, control and naltrexone, respectively). A typical Scatchard plot is shown in Fig. 3.

4. Discussion

In the present study, naltrexone produced a significant increase in μ -opioid receptor density in spinal cord and decreased the abundance of GRK-2 and dynamin-2. These data raise the possibility that down-regulation of cellular trafficking proteins may mediate opioid receptor antagonist-induced receptor up-regulation.

Opioid receptor antagonist-induced receptor up-regulation is a robust phenomenon in the whole animal and in cell culture (Zukin et al., 1982, 1984; Yoburn et al., 1985, 1986; Yoburn, 1988; Unterwald et al., 1995). Up-regulation of μ -opioid receptors has been demonstrated in many CNS regions (e.g., Unterwald et al., 1995), including the spinal

cord (Shah et al., 1997). Although several mechanisms have been proposed, to date, the basis of this effect has remained unknown. Among the possible substrates for opioid receptor antagonist-induced up-regulation are changes in transcription, recruitment of "cryptic" receptors and reduction in receptor degradation in association with changes in receptor cycling.

Regulatory mechanisms involving increased transcription are unlikely, since antagonist-induced μ -opioid receptor up-regulation is not associated with changes in μ -opioid receptor mRNA levels (Duttaroy et al., 1999; Castelli et al., 1997; Jenab et al., 1995; Unterwald et al., 1995; however, see Brodsky et al., 1995). Similarly, up-regulation is probably independent of translational changes since an antisense oligodeoxynucleotide to the μ -opioid receptor does not block opioid receptor antagonist-induced μ -opioid receptor up-regulation or supersensitivity in vivo (Shah et al., 1997); and the protein synthesis inhibitor, cycloheximide, does not interfere with receptor up-regulation in tissue culture (Temple et al., 1986). Taken together, these data do not support a role for gene expression or protein synthesis in antagonist-induced opioid receptor up-regulation.

Activation of a pre-existing pool of "cryptic" receptors (Candido et al., 1992; Chan et al., 1995; Moudy et al., 1985; Unterwald et al., 1998) has also been suggested to account for opioid receptor antagonist-induced up-regulation. The cryptic receptor hypothesis was addressed using combined receptor autoradiography and immunohistochemistry in brain slices (Unterwald et al., 1998). The results are generally consistent with a cryptic receptor hypothesis, since antagonist treatment induced greater magnitude increases in radioligand binding in more brain areas than in immunoreactive protein. However, there were significant increases in immunoreactive protein, and all changes in binding could not be accounted for by unmasking of cryptic receptors.

A preliminary study of opioid receptor antagonist-induced up-regulation in cells expressing μ -opioid receptors raised the possibility of a reduction in constitutive receptor cycling (Evans et al., 1997). More recently, studies of μ -opioid receptor splice variants found that naloxone may act to stabilize membrane opioid receptors by interfering with constitutive internalization (Koch et al., 2001). If opioid receptor antagonist-induced receptor up-regulation involves a change in constitutive internalization, then it might be expected that trafficking proteins would be regulated by chronic antagonist treatment. Both GRK and dynamin have been shown to be involved in internalization of the constitutively active mutants of many G-protein-coupled receptors in vitro (Mhaouty-Kodja et al., 1999; Li et al., 2001; Pei et al., 1994). Furthermore, numerous studies have shown that both GRK and dynamin play an important role in agonist-mediated receptor internalization of G-protein-coupled receptors including μ -opioid receptors (Whistler and von Zastrow, 1998; Zhang et al., 1998).

Our data demonstrating down-regulation of GRK-2 and dynamin-2 by naltrexone support the role of inhibition of constitutive cycling in up-regulation. While opioid receptor antagonist treatment may act to alter GRK-2 and dynamin-2 abundance, it is unknown if this is mediated by a change in transcription or degradation rate of these proteins. Furthermore, we recognize that several different mechanisms might contribute simultaneously to opioid receptor antagonist-induced receptor up-regulation in vivo. For example, activation of a pre-existing pool of cryptic receptors (Candido et al., 1992; Chan et al., 1995; Moudy et al., 1985; Unterwald et al., 1998) or a decrease in receptor degradation (Belcheva et al., 1991) are also likely candidates that would support up-regulation.

In summary, down-regulation of GRK-2 and dynamin-2 abundance as demonstrated in the present study may help mediate opioid receptor antagonist-induced up-regulation. In the future, it would be of interest to examine the effect of regulating GRK-2 and dynamin-2 either alone or together on μ -opioid receptor density and functional potency of opioids.

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