MOL PHARM

MOLECULAR PHARMACOLOGY

Opioid Agonists Differentially Regulate μ-Opioid Receptors and Trafficking Proteins in Vivo

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Received June 3, 2002; accepted September 18, 2002   This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Chronic opioid agonist treatment produces tolerance and in some cases opioid receptor internalization and down-regulation. Both morphine and etorphine induce tolerance; however, only etorphine produces μ-opioid receptor (μOR) down-regulation. In vitro studies implicate dynamin-2 (DYN-2) and G-protein receptor kinase-2 (GRK-2) in these processes. Therefore, we examined etorphine and morphine effects on regulation of GRK-2 and DYN-2 in mouse spinal cord. Mice were treated for 7 days with etorphine (200 μg/kg/day infusion) or morphine (40 mg/kg/day infusion + one 25-mg implant pellet). Controls were implanted with a placebo pellet. On the 7th day after implantation mice were tested for i.t. [D-Ala^2,N-Me-Phe^4,Gly^5-ol]-enkephalin (DAMGO) analgesia. In other mice, spinal cord was removed for [^{3}H]DAMGO binding studies or GRK-2 and DYN-2 protein and mRNA abundance were determined. Both etorphine and morphine produced significant tolerance (ED$_{50}$ shift = 7.6- and 7.3-fold for morphine and etorphine, respectively). Etorphine decreased spinal μOR density by ≈30%, whereas morphine did not change μOR density. Etorphine increased (≈70%) DYN-2 protein abundance and decreased its mRNA (31%), whereas it had no effect on GRK-2 protein and mRNA abundance. Morphine had no effect on either DYN-2 or GRK-2 protein or mRNA abundance. These data raise the possibility that unequal receptor regulation by etorphine and morphine might be due to differential regulation of trafficking proteins. Overall, receptor down-regulation associated with chronic etorphine treatment may accelerate dynamin-related activity. Finally, the decrease in DYN-2 mRNA may be related to stabilization of DYN-2 protein abundance, which might inhibit transcription.

Opioid agonists have been shown to produce tolerance and in some cases internalization and down-regulation of opioid receptors (Duttaroy and Yoburn, 1995; Keith et al., 1996; Yabaluri and Medzihradsky, 1997; Whistler et al., 1999; Shen et al., 2000; Zaki et al., 2000). The ability of opioid agonists to regulate μOR has been suggested to be related to the intrinsic efficacy of the agonist. For example, chronic treatment with low intrinsic efficacy agonists (e.g., morphine) may cause blunting of receptor signaling (desensitization) without any change in surface receptor number (Yoburn et al., 1993; Burford et al., 1998; Van Bockstaele and Commons, 2001). Conversely, chronic exposure to high intrinsic efficacy opioid agonists (e.g., etorphine) can internalize and down-regulate opioid receptors, as well as produce desensitization (Duttaroy and Yoburn, 1995; Yabaluri and Medzihradsky, 1997; Whistler et al., 1999; Shen et al., 2000; Zaki et al.,

2000). Internalization of μOR has also been noted after acute etorphine and opioid peptide treatment (Trafton et al., 2000; Van Bockstaele and Commons, 2001). Although decreases in receptor density depend upon the agonist used, recent studies suggest that down-regulation of μOR, although not required for opioid tolerance, can contribute to opioid tolerance (Stafford et al., 2001).

Chronic agonist treatment has been shown to regulate G-protein-coupled receptor (GPCR) density in cell culture studies. Agonist-induced activation of G-protein receptor kinase (GRK) seems to precede receptor phosphorylation. Once the receptor has been phosphorylated, β-arrestin translocates to the plasma membrane and uncouples the receptor/G-protein complex, which may be followed by congregation of receptors in clathrin-coated pits and subsequent dynamin (DYN)-mediated internalization (Carman and Benovic, 1998; Krupnick and Benovic, 1998). Both GRK-2 and DYN-2 have been shown to play an important role in agonist-mediated internalization of μOR (Zhang et al., 1998; Whistler and von Zastrow, 1998). Dominant negative mutants of GRK-2 and DYN-2 inhibit agonist-induced internalization of μOR (Whistler and von Zastrow, 1998; Zhang et al., 1998; Lazari et al., 1999; Gaborik et al., 2001). Furthermore, overexpression of

ABBREVIATIONS: μOR, μ-opioid receptor; GPCR, G-protein-coupled receptor; GRK, G-protein receptor kinase; DYN, dynamin; DAMGO, [D-Ala^2,N-MePhe^4,Gly^5-ol]-enkephalin; bp, base pair(s).

GRK-2 or β-arrestin 2 confers on morphine the ability to induce receptor internalization in vitro (Whistler and von Zastrow, 1998; Zhang et al., 1998). It is possible that some agonists (e.g., etorphine) may effectively regulate these trafficking proteins more than other agonists (e.g., morphine).

Differences in regulation of opioid receptor density by agonists could be due to variations in the characteristics of the agonist-receptor conformation. For example, morphine, DAMGO, and endomorphin I activate cognate heterotrimeric G-proteins with the same profile; however, only DAMGO and endomorphin I induced μOR internalization (Burford et al., 1998). Furthermore, μOR down-regulation in vivo has been demonstrated to be independent of functional G-protein signaling (Gomes et al., 2002). These results suggest that different agonists activate G-proteins similarly but that some agonists render receptors into conformations that are more suitable as substrates for trafficking proteins (e.g., β-arrestin, GRK, and DYN) (Burford et al., 1998; Whistler and von Zastrow, 1998).

Taken together, these data support suggestions that proteins identified in cell culture studies, such as GRK-2 and DYN-2, may play an important role in trafficking of μOR in vivo. In the present study, we explored whether opioid agonists that differ in their ability to regulate μOR also differ in regulation of trafficking proteins in vivo. To our knowledge this is the first in vivo study that examines regulation of μOR and intracellular trafficking proteins by agonists of different intrinsic efficacy.

Materials and Methods

Subjects. Male Swiss-Webster mice (22–24 g) (Taconic Farms, Germantown, NY) were used in all experiments. The animals were housed 10 per cage for at least 24 h with free access to food and water before experimentation. Mice were used only once.

General Procedure. Mice were implanted subcutaneously with an osmotic minipump (ALZET model 2001; Alza, Palo Alto, CA) that infused etorphine (200 μg/kg/day, 1 μl/h). For morphine, mice were implanted subcutaneously with an osmotic minipump that infused morphine (40 mg/kg/day) plus one 25-mg implant pellet. The morphine implant pellet and the minipump infusion protocol were based on previous studies that demonstrated that this treatment produced reliable and significant tolerance (Stafford et al., 2001). All controls were implanted with inert placebo pellets. The pumps and pellets were implanted at the nape of the neck while mice were lightly anesthetized with halothane/oxygen (4:96). Seven days after etorphine, morphine, or placebo treatment, mice were tested for antinociception or sacrificed and spinal cords were collected for receptor binding studies, Western blotting, or for reverse transcription-polymerase chain reaction (RT-PCR) assays. In the case of antinociception and binding studies, pumps and pellets were removed on day 7 and 4 h after morphine and 16 h after etorphine assays were conducted.

μ-Opioid Receptor Binding Assay. Binding was performed as described by Yoburn et al. (1995). Briefly, pumps and pellets were removed on day 7 of etorphine, morphine, or placebo treatment. Mice (n = 12/treatment) were sacrificed 4 h after termination of morphine and 16 h after termination of etorphine treatment (Yoburn et al., 1993). Controls were treated similarly. Spinal cords were removed and homogenized in 80 volumes of ice-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 for 30 min at 25°C in ice-cold 50 mM Tris buffer (pH 7.4). Homogenates were centrifuged again and the pellets were finally resuspended in 20 to 80 volumes of 50 mM phosphate buffer (pH 7.2).

An aliquot (200 μl) of homogenate was assayed in triplicate in tubes containing 0.03 to 5 nM [³H]DAMGO (μ-ligand; PerkinElmer Life Sciences, Boston, MA). Nonspecific binding was determined in the presence of 1000 nM 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 Inc., Gaithersburg, MD). Filters were washed three times with cold phosphate buffer and transferred to vials containing scintillation cocktail and then counted. The cpm values were converted to dpm values using the external standard method. Protein was assayed by the Bradford method (Bradford, 1976) with reagent purchased from Bio-Rad (Hercules, CA).

Analgesia Assay. Analgesia (antinociception) was determined using the tail-flick assay in which a beam of light was focused on the dorsal tail surface approximately 2 cm from the tip of the tail. The intensity of the light was adjusted so that baseline tail-flick latencies were 2 to 4 s. Mice (n = 6/dose/treatment) were then briefly anesthetized using halothane and injected intrathecally with DAMGO (0.2–15 ng/4 μl/mouse for placebo; 3.8–60 ng/4 μl/mouse for etorphine; and 15–120 ng/4 μl/mouse for morphine) and tested for analgesia 30 min later. If a mouse failed to flick by 10 s after DAMGO administration, the test was terminated and a latency of 10 s was recorded. Mice that had a latency of 10 s were defined as analgesic. All testing was conducted in a blind manner.

Western Blotting. Mice (n = 12/treatment) were sacrificed 7 days after etorphine, morphine, or placebo treatment. Spinal cords were removed and homogenized (Polytron homogenizer at 20,000 rpm for 30 s) in 2 ml of lysis buffer (2% SDS, 1 mM sodium orthovanadate, and 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 μl, 0.6–12 μg of protein) was loaded on polyacrylamide gels (Pager Gels 10% TG; BioWhittaker, Rockland, ME) and samples were separated by electrophoresis (150 V for 60 min). Proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad) using the miniprotean II (Bio-Rad) at 100 V for 75 min. Nonspecific binding sites on the membrane were blocked by incubation (1 h, 24°C) in blocking buffer [0.2% Aurora Blocking Reagent (ICN Pharmaceuticals, Costa Mesa, CA), 1× phosphate-buffered saline, and 0.1% Tween 20) 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, 24°C) with secondary antibody in blocking buffer [anti-rabbit IgG-alkaline phosphatase for GRK-2 (1:5000); anti-goat IgG-alkaline phosphatase for DYN-2 (1:5000); Santa Cruz Biotechnology]. Membranes were then washed three times with blocking buffer followed by two quick rinses with assay buffer (200 mM Tris-HCl, pH 9.8, and 10 mM MgCl₂). Membranes were then incubated for 5 min in Chemiluminescent Substrate Solution containing Optimembrane (150 μl Optimembrane in 3 ml of Starlight ready-to-use solution; ICN Pharmaceuticals). Target proteins were visualized by capturing chemiluminescence using a FluorChem version 2.0 Imaging System (Alpha Innotech Corporation, San Leandro, CA). The images were digitized and analyzed for optical density using GelPro image analysis software (version 3.0; Media Cybernetics, Silver Spring, MD).

A standard curve using increasing amounts (0.2–20 μg) of spinal cord sample was included in each gel assay. This allowed conversion of optical density into valid estimates of percentage of changes in protein. All data are expressed as percentage of control.

RT-PCR Assay. Total RNA was extracted from mouse spinal cord (n = 3/treatment) using TRIzol reagent (Invitrogen, Carlsbad, CA) and precipitated in 100% isopropyl alcohol. Yeast tRNA (25 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. 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 and 280 nm).

The RT-PCR assay was based on the Thermostable rTth RNA PCR kit (Applied Biosystems, Foster City, CA). The primers for mouse DYN-2 were CAG TTT GGA GTG GAC TTT GAG AAGC (forward) and AGA CAG GGC TCT TTC AGC TTG ACC (backward) corresponding to bases 131 to 155 and 1379 to 1402 (272-bp product). The primers for mouse GRK-2 were AGA AGT ATC TGG AGG ACC GAGG (forward) and TCT TTG AAA AGG GAT GTG AGC AGGC (backward) corresponding to bases 131 to 152 and 355 to 379 (249-bp product).

The reverse transcription reaction used 0.18 μg of spinal RNA. The initial reaction mixture contained backward primers 1 μM for murine DYN-2 and 0.75 μM for GRK-2 mRNAs, 200 μM dNTPs, 1 mM $MnCl_2$, and 5 units of rTth DNA polymerase in 10× rTth reverse transcriptase buffer (100 mM Tris-HCl, pH 8.3, and 900 mM KCl). Total volume was brought to 10 μl with diethyl pyrocarbonate water. Tubes were then placed in a Techne Progene thermal cycler (Techne, Princeton, NJ) at 70°C for 10 min for reverse transcription reaction. Next, 10× chelating buffer [4% (v/v) glycerol, 8 mM Tris-HCl, pH 8.3, 0.08 M KCl, 0.04% (w/v) Tween 20, 0.6 mM EGTA, and 20 mM $MgCl_2$], 25 mM $MgCl_2$, and forward primers 1 μM for DYN-2 and 0.75 μM for GRK-2 were added into the reaction tubes. Total volume was brought to 50 μl with diethyl pyrocarbonate water. PCR was then carried out in the thermocycler under the following conditions: a 60-s incubation at 95°C, followed by amplification for 35 cycles at 95°C for 10 s and 60°C for 15 s, followed by a final extension at 60°C for 7 min. Tubes were brought to 4°C, 1 μl of loading dye (Ambion) was added to each tube, and then a 5-μl aliquot containing amplified product was loaded on 1.8% agarose gel and electrophoresed (100 V, 55 min). DNA products were then visualized with UV light and captured using FluorChem version 2.0 Imaging System (Alpha Innotech Corporation). The RT-PCR assay yielded the predicted size amplification products for DYN-2 (272 bp) and GRK-2 (249 bp). The gel image was digitized (Gel-Pro version 3.0) and bands analyzed for optical density. A standard curve for DYN-2 and GRK-2 mRNA using 0.05, 0.1, 0.2, and 0.4 μg of total RNA was included in each assay so as to reliably estimate percentage of changes in target mRNAs. The standard curves were used to convert optical densities to arbitrary RNA units for both DYN-2- and GRK-2-amplified products.

Drugs and Chemicals. Etorphine hydrochloride, DAMGO, morphine pellets, and corresponding placebo pellets were obtained from Research Triangle Institute (Research Triangle Park, NC) through the Research Technology Branch of the National Institute on Drug Abuse. Morphine sulfate was obtained from Penick Laboratories (Newark, NJ). [3H]DAMGO was obtained from PerkinElmer Life Sciences (Boston, MA). Etorphine and morphine were dissolved in 0.9% saline. The pellets were wrapped in nylon mesh before subcutaneous implantation.

Data Analysis. Western blot analysis and RT-PCR data were analyzed by t tests. Quantal dose-response data were analyzed by Probit Analysis using a computerized program (BLISS 21; Department of Statistics, University of Edinburg, Edingburg, Scotland) that estimated ED_{50} values (95% confidence interval) and relative potencies. B_{max} and K_D were estimated from saturation studies using nonlinear regression (Prism version 3.02; GraphPad Software, San Diego, CA). Binding data were best fit by a one-site model.

Results

Saturation Binding Study. In saturation binding studies, chronic etorphine treatment produced significant μOR down-regulation (31%) without altering affinity (Fig. 1). The reduction in B_{max} with no change in K_D suggests that residual agonist is not present in the binding assay. Morphine treatment did not produce any change in μOR density (Fig.

1). Typical Scatchard plots are shown in Fig. 1. Similar results were observed in other independent studies.

DAMGO Potency. After termination of etorphine and morphine treatment, baseline tail-flick latencies were determined before i.t. DAMGO injection. There were no significant ($p > 0.05$) differences in the baseline tail-flick latencies among all groups (mean ± S.E.M. for placebo, etorphine, and morphine is 1.9 ± 0.1, 1.9 ± 0.1, and 1.7 ± 0.1 s, respectively). Seven days of treatment with both etorphine (ED_{50} shift = 7.3-fold) and morphine (ED_{50} shift = 7.6-fold) caused a reduction in i.t. DAMGO analgesic potency (Fig. 2).

Effects on GRK-2 and DYN-2 Abundance. Changes in the abundance of GRK-2 and DYN-2 in spinal cord were determined after chronic etorphine and morphine treatment. Standard curves for GRK-2 (mean ± S.E.M., r^2 = 0.96 ±
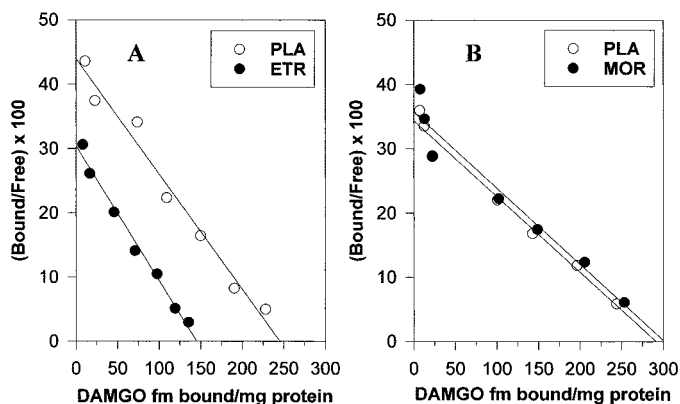

Fig. 1. Scatchard plot of specific [3H]DAMGO binding in spinal cord homogenate from mice (n = 12/group) chronically treated subcutaneously with etorphine, morphine, or placebo for 7 days. A, effect of etorphine on μOR density: B_{max} ± S.E.M. = 251 ± 9; 149 ± 4 fmol/mg protein, K_D ± S.E.M. = 0.6 ± 0.07; 0.5 ± 0.04 nM, placebo, and etorphine, respectively. B, effect of morphine on μOR density: B_{max} ± S.E.M. = 299 ± 7; 310 ± 11 fmol/mg protein, K_D ± S.E.M. = 0.9 ± 0.06; 0.9 ± 0.08 nM, placebo, and morphine, respectively. The results are representative data from one of two (morphine) or three (etorphine) experiments. PLA, placebo; ETR, etorphine; MOR, morphine.
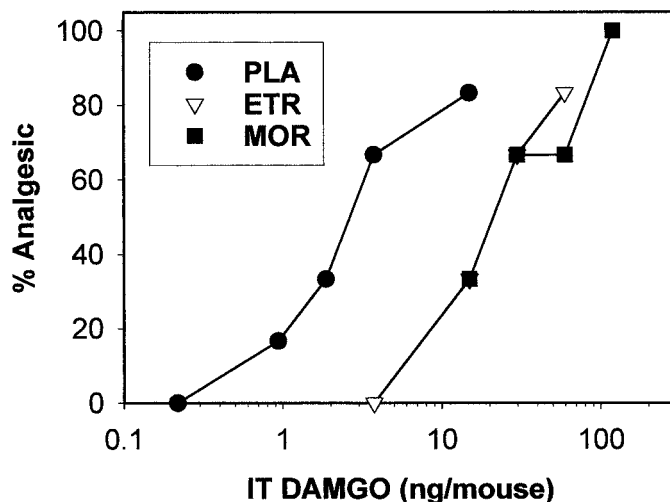

Fig. 2. Effects of etorphine and morphine on i.t. DAMGO analgesic potency. Mice (n = 6/dose/treatment) were treated with etorphine (200 μg/kg/day), morphine (40 mg/kg/day + one 25-mg pellet) or placebo pellets for 7 days. Sixteen hours after etorphine treatment and 4 h after morphine treatment i.t. DAMGO dose-response studies were conducted. The results are representative data from a single experiment. PLA, placebo; ETR, etorphine; MOR, morphine).

0.03, 12 assays) and DYN-2 (mean ± S.E.M., $r^2 = 0.95 ±$ 0.05, 10 assays) Western blotting assays were linear and included the range of optical densities for the unknowns. Chronic etorphine treatment significantly increased the abundance of DYN-2 ($\approx 70\%$) in spinal cord, whereas it had no effect on the abundance of GRK-2 compared with placebo group (Fig. 3). Morphine treatment had no effect on either DYN-2 or GRK-2 abundance (Fig. 4). A representative blot for GRK-2 and DYN-2 after etorphine and morphine treatment is shown in the inset of Figs. 3 and 4.

Changes in DYN-2 and GRK-2 mRNA Levels. Effect of chronic etorphine and morphine treatment on DYN-2 and GRK-2 mRNA levels was determined using RT-PCR. Standard curves were linear and included the range of optical densities for unknowns (GRK-2: mean ± S.E.M. $r^2 = 0.98 ±$ 0.03, seven assays; DYN-2: mean ± S.E.M. $r^2 = 0.97 ± 0.01$, six assays). RT-PCR analysis indicated that chronic etorphine treatment significantly ($p < 0.05$) reduced DYN-2

mRNA abundance (31%) in mouse spinal cord but had no effect on GRK-2 mRNA (Fig. 5). Morphine treatment had no effect on either DYN-2 or GRK-2 mRNA (Fig. 6).

Discussion

Cell culture studies have implicated GRK-2 and DYN in the trafficking of GPCRs, including μOR (Gagnon et al., 1998; Kato et al., 1998; Whistler and von Zastrow, 1998; Zhang et al., 1998; Lazari et al., 1999; Li et al., 2000; Gaborik et al., 2001). Although dominant negative mutants of GRK-2 and DYN-2 inhibited opioid agonist-induced internalization of μOR (Zhang et al., 1998; Whistler and von Zastrow, 1998; Li et al., 2000), the significance of these effects in the whole animal remains unknown. In support of the role of these trafficking proteins in μOR receptor regulation in vivo, a recent study demonstrated that chronic opioid antagonist-induced up-regulation of μOR was associated with a reduc-
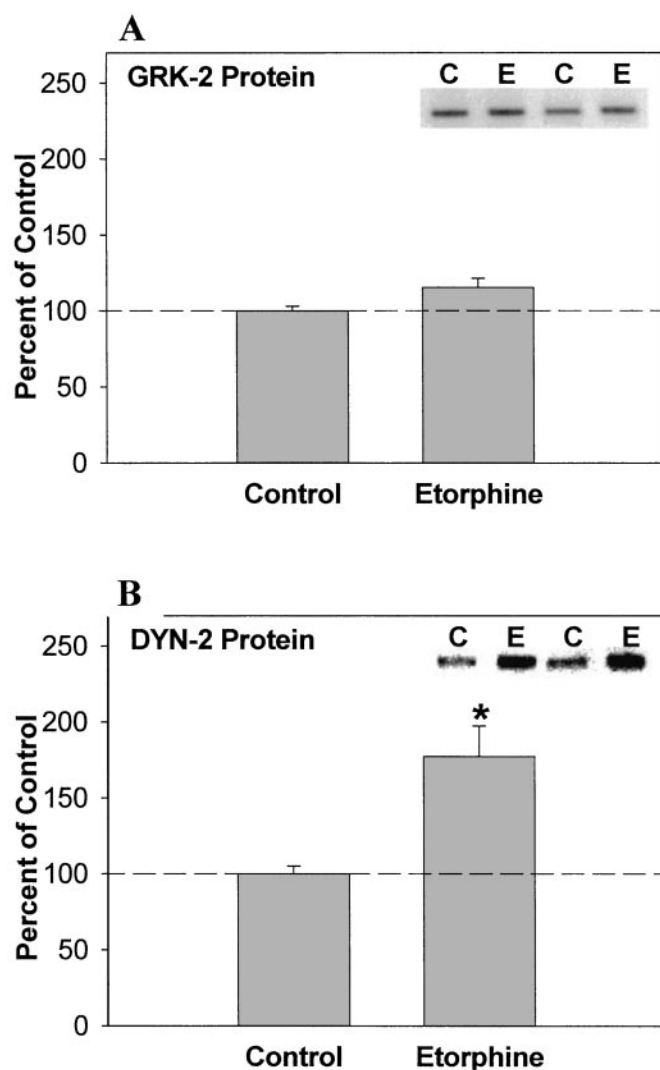

Fig. 3. Effect of chronic etorphine on GRK-2 (A) and DYN-2 (B) abundance in spinal cord. Mice were treated with etorphine (200 μg/kg/day) or placebo for 7 days. At the end of treatment spinal cords were removed and levels of GRK-2 and DYN-2 were determined using Western blotting. Insets are representative blots for GRK-2 and DYN-2 (C, control; E, etorphine). Data are mean ± S.E.M. from five independent experiments. *, Significantly different from placebo ($p < 0.05$).
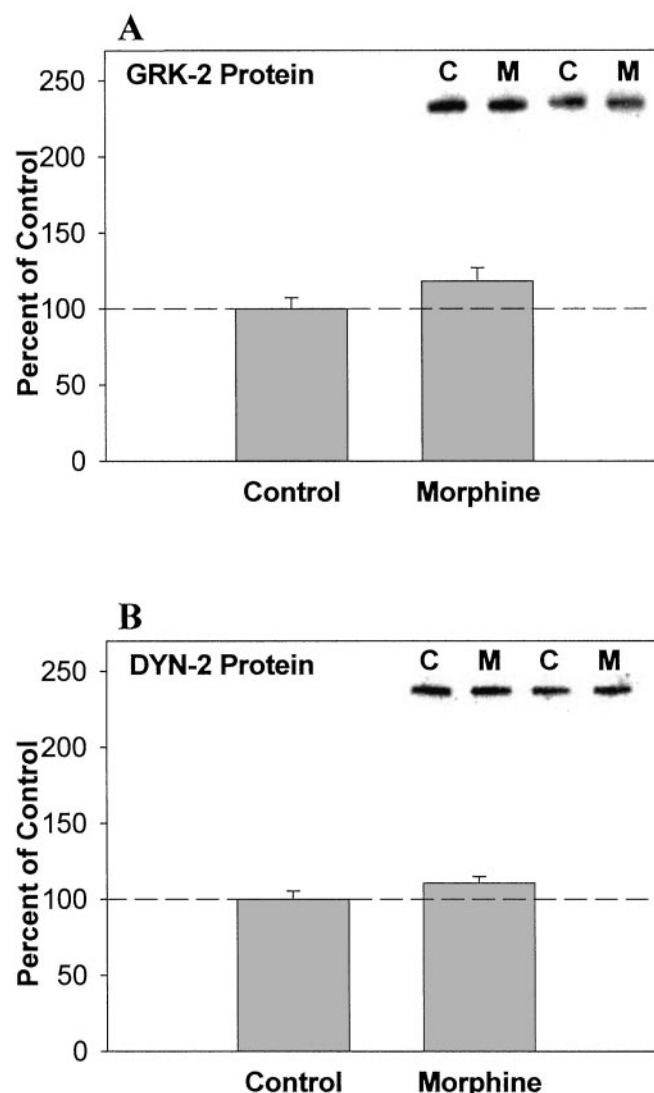

Fig. 4. Effect of chronic morphine on GRK-2 (A) and DYN-2 (B) abundance in spinal cord. Mice were treated with morphine (40 mg/kg/day + one 25-mg pellet) or placebo for 7 days. At the end of treatment spinal cords were removed and levels of GRK-2 and DYN-2 were determined using Western blotting. Insets are representative blots for GRK-2 and DYN-2 (C, control; M, morphine). Data are mean ± S.E.M. from five or more independent experiments.

tion in GRK-2 and DYN-2 protein abundance in vivo (Patel et al., 2002). However, the mechanisms involved in opioid receptor regulation after opioid agonist and antagonist treatment are different. For example, opioid agonist treatment is associated with changes in gene expression of opioid receptors (Kim et al., 1995; Sehba et al., 1997; Duttaroy and Yoburn, 2000) but antagonist treatment is not (Jenab et al., 1995; Unterwald et al., 1995; however, see Brodsky et al., 1995; Castelli et al., 1997; Duttaroy et al., 1999). Thus, it was not certain that GRK-2 and DYN-2 would be involved in opioid agonist-induced μOR regulation in vivo.

The present results indicate that differential regulation of DYN-2 by etorphine and morphine may account for differences in agonist-induced μOR regulation. Etorphine-induced down-regulation was associated with an increase in spinal DYN-2 protein and a decrease in DYN-2 mRNA. Etorphine

did not change GRK-2 protein or mRNA abundance. On the other hand, morphine, which does not down-regulate μOR density in intact animals (Yoburn et al., 1993), had no effect on either DYN-2 or GRK-2 protein or mRNA abundance. Both agonists produced tolerance to spinal DAMGO. Because DYN is involved in down-regulation of many GPCRs, including opioid receptors (Gagnon et al., 1998; Li et al., 2000), the increase in DYN-2 levels after chronic etorphine treatment may accelerate sequestration of receptors and subsequent receptor degradation. The reduction in DYN-2 mRNA after etorphine treatment may be related to stabilization and increased abundance of DYN-2 protein, followed by feedback inhibition of transcription.

Although opioid agonists produce tolerance, it has been suggested that only high intrinsic efficacy agonists produce
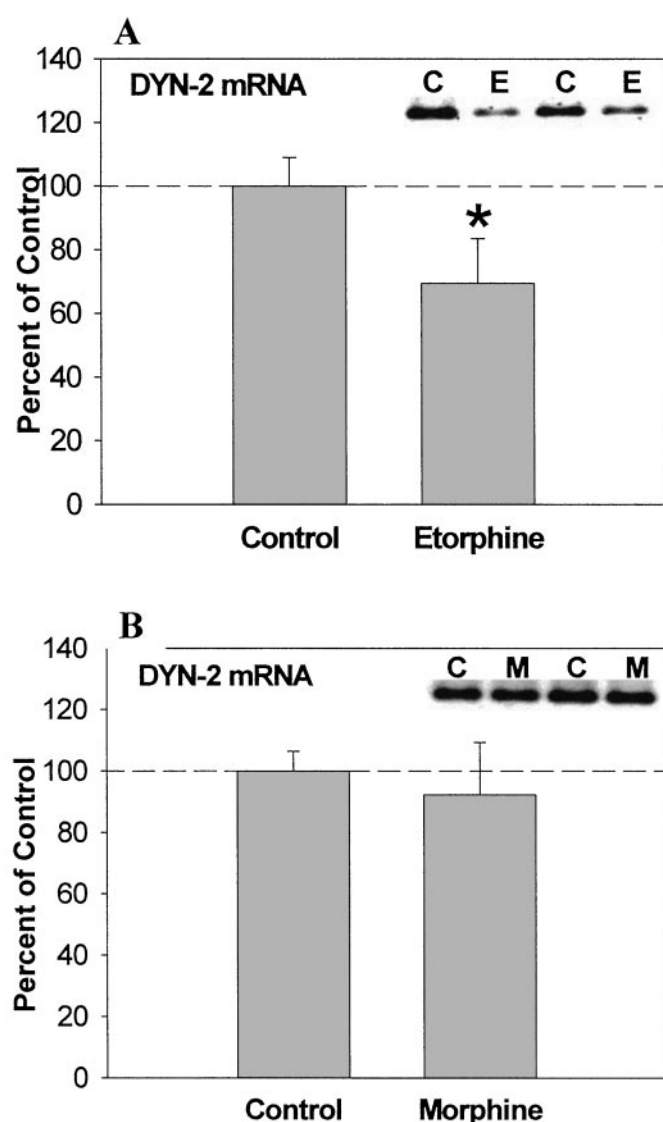

Fig. 5. Effect of chronic etorphine (A) and morphine (B) on DYN-2 mRNA abundance in spinal cord. Mice were treated with etorphine (200 μg/kg/day), morphine (40 mg/kg/day + one 25-mg pellet), or placebo for 7 days. At the end of treatment spinal cords were removed and mRNA levels of DYN-2 were determined using RT-PCR. Insets are representative blots for DYN-2 after etorphine and morphine (C, control; E, etorphine; M, morphine). Data are mean ± S.E.M. from three independent experiments. * Significantly different from placebo ($p < 0.05$).
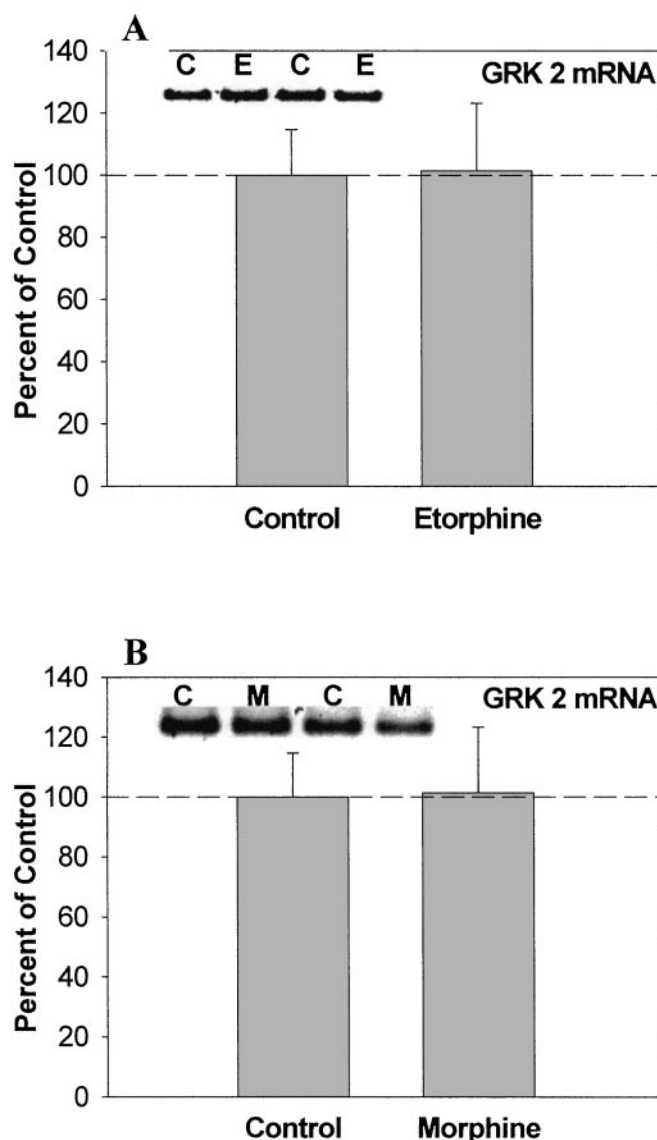
Fig. 6. Effect of chronic etorphine (A) and morphine (B) on GRK-2 mRNA abundance in spinal cord. Mice were treated with etorphine (200 μg/kg/day), morphine (40 mg/kg/day + one 25-mg pellet) or placebo for 7 days. At the end of treatment spinal cords were removed and mRNA levels of GRK-2 were determined using RT-PCR. Insets are representative blots for GRK-2 after etorphine and morphine (C, control; E, etorphine; M, morphine). Data are mean ± S.E.M. from three to four independent experiments.

opioid receptor internalization and down-regulation (Yoburn et al., 1993; Duttaroy and Yoburn, 1995; Yabaluri and Medzi-hradsky, 1997; Burford et al., 1998; Shen et al., 2000; Zaki et al., 2000; Stafford et al., 2001; however, see Alvarez et al., 2002). The present results support the role for intrinsic efficacy in μOR regulation, although morphine and etorphine differ in several other respects (e.g., receptor selectivity and kinetics) in addition to intrinsic efficacy. Nevertheless, several cellular mechanisms might mediate the differences in opioid receptor regulation by opioid agonists. For example, different agonist-receptor conformations may render the receptor a weak or strong substrate for trafficking proteins such as GRK-2 and β-arrestin (Whistler and von Zastrow, 1998; Zhang et al., 1998). Recently, an in vivo study has demonstrated that G-protein signaling is not required for agonist-induced down-regulation of μOR (Gomes et al., 2002). These results suggest that even if morphine and etorphine equivalently activate μOR signaling, receptor conformations induced by some opioid agonists (e.g., etorphine) may be better substrates for trafficking proteins than others (Whistler and von Zastrow, 1998; Zhang et al., 1998).

The present data are consistent with suggestions that certain agonist-receptor conformations are superior substrates for trafficking proteins. However, up-regulation of DYN-2 by etorphine suggests that internalization and down-regulation may be accelerated by increases in some trafficking proteins. The failure of morphine to alter GRK-2, DYN-2, or μ-opioid receptor density, raises the possibility that opioid agonist-induced μOR down-regulation in the mouse spinal cord may require differential regulation of trafficking proteins (Patel et al., 2002). The failure of etorphine and morphine to regulate GRK-2 does not necessarily rule out a role for this protein in either tolerance or μ-receptor down-regulation. It is possible that sufficient levels of GRK-2 are present to support receptor internalization after etorphine, but that receptor trafficking would be inhibited by GRK-2 depletion. This suggestion is supported by decreases in GRK-2 associated with opioid antagonist-induced μOR up-regulation (Patel et al., 2002).

Although GRK-mediated phosphorylation may be required for internalization of opioid receptors (Zhang et al., 1998; Hasbi et al., 2000), it is not known whether phosphorylation is obligatory for lysosomal degradation of opioid receptors. Whistler et al. (2001) have shown that trafficking of mutant δ-opioid receptors to lysosomes does not require phosphorylation of the receptor protein, even though phosphorylation controls endocytic trafficking of mutant δ-opioid receptors. In addition, Burd et al. (1998) have proposed that phosphorylation is not an obligatory event for etorphine-induced down-regulation. In that study, agonist-mediated down-regulation of the mutated μOR was attenuated compared with wild-type μOR. However, phosphorylation of the mutated μOR was similar to that of the wild-type μOR. In the present study, failure of etorphine to regulate GRK-2 further supports the notion that phosphorylation might not be an obligatory event for etorphine-induced μOR down-regulation in mouse spinal cord. However, this does not rule out opioid agonist-induced changes in GRK-2 in supraspinal regions. Previous studies have reported increases in some GRKs in some rat brain regions after development of tolerance (Terwilliger et al., 1994; Hurle, 2001). The differences between our results and

these previous reports could be due to the central nervous system region studied or the species differences.

In summary, the present study indicates that opioid agonists that differentially regulate μOR also selectively induce changes in expression and abundance of a protein involved in μOR internalization/down-regulation. Agonist-induced down-regulation may be mediated by an increase in dynamin-related activity that accelerates receptor down-regulation. In the future, it would be of interest to examine the effect of directly regulating dynamin activity on μOR density and the functional potency of opioids in the whole animal.

References

Alvarez VA, Arttamangkul S, Dang V, Salem A, Whistler JL, Von Zastrow M, Grandy DK, and Williams JT (2002) μ-Opioid receptors: ligand-dependent activation of potassium conductance, desensitization and internalization. *J Neurosci* **22:**5769–5776.
Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:**248–254.
Brodsky M, Elliott K, Hynansky A, and Inturrisi CE (1995) CNS levels of mu-opioid receptor (MOR-1) mRNA during chronic treatment with morphine or naltrexone. *Brain Res Bull* **38:**135–141.
Burd AL, El-Kouhen R, Erickson LJ, Loh HH, and Law PY (1998) Identification of serine 356 and serine 363 as the amino acids involved in etorphine-induced down-regulation of the μ-opioid receptor. *J Biol Chem* **273:**34488–34495.
Burford NT, Tolbert LM, and Sadee W (1998) Specific G protein activation and μ-opioid receptor internalization caused by morphine, DAMGO and endomorphine I. *Eur J Pharmacol* **342:**123–126.
Carman CV and Benovic JL (1998) G-protein-coupled receptors: turn-ons and turn-offs. *Curr Opin Neurobiol* **8:**335–344.
Castelli MP, Melis M, Mameli M, Fadda P, Diaz G, and Gessa GL (1997) Chronic morphine and naltrexone fail to modify μ-opioid receptor mRNA levels in the rat brain. *Brain Res Mol Brain Res* **45:**149–153.
Duttaroy A and Yoburn BC (1995) The effect of intrinsic efficacy on opioid tolerance. *Anesthesiology* **82:**1226–1236.
Duttaroy A, Shen J, Shah S, Chen B, Sehba F, Carroll J, and Yoburn BC (1999) Opioid receptor upregulation in μ-opioid receptor deficient CXBK and outbred Swiss Webster mice. *Life Sci* **65:**113–123.
Duttaroy A and Yoburn BC (2000) In vivo regulation of μ-opioid receptor density and gene expression in CXBK and outbred swiss webster mice. *Synapse* **37:**118–124.
Gaborik Z, Szaszak M, Szidonya L, Balla B, Paku S, Catt KJ, Clark AJ, and Hunyady L (2001) β-Arrestin- and dynamin-dependent endocytosis of the AT1 angiotensin receptor. *Mol Pharmacol* **59:**239–247.
Gagnon AW, Kallal L, and Benovic JL (1998) Role of clathrin-mediated endocytosis in agonist-induced down-regulation of the β2-adrenergic receptor. *J Biol Chem* **273:**6976–6981.
Gomes BA, Shen J, Stafford K, Patel M, and Yoburn BC (2002) μ-Opioid receptor down-regulation and tolerance are not equally dependent upon G-protein signaling, *Pharmacol Biochem Behav* **72:**273–278.
Hasbi A, Allouche S, Sichel F, Stanasila L, Massotte D, Landemore G, Polastron J, and Jauzac P (2000) Internalization and recycling of δ-opioid receptor are dependent on a phosphorylation-dephosphorylation mechanism. *J Pharmacol Exp Ther* **293:**237–247.
Hurle MA (2001) Changes in the expression of G protein-coupled receptor kinases and β-arrestin 2 in rat brain during opioid tolerance and supersensitivity. *J Neurochem* **77:**486–492.
Jenab S, Kest B, and Inturrisi CE (1995) Assessment of delta opioid antinociception and receptor mRNA levels in mouse after chronic naltrexone treatment. *Brain Res* **691:**69–75.
Kato S, Fukuda K, Morikawa H, Shoda T, Mima H, and Mori K (1998) Adaptations to chronic agonist exposure of μ-opioid receptor-expressing Chinese hamster ovary cells. *Eur J Pharmacol* **345:**221–228.
Keith DE, Murray SR, Zaki PA, Chu PC, Lissin DV, Kang L, Evans CJ, and von Zastrow M (1996) Morphine activates opioid receptors without causing their rapid internalization. *J Biol Chem* **9:**19021–19024.
Kim DS, Chin H, and Klee WA (1995) Agonist regulation of the expression of the delta opioid receptor in NG108-15 cells. *FEBS Lett* **376:**11–14.
Krupnick JG and Benovic JL (1998) The role of receptor kinases and arrestins in G protein-coupled receptor regulation. *Annu Rev Pharmacol Toxicol* **38:**289–319.
Lazari MF, Liu X, Nakamura K, Benovic JL, and Ascoli M (1999) Role of G protein-coupled receptor kinases on the agonist-induced phosphorylation and internalization of the follitropin receptor. *Mol Endocrinol* **13:**866–878.
Li JG, Benovic JL, and Liu-Chen LY (2000) Mechanisms of agonist-induced down-regulation of the human κ-opioid receptor: internalization is required for down-regulation. *Mol Pharmacol* **58:**795–801.
Patel M, Gomes B, Patel C, and Yoburn BC (2002) Antagonist-induced μ-opioid receptor upregulation decreases G-protein receptor kinase-2 and dynamin-2 abundance in mouse spinal cord. *Eur J Pharmacol* **446:**37–42.
Sehba F, Duttaroy A, Shah S, Chen B, Carroll J, and Yoburn BC (1997) In vivo

homologous regulation of μ-opioid receptor gene expression in the mouse. *Eur J Pharmacol* **339:**33–41.

Shen J, Benedict GA, Gallagher A, Stafford K, and Yoburn BC (2000) Role of cAMP-dependent protein kinase (PKA) in opioid agonist-induced μ-opioid receptor down-regulation and tolerance in mice. *Synapse* **38:**322–327.

Stafford K, Gomes BA, Shen J, and Yoburn BC (2001) μ-Opioid receptor down-regulation contributes to opioid tolerance in vivo. *Pharmacol Biochem Behav* **69:**233–237.

Terwilliger RZ, Ortiz J, Guitart X, and Nestler EJ (1994) Chronic morphine administration increases β-adrenergic receptor kinase (βARK) levels in the rat locus coeruleus. *J Neurochem* **63:**1983–1986.

Trafton JA, Abbadie C, Marek K and Basbaum (2000) AI. Postsynaptic signaling via the μ-opioid receptor: responses of dorsal horn neurons to exogenous opioids and noxious stimulation. *J Neurosci* **20:**8578–8584.

Unterwald EM, Rubenfeld JM, Imai Y, Wang JB, Uhl GR, and Kreek MJ (1995) Chronic opioid antagonist administration upregulates μ opioid receptor binding without altering μ opioid receptor mRNA levels. *Brain Res Mol Brain Res* **33:**351–355.

Van Bockstaele EJ and Commons KG (2001) Internalization of μ-opioid receptors produced by etorphine in the rat locus coeruleus. *Neuroscience* **108:**467–477.

Whistler JL and von Zastrow M (1998) Morphine-activated opioid receptors elude desensitization by β-arrestin. *Proc Natl Acad Sci USA* **95:**9914–9919.

Whistler JL, Chuang H, Chu P, Jan LY, and von Zastrow M (1999) Functional dissociation of opioid receptor signaling and endocytosis: implications for the biology of opiate tolerance and addiction. *Neuron* **23:**737–746.

Whistler JL, Tsao P, and von Zastrow M (2001) A phosphorylation-regulated brake mechanism controls the initial endocytosis of opioid receptors but is not required for post-endocytic sorting to lysosomes. *J Biol Chem* **276:**34331–34338.

Yabaluri N and Medzihradsky F (1997) Down-regulation of μ-opioid receptor by full but not partial agonists are independent of G protein coupling. *Mol Pharmacol* **52:**896–902.

Yoburn BC, Billings B, and Duttaroy A (1993) Opioid receptor regulation in mice. *J Pharmacol Exp Ther* **265:**314–320.

Yoburn BC, Shah S, Chan K, Duttaroy A, and Davis T (1995) Supersensitivity to opioid analgesics following chronic opioid antagonist treatment: relationship to receptor selectivity. *Pharmacol Biochem Behav* **51:**535–539.

Zaki PA, Keith DA Jr, Brine GA, and Carroll FI (2000) Ligand-induced changes in surface μ-opioid receptor number: relationship to G protein activation? *J Pharmacol Exp Ther* **292:**1127–1134.

Zhang J, Ferguson SG, Barak LS, Bodduluri SR, Laporte SA, Law PY, and Caron MG (1998) Role for G-protein-coupled receptor kinase in agonist-specific regulation of μ-opioid receptor responsiveness. *Proc Natl Acad Sci USA* **95:**7157–7162.

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