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Journal

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Publication Date

DOI

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Prolonged Morphine Treatment Targets δ Opioid Receptors to Neuronal Plasma Membranes and Enhances δ-Mediated Antinociception

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Opioid receptors are known to undergo complex regulatory changes in response to ligand exposure. In the present study, we examined the effect of morphine on the *in vitro* and *in vivo* density and trafficking of δ opioid receptors (δORs). Prolonged exposure (48 hr) of cortical neurons in culture to morphine (10 μM) resulted in a robust increase in the internalization of Fluo-deltorphin, a highly selective fluorescent δOR agonist. This effect was μ-mediated because it was entirely blocked by the selective μ opioid receptor antagonist D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH$_2$ and was reproduced using the selective μ agonist fentanyl citrate. Immunogold electron microscopy revealed a marked increase in the cell surface density of δORs in neurons exposed to morphine, indicating that the increase in Fluo-deltorphin internalization was caused by increased receptor availability. Prolonged morphine exposure had no effect on δOR protein levels, as assessed by immunocytochemistry and Western blotting, suggesting that the increase in bioavailable δORs was caused by recruitment of reserve receptors from intracellular stores and not from receptor neosynthesis. Complementary *in vivo* studies demonstrated that chronic treatment of adult rats with morphine (5–15 mg/kg, s.c., every 12 hr) similarly augmented targeting of δORs to neuronal plasma membranes in the dorsal horn of the spinal cord. Furthermore, this treatment markedly potentiated intrathecal D-[Ala^2]deltorphin II-induced antinociception. Taken together, these results demonstrate that prolonged stimulation of neurons with morphine markedly increases recruitment of intracellular δORs to the cell surface, both *in vitro* and *in vivo*. We propose that this type of receptor subtype cross-mobilization may widen the transduction repertoire of G-protein-coupled receptors and offer new therapeutic strategies.

Key words: opiate; trafficking; narcotic; internalization; analgesia; receptor recruitment

Endogenous as well as exogenous opioids are known to act through at least three distinct opioid receptor subtypes referred to as μ, δ, and κ. These three receptor subtypes have been cloned and were shown to belong to the G-protein-coupled receptor (GPCR) family (Evans et al., 1992; Kieffer et al., 1992; Chen et al., 1993; Fukuda et al., 1993; Meng et al., 1993; Thompson et al., 1993; Wang et al., 1993; Yasuda et al., 1993). Activation of one or more of these receptors by opioid ligands has been demonstrated to affect various physiological functions, including pain perception, locomotion, motivation, reward, autonomic function, immunomodulation, and hormone secretion.

Although each subtype of opioid receptor can transduce its effects independently, evidence has been accumulating for the existence of cellular and/or molecular interactions between them. Thus, cross-talk between μ and δ receptors was proposed on the

basis of pharmacological studies demonstrating both competitive and noncompetitive changes in the binding of δ-selective radioligands on exposure to μ-selective ones (Rothman et al., 1986; Gouardères et al., 1993). Conversely, administration of δ opioid receptor (δOR) antagonists, or of antisense oligonucleotides directed against the δOR, were shown to reduce the development of tolerance to the antinociceptive effects of morphine (Miyamoto et al., 1993; Bilsky et al., 1996; Kest et al., 1996). Accordingly, δOR knock-out mice maintained μOR-mediated analgesia but showed a decrease in the development of tolerance to morphine (Zhu et al., 1999). Recent evidence using transfected cell systems demonstrated direct molecular interactions between different members of the opioid receptor family, with reports of heterodimerization of the δOR with either the μOR (George et al., 2000; Gomes et al., 2000) or κOR (Jordan and Devi, 1999). However, the time frame of these molecular associations cannot account for all of the reported interactions between μOR and δOR. Furthermore, these observations in artificial cell systems are difficult to reconcile with reports that in mammalian CNS, μORs are found mainly on the cell surface, whereas δORs are almost exclusively intracellular (Arvidsson et al., 1995a,b; Cheng et al., 1997; Cahill et al., 2001).

In the present study, we describe a new mechanism that could underlie interactions between μORs and δORs *in vivo* and might potentially be exploited to enhance the analgesic effects of δOR

agonists. This was accomplished using both neuroanatomical and molecular approaches *in vitro* and *in vivo* to demonstrate that stimulation of μORs induces the targeting of δORs from intracellular compartments to the plasma membrane.

MATERIALS AND METHODS

Cortical neuronal culture. Cerebral cortices were isolated from the brains of newborn (P0) Sprague Dawley rats, washed with HBSS (Life Technologies-BRL, Grand Island, NY), and incubated in the presence of trypsin-EDTA (Life Technologies-BRL) for 15 min at 37°C. After washing, the cells were mechanically separated by gentle trituration through fire-polished Pasteur pipettes of decreasing bore diameter. The cell suspension was filtered through a 70 μm sterile filter, and cells were plated onto poly-L-lysine-coated coverslips at a density of 2×10^5 cells or onto poly-L-lysine-coated 100 mm Petri dishes at a density of $2-4 \times 10^6$ cells. The growth medium was composed of DMEM (Life Technologies-BRL) supplemented with 20 mM KCl, 110 mg/l sodium pyruvate, 2 mM glutamine, 0.9% glucose, 0.1% penicillin and streptomycin (Life Technologies-BRL), 0.5% fungizone, 2% B27 (Life Technologies-BRL), and 1–2% fetal bovine serum (Harlan, Indianapolis, IN). Neurons were routinely maintained in culture for 10 d without any change of growth medium, in a humidified incubator at 37°C with 5% CO_2.

Chronic treatment of neurons in vitro. Six to 8 d after plating, fully differentiated neurons were treated, or not, with a single application of the specified drug(s) for 48 hr. Stock solutions of naloxone hydrochloride (Sigma, St. Louis, MO), morphine sulfate (Sabex, Boucherville, Québec, Canada), fentanyl citrate (Sabex), and D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH$_2$ (CTOP) (RBI, Natick, MA) were prepared in distilled water. Naloxone, morphine, and morphine with CTOP were added to the growth medium to yield a final concentration of 10 μM for each drug, whereas fentanyl was added to yield a final concentration of 100 nM. At the end of the 48 hr incubation, cells were washed to remove residual drug(s) before subsequent manipulations (internalization assay, immunostaining, and Western blotting).

Binding of ω-Bodipy red-deltorphin in primary cortical cultures. Internalization of the fluorescent δOR agonist, ω-Bodipy 576/589 deltorphin-I 5APA (Fluo-DLT) was visualized and quantified in primary cortical neurons using confocal microscopy. For this purpose, neurons were preincubated for 10 min at 37°C in Earles-HEPES (140 mM NaCl, 5 mM KCl, 1.8 mM CaCl$_2$, 0.9 mM MgCl$_2$, and 25 mM HEPES) binding buffer supplemented with 0.8 mM of the protease inhibitor 1,10 orthophenanthroline (Sigma), 0.09% glucose, and 0.2% BSA before the incubation with Fluo-DLT in the same buffer for 30 min. Identical results were obtained whether or not morphine was added to the preincubation medium, indicating that the observed effects were not caused by the removal of morphine from the culture medium. To determine the specificity of Fluo-DLT binding, additional cells were labeled in the presence of the specific δOR antagonist, ICI-174,864 (RBI). At the end of the incubation, cells were subjected to a hypertonic acid wash, pH 4.0, to dissociate surface-bound ligand, and subsequently fixed with 4% paraformaldehyde (PFA). They were then rinsed with ice-cold Earles-HEPES buffer and examined under a Zeiss laser scanning microscope attached to an Axiovert 100 inverted microscope (Carl Zeiss Canada Ltd., Toronto, Ontario). Single optical sections were acquired through a *trans*-nuclear plane at eight scans per frame. Cellular morphology, as visualized by phase-contrast confocal microscopy, was used to identify neuronal phenotype. Acquired images were processed using Photoshop version 4.0.1 or 5.5 (Adobe Systems, San Jose, CA) on an IBM-compatible computer. Fluorescence intensity of acquired confocal images was quantified by converting the images to a gray scale and subsequently calculating the integrated density per unit area [in arbitrary units (AU)] using NIH ScionImage software program (Scion Corporation). Calculations and statistical analyses were performed using Excel 97 (Microsoft, San Francisco, CA) and Prism 3.02 (Graph Pad Software, San Diego, CA).

Immunofluorescence detection of δOR in cultured neurons. Cultured neurons were quickly washed with 0.1 M phosphate buffer (PB), pH 7.4, and immediately fixed with 4% PFA for 20–30 min at 37°C. They were further washed with 0.1 M PB and 0.1 M Tris-buffered saline (TBS), pH 7.4, and incubated overnight at 4°C with a blocking solution consisting of 10% normal goat serum (NGS), 0.1% Triton X-100, and 2% BSA in 0.1 M TBS. They were then incubated for 48 hr at 4°C with an N-terminally directed δOR antiserum (Chemicon, Temecula, CA; AB1560 lot numbers 17080164 and 20010505), diluted to a concentration of 0.2–0.5 μg/ml

in 0.5% NGS, 0.1% Triton X-100 in 0.1 M TBS, pH 7.4. Specificity of this antibody toward the rat δOR has been thoroughly characterized by both immunohistochemistry and Western blotting (Cahill et al., 2001). After extensive washing with 0.1 M TBS, cells were incubated with either a Cy3- or Texas Red-conjugated goat anti-rabbit secondary antibody (Molecular Probes, Eugene, OR) for 30 min at 37°C. Finally, cells were washed, and the coverslips were mounted onto gelatin-coated slides using Aquamount. Specificity controls were obtained by replicating the experimental conditions in the absence of primary antibody or using δOR antiserum preabsorbed with antigenic peptide. Images were acquired as single *trans*-nuclear optical sections at eight scans per frame and processed by using Photoshop version 4.0.1/5.5 (Adobe Systems) on an IBM-compatible computer. Fluorescence intensity of acquired confocal images was quantified as described above. Calculations and statistical analyses were performed using Excel 97 (Microsoft) and Prism 3.02 (Graph Pad Software).

Western blotting experiments. On ice, primary cortical cells were quickly washed with 0.1 M PB, pH 7.4, collected in 25 mM Tris, 1 mM EDTA, and 250 mM sucrose with protease inhibitors (Complete Protease inhibitor tablets, Roche Molecular Biochemicals, Laval, Québec, Canada), and pelleted by centrifuging at 10,000 rpm for 10 min at 4°C. The pellet was sonicated for 15 sec in 5 mM Trisma base, pH 7.4, with protease inhibitors. Samples were centrifuged at $20,000 \times g$ for 20 min at 4°C, and the pellet was resuspended in 5 mM Trisma Base, pH 7.4, with protease inhibitors. The membranes were subsequently denatured using 6× Laemmli sample buffer (0.375 mM Trisma base, pH 6.8, 12% w/v SDS, 30% v/v glycerol, 12% v/v 2-mercaptoethanol, 0.2% w/v bromophenol blue). Samples were resolved using 10% Tris-glycine gels (Novex, San Diego, CA), and the proteins were electroblotted onto nitrocellulose membranes. Molecular mass markers (Bio-Rad, Richmond, CA) were used to calibrate the gels. Nitrocellulose membranes were incubated with 1% BSA and 1% chicken egg albumin in 25 mM Tris with 150 mM sodium chloride containing 0.075% Tween 20 (TBST) at 4°C overnight to block nonspecific sites. Nitrocellulose membranes were then immunoblotted for 48 hr at 4°C with the same δOR antisera as used for immunocytochemistry at a concentration of 0.2 μg/ml in TBST containing 1% BSA and 1% chicken egg albumin. Bound antibody was visualized using an HRP-conjugated goat anti-rabbit secondary antibody (Amersham Pharmacia Biotech, Baie D'Urfé, Québec, Canada) diluted 1:4000 in TBST and 5% milk powder followed by chemiluminescent reagents (NEN Life Science Products, Boston, MA). Blots were digitized by scanning with an Agfa Duoscan T1200, and image processing was performed using Photoshop version 4.0.1/5.5 imaging software (Adobe Systems) on an IBM-compatible computer.

Immunogold electron microscopic detection of δORs in cultured neurons. For electron microscopic localization of δORs, cells were washed quickly to remove growth medium and immediately fixed with a mixture of 2% acrolein/2% PFA in 0.1 M PB and subsequently post-fixed with 2% PFA. Cells were washed thoroughly with 0.1 M TBS, exposed to 3% NGS in 0.1 M TBS for 30 min, and incubated for 24–48 hr at 4°C with the anti-δOR antibody (Chemicon) diluted to a concentration of 0.2–0.5 μg/ml in 0.1 M TBS along with 0.2% Triton X-100 and 0.5% NGS. After incubation with the primary antibody, cells were washed repeatedly with 0.01 M PBS, and nonspecific binding sites were blocked using 0.1% gelatin and 0.1% BSA diluted in 0.01 M PBS. Cells were then incubated with a 1:50 dilution of goat anti-rabbit IgG-gold (AuroProbe One GAR, Amersham Pharmacia Biotech) at room temperature for 2 hr. After thorough washing, cells were fixed with 2% glutaraldehyde, and immunogold deposits were enhanced by incubation with ionic silver in citrate buffer (IntenSE M Silver Enhancement Kit, Amersham Pharmacia Biotech). Subsequent to reaction amplification, cells were rinsed in buffer, post-fixed with 2% OsO$_4$, dehydrated in graded alcohols, embedded in Epon, and sectioned at 80 nm thickness on an ultramicrotome. Sections were stained with uranyl acetate and lead citrate and examined with a JEOL 100CX transmission electron microscope (JEOL USA, Peabody, MA). Negatives were scanned using an AGFA Duoscan T1200, and images were processed using Photoshop version 4.0.1 (Adobe Systems) on an IBM-compatible computer.

For immunogold particle quantification, neurons were pooled from three separate experiments. Gold particles were designated as membrane-associated only if in actual contact with the plasma membrane; gold particles in close proximity to the plasma membrane were considered to be intracellular. Neurons with <50 gold grains were eliminated. Only neurons with a surface area between 30 and 100 μm^2, as measured by computer-assisted morphometry (BioCom, Les Ulis,

France), were included in the analysis. The density of immunoreactive δOR per unit length of membrane was calculated by dividing the number of gold particles detected at the surface of each neuron by its respective perimeter (measured by computer-assisted morphometry). The number of δORs per unit area (micrometers squared) was calculated by dividing the total number of gold particles detected over the entire cross-sectional profile of the neuron (including the nucleus) by its surface area (as measured by computer-assisted morphometry). The ratio of membrane-associated to intracellular gold particles was calculated for each labeled neuron by dividing the number of gold particles found at the cell surface by that detected as intracellular for each individual neuron. Statistical significance was verified using a Mann–Whitney *U* test (two tailed). Calculations and statistical analyses were performed using Excel 97 (Microsoft) and Prism 3.02 (Graph Pad Software).

Chronic morphine treatment in vivo. Experiments were performed on adult male Sprague Dawley rats (220–250 gm; Charles River, Québec, Canada) housed in groups of two per cage. Rats were maintained on a 12 hr light/dark cycle and were allowed *ad libitum* access to food and water. Experiments were performed according to a protocol approved by the animal care committee at McGill University and in accordance with the policies and guidelines of the Canadian Council on Animal Care. Rats were injected with increasing doses of morphine sulfate in saline over a 48 hr period (5, 8, 10, and 15 mg/kg, s.c., every 12 hr). Rats were used 8–12 hr after morphine for thermal nociceptive testing and electron microscopy experiments, and 12–20 hr after morphine for formalin testing. An additional electron microscopy experiment was performed 1 hr after morphine treatment to ensure that the observed effects were not caused by opiate withdrawal. The results were identical to those obtained 8–12 hr after morphine.

Electron microscopic immunodetection of δORs in vivo. Rats (*n* = 3–4 per group) were anesthetized with sodium pentobarbitol (70 mg/kg) and perfused through the aortic arch with 50 ml of heparin (75 U/ml heparin in 0.9% saline) followed by 50 ml of a mixture of 3.75% acrolein and 2% PFA in 0.1 M PB, pH 7.4, and then by 400 ml of 2% PFA in 0.1 M PB, pH 7.4. Lumbar spinal cords were removed and post-fixed in 2% PFA in 0.1 M PB for 30 min at 4°C. Transverse sections (40 μm) were cut using a Vibratome series 1000 (Technical Products International, St. Louis, MO) and collected in PB. Spinal cord sections were incubated for 30 min with 1% sodium borohydride in PB followed by copious rinses with PB. Sections were then incubated for 30 min in a cryoprotectant solution consisting of 25% sucrose and 3% glycerol in PB before snap freezing with isopentane (−70°C), immersion in liquid nitrogen, and thawing in PB. Sections were rinsed with 0.1 M TBS and preincubated for 1 hr at room temperature in blocking solution consisting of 3% NGS in TBS. They were then incubated overnight at 4°C in δOR antiserum (Chemicon) at a concentration of 0.2 μg/ml in TBS containing 0.5% NGS. After washing, sections were incubated for 2 hr at room temperature with 1 nm colloidal gold-conjugated goat anti-rabbit IgG (1:50, Amersham Pharmacia Biotech) diluted in 0.1 M PBS containing 2% gelatin and 8% BSA. Sections were then fixed for 10 min with 2% glutaraldehyde in PB and rinsed with 0.2 M citrate buffer, pH 7.4. Immunogold particles were intensified with silver for 7 min using an IntenSE M kit (Amersham Pharmacia Biotech) and rinsed with citrate buffer to stop the reaction. Sections were post-fixed by incubation for 40 min at room temperature with 2% OsO$_4$ in PB, rinsed, and dehydrated with increasing concentrations of ethanol. Sections were embedded in plastic by preincubation with Epon 812 and polypropylene oxide (1:1 then 3:1, respectively). The plastic mixture was replaced by 100% Epon 812 and incubated overnight at 4°C followed by placement between plastic coverslips at 60°C for 24 hr. Ultrathin sections (80 nm) were collected and counterstained with lead citrate and uranyl acetate for examination with a JEOL 100CX transmission electron microscope.

Immunolabeled δOR distribution was assessed in ~200 immunopositive dendrites photographed of three to four grids from three independent experiments for each condition. The distribution of immunogold particles was analyzed using computer-assisted morphometry (Biocom). First, the total number of immunogold particles per unit area was calculated for each labeled dendritic profile detected in both saline- and morphine-treated rats. A profile was considered labeled if it had more than one immunogold particle associated with it. Second, dendrite-associated immunogold particles were classified for each profile as being either intracellular or plasma membrane-associated. A gold particle was considered to be associated with the plasma membrane when it either contacted or overlaid it. Particles not in contact with the plasma membrane, even if in close proximity, were classified as intracellular. Intra-

cellular particles were further categorized according to their distance (200 nm bins) from the plasma membrane. The total number of gold particles associated with the plasmalemma and those within various distances from the plasma membrane in the intracellular compartment were then expressed as a percentage of the total number of immunogold particles per dendrite. Statistical significance was verified using a Mann–Whitney *U* test (two tailed) using Prism 3.02 (Graph Pad Software). Negatives were scanned using an AGFA Duoscan T1200, and images were processed using Photoshop version 4.0.1/5.5 (Adobe Systems) on an IBM-compatible computer.

Acute and persistent pain models. The hot plate test was used as an acute pain test whereby rats are placed on a fixed temperature hot plate (52°C). Latency to response was determined by licking or vigorous shaking of either hind paw, at which point the rat was removed from the plate. A cutoff of 50 sec was imposed to minimize tissue damage in the event that the rat did not respond. The formalin test was used as a model of persistent pain, whereby an intraplantar injection of formalin (2.5%) produced a characteristic biphasic nociceptive response. Nocifensive behaviors were assessed using a weighed score as described previously (Coderre et al., 1993). Briefly, the nociceptive behavior was assessed as follows: (1) no favoring of the injected hind paw, (2) favoring, (3) complete elevation of the hind paw from the floor, and (4) licking or flinching. The behavior was evaluated in 5 min intervals, and the severity of the response was determined by the following formula: (0 × the time spent in category 1, + 1 × the time spent in category 2, + 2 × the time spent in category 3, + 3 × the time spent in category 4). Dose–response curves were generated for each phase of the formalin test by calculating the area under the curve for each dose and expressing the results as a percentage of the area under the curve for the control. Phase 1 values were calculated between 0 and 10 min. Phase 2 values were calculated between 15 and 45 min. ED$_{50}$ values for each dose–response curve were calculated using Prism 3.02 (Graph Pad Software).

RESULTS

In vitro studies

Incubation of neuronal cultures for 30 min at 37°C with the highly selective fluorescent δOR agonist, Fluo-DLT, followed by hypertonic acid wash to dissociate surface-bound ligand, resulted in the weak fluorescent labeling of a small subset of neuronal cells (~15% of neurons) (Fig. 1*A*). Coadministration of Fluo-DLT with the selective δOR antagonist, ICI-174,864, completely abolished this fluorescent labeling, verifying the specificity of our fluorescent probe for δORs (data not shown). No specific (i.e., ICI-174,864-displaceable) staining was observed over glial cells. Fluo-DLT internalization was abolished when the incubation was performed in the presence of the endocytosis inhibitor, phenylarsine oxide, confirming that the fluorescence visualized by confocal microscopy corresponded to internalized ligand (data not shown).

Prolonged treatment of primary neuronal cultures with 10 μM morphine before the internalization assay induced a significant increase in the amount of internalized Fluo-DLT when compared with untreated neurons (~234% of the control; $p < 0.001$) (Fig. 1*B,D*). When neurons were concomitantly pretreated with morphine and the selective μOR antagonist, CTOP, there was no significant difference in Fluo-DLT internalization when compared with control, indicating that the morphine-induced increase in Fluo-DLT internalization was dependent on the interaction of morphine with μOR (Fig. 1*C,D*). Accordingly, treatment of primary cultures for 48 hr with 100 nM fentanyl citrate, a highly selective μOR agonist, produced an increase in the internalization of Fluo-DLT that was not significantly different from that elicited by morphine ($p > 0.05$; data not shown). Treatment of neurons with 10 μM somatostatin did not lead to any detectable increase in Fluo-DLT internalization (data not shown), indicating that this effect could not be produced by activation of any G-protein-coupled receptor.
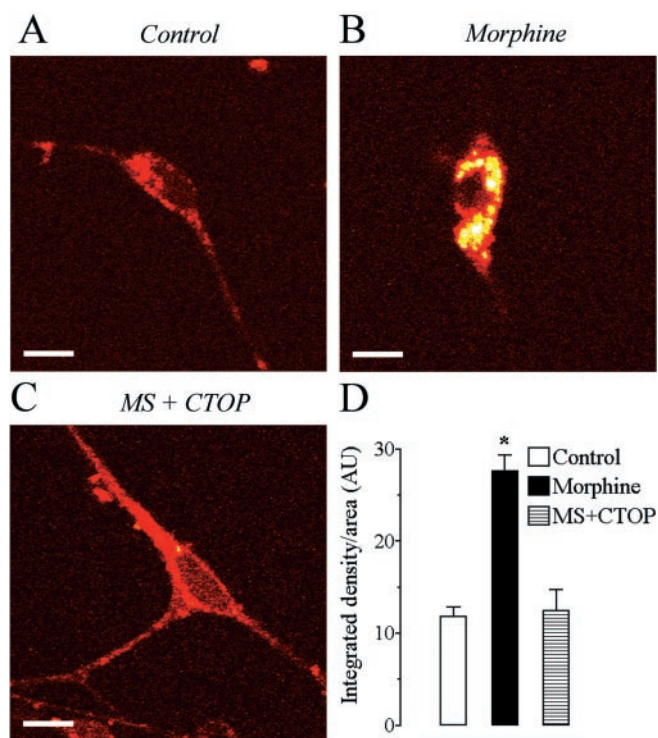
Figure 1. Chronic stimulation with morphine leads to μOR-induced increase in bioavailable δORs at the plasma membrane. Internalization of Fluo-DLT, a selective δOR agonist, in primary cortical neurons either untreated (*A, Control*) or treated with 10 μM morphine sulfate for 48 hr (*B*) or treated with 10 μM morphine sulfate (*MS*) in the presence of 10 μM of the μOR antagonist CTOP (*C*). Images are displayed in pseudocolor, where *white* represents the highest fluorescence intensity and *red* represents the lowest. Internalized Fluo-DLT can clearly be seen intracellularly, especially in morphine-treated cells. Note the absence of internalized ligand in the nucleus. Scale bar, 10 μm. *D*, Internalization of Fluo-DLT is significantly increased (*p* < 0.001) after treatment with morphine for 48 hr. This augmentation is no longer observed when morphine is administered in the presence of CTOP. Each *bar* in the graph represents the integrated density per area (±SEM) pooled from at least three different experiments, with *n* = 13–38 for each group. Statistical significance was determined using the Kruskal–Wallis test, followed by Dunn's multiple comparison test. The *asterisk* denotes significant differences between morphine-treated and untreated neurons (*p* < 0.001), as well as between morphine- and *MS+CTOP*-treated neurons (*p* < 0.001).

To determine whether the morphine-induced increase in the amount of internalized Fluo-DLT reflected an increase in cell surface δORs available for internalization, immunogold electron microscopy was used to monitor cell surface δOR density. As can be seen in Figure 2, silver-intensified immunogold particles, corresponding to immunoreactive δORs, were evident both intracellularly and on the plasma membrane in untreated and morphine-treated primary cortical cells. However, the density of membrane-associated δORs per unit length of membrane was significantly higher in morphine-treated than in untreated neurons (Fig. 2C), suggesting that prolonged treatment with morphine increased cell surface δORs.

To investigate whether the morphine-induced increase in plasma membrane-associated δORs was the result of receptor upregulation (i.e., of an increase in the synthesis of δOR proteins), total δOR proteins were measured by Western blotting of membranes prepared from untreated and morphine-treated (10 μM) cortical cells. Treatment of neuronal cultures with naloxone, a nonspecific opioid receptor antagonist documented to lead to
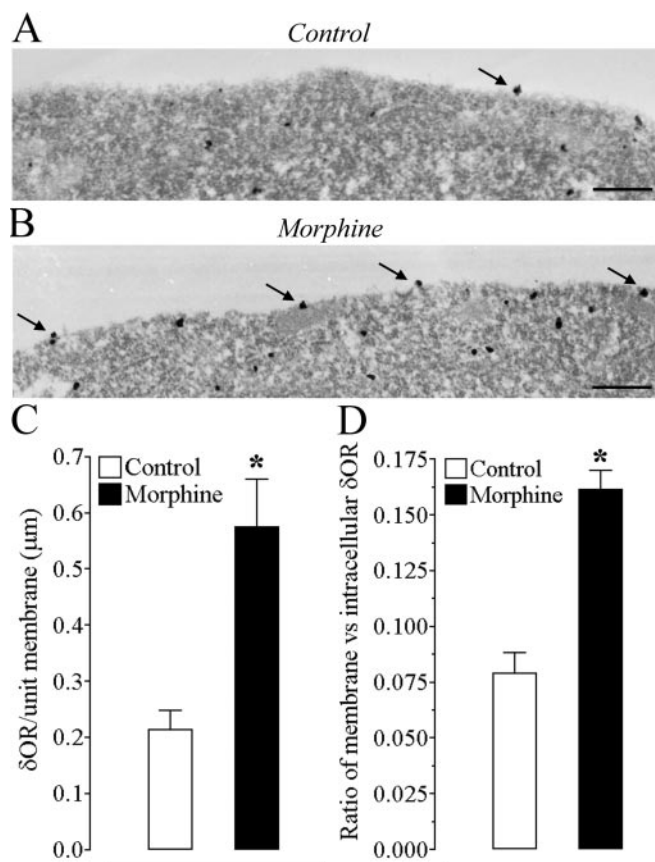
Figure 2. Morphine leads to increased plasma membrane-associated δORs as assessed by immunogold electron microscopy. Primary cortical cells were maintained in culture for 6–8 d before treatment, or not, with 10 μM morphine sulfate for 48 hr, and subsequent processing for immunogold staining of δORs. In untreated (*A, Control*) cortical neurons, immunogold particles are rarely associated with the cell surface (*arrow*), whereas in morphine-treated neurons (*B*), cell surface-associated immunogold particles are more numerous (*arrows*). Scale bar, 0.5 μm. *C*, The number of membrane-associated gold particles per length of plasma membrane is significantly increased in primary cortical neurons treated for 48 hr with 10 μM morphine when compared with untreated controls. The *asterisk* indicates significant difference (two-tailed Mann–Whitney *U* test; *p* < 0.002) between morphine-treated and untreated neurons. *D*, The proportion of membrane-associated gold particles (expressed as the ratio of membrane-associated versus intracellular δORs per neuron) is significantly increased in primary cortical neurons treated for 48 hr with 10 μM morphine when compared with untreated controls, indicating a shift in receptors from an intracellular location toward the cell surface. The *asterisk* in *D* indicates significant difference (two-tailed Mann–Whitney *U* test, *p* < 0.0001) between morphine-treated and untreated neurons.

increased δOR protein expression (Belcheva et al., 1994; Zadina et al., 1994), was used as a positive control. In all cases, immunoreactive bands were observed at estimated molecular weights of 52, 59, 105, and 180 kDa (Fig. 3). Bands at the lower molecular weights (52 and 59 kDa) most likely represent the monomeric form of the receptor, whereas the higher molecular weight forms (105 and 180 kDa) presumably correspond to protein-associated or oligomeric forms of the receptor (Cahill et al., 2001). A reproducible increase in the signal intensity of the 105 kDa band was detected in neurons treated with naloxone but not in those treated with morphine when compared with the untreated cells (Fig. 3). By contrast, no reproducible change was detected in the intensity of any of the other immunoreactive bands after treatment with either 10 μM naloxone or morphine for 48 hr (Fig. 3).
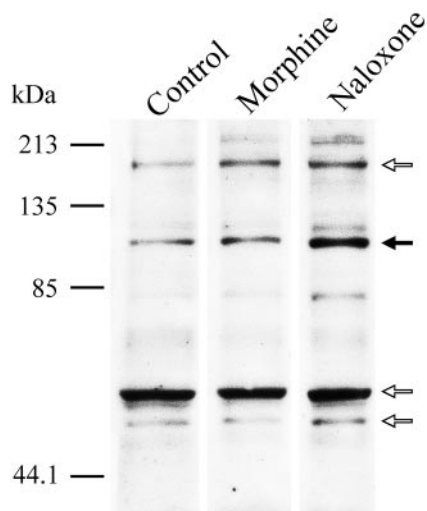
Figure 3. Comparison of the effect of naloxone and morphine on total δOR protein expression. Dissociated primary cortical neurons were maintained in culture for 6–8 d and treated, or not, with 10 μM naloxone or 10 μM morphine sulfate for 48 hr. Cell membranes were isolated, and the samples were resolved and immunoblotted with the δOR antisera. Major immunoreactive bands were observed at estimated molecular weights of 52, 59, 105, and 180 kDa (*arrows*). Specificity of this antibody has been characterized previously (Cahill et al., 2001). Immunoblot analysis reveals that treatment of cortical cells with 10 μM naloxone for 48 hr, but not 10 μM morphine for 48 hr, leads to an increased signal intensity of the band at 105 kDa (*filled arrow*), indicating augmented δOR protein expression. This increase was reproduced in three experiments.

To assess δOR protein concentrations at the single-cell level, both fluorescence and electron microscopic immunocytochemistry were used to complement the immunoblotting data. By confocal microscopy, δOR immunolabeling appeared characteristically punctate and was more pronounced at the level of cell bodies than processes (Fig. 4*A–C*). This labeling was specific, because it was no longer observed after either omission of the primary antibody or preabsorption of the antibody with antigenic peptide (data not shown). No increase in δOR immunolabeling density was detected either visually or by microdensitometry after 48 hr exposure of cultured neurons to 10 μM morphine when compared with untreated controls (Fig. 4*A,B,D*). By contrast, after 48 hr treatment with naloxone, there was a significant augmentation of δOR immunoreactivity ($p < 0.0005$) when compared with either morphine-treated or untreated neurons (Fig. 4*A,C,D*), indicating that the technique was sensitive enough to detect changes in δOR protein levels in our culture system. By electron microscopy, there was no significant increase in the overall density of immunogold particles (per unit area) in morphine-treated as compared with untreated neurons, although a trend toward an increased density was observed (1.30 ± 0.15 vs 2.11 ± 0.31 grains/μm^2 for untreated and morphine-treated neurons, respectively; $p > 0.05$). Immunoblotting and immunocytochemical data therefore concur in suggesting that increased protein synthesis is not the primary mechanism responsible for augmented plasma membrane-associated δORs.

To address the possibility that recruitment of intracellular reserve receptors to the cell surface was responsible for the increase in δOR plasma membrane density, the proportion of membrane-associated versus intracellular δORs was determined from our electron microscopic data. As can be seen in Figure 2*D*, the cell surface to intracellular immunoreactive receptor ratio
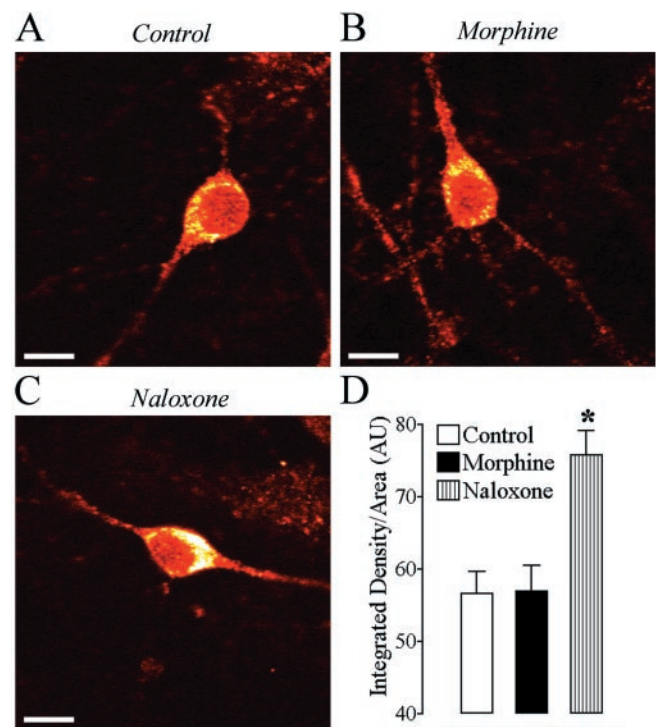
Figure 4. Immunocytochemical determination of the effects of morphine and naloxone on δOR protein expression. Treatment with 10 μM naloxone (*C*) for 48 hr leads to a significant increase in fluorescent immunolabeling intensity when compared with either untreated (*A*) or morphine-treated neurons (*B*). Dissociated primary cortical cells were maintained in culture for 6–8 d, treated or not with 10 μM naloxone or 10 μM morphine sulfate for 48 hr, and stained with the anti-δOR antibody and a Cy3-linked secondary antibody. *Control* refers to untreated neurons. Fluorescent images were acquired using a confocal microscope as described in Figure 1. Scale bar, 10 μm. *D*, As detected by immunocytochemistry, treatment with naloxone results in a significant increase in δOR protein levels when compared with either morphine-treated or untreated neurons. Each *bar* in the graph represents the integrated density per area ($\pm$SEM) pooled from three different experiments, with $n = 8$ for each condition in each experiment. A two-tailed Mann–Whitney *U* test with a Bonferroni correction was used to determine statistical significance. The *asterisk* denotes significant differences between naloxone-treated and untreated neurons (*U* statistic = 106; $p < 0.0005$), as well as between morphine- and naloxone-treated neurons (*U* statistic = 107; $p < 0.0005$). There was no significant difference between untreated and morphine-treated neurons.

was significantly greater in neurons treated with 10 μM morphine for 48 hr when compared with untreated neurons (0.161 vs 0.079, respectively; $p < 0.0001$), indicating that intracellular δORs were targeted to the cell surface in response to prolonged morphine stimulation.

In vivo studies

The study was subsequently extended to an *in vivo* animal model to assess the physiological relevance and possible pharmacological implications of our *in vitro* results. In a first set of experiments, electron microscopy was used to determine the subcellular distribution of δOR immunolabeling in the superficial dorsal horn of the lumbar spinal cord of both saline- and morphine-treated rats.

In both groups of animals, the vast majority of immunolabeled δORs was detected in association with perikarya and dendrites of small intrinsic neurons. Within these neurons, most of the immunoreactive δORs were associated with intracellular compartments rather than with the plasma membrane (Fig. 5*A,B*). In accordance with our neuronal culture results, no significant increase in
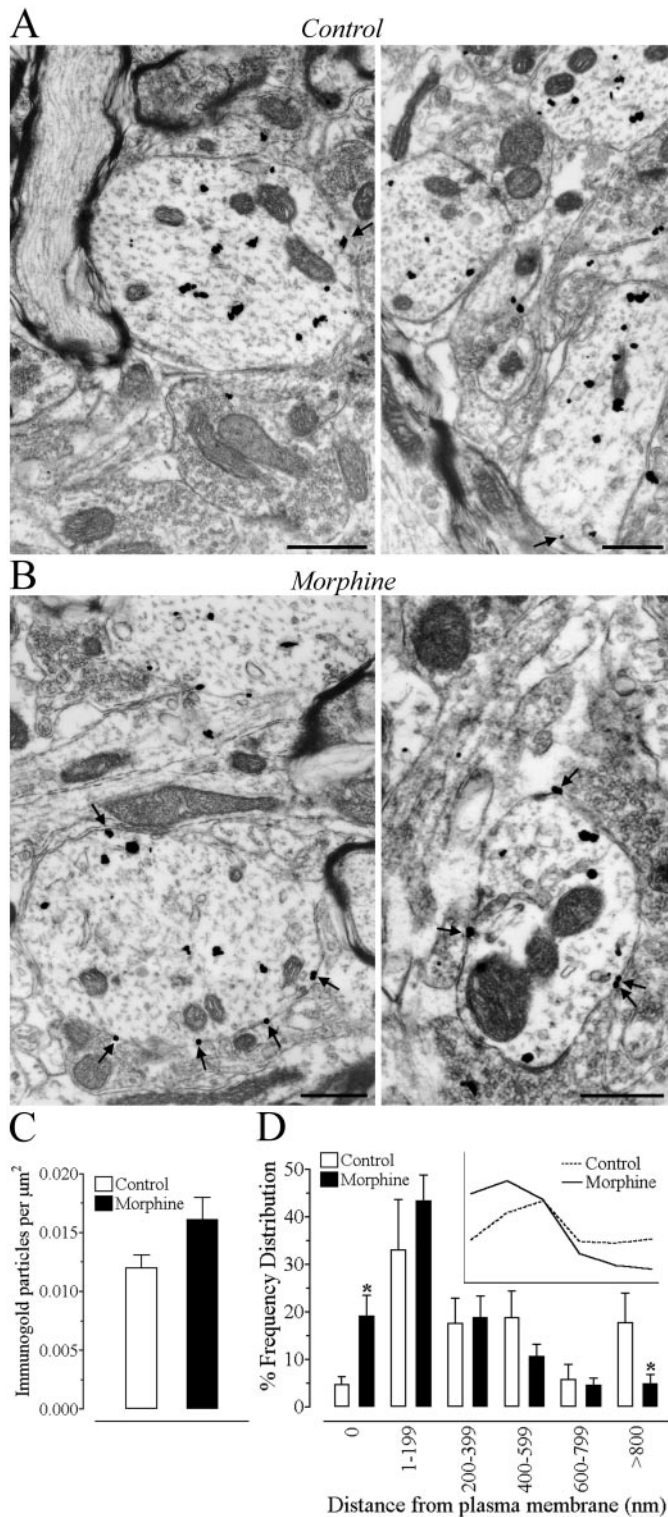
Figure 5. Electron micrographs of δOR-immunolabeled dendrites in the superficial dorsal horn of the spinal cord in saline- and chronic morphine-treated rats (*n* = 3–4 per group). In saline-treated rats (*A*), few immunogold particles are evident on the plasma membrane (*arrows*), whereas in morphine-treated rats (*B*), several immunogold particles are associated with it (*arrows*). Ultrastructural analysis reveals no significant difference in the number of gold particles per unit area of labeled dendritic profiles between treatment groups (*C*). However, the percentage of gold particles associated with the plasma membrane is significantly higher in rats injected with morphine compared with saline-treated rats (*D, first column*). A shortening of the mean distance separating intracellular immunogold

the total number of immunogold particles was evident in morphine-treated compared with saline-injected rats, although a trend toward an increased number of δORs was suggested (Fig. 5*C*). However, all labeled dendrites showed a significantly higher ratio of plasma membrane-associated over total immunogold particles in morphine-treated as compared with saline-injected rats (Fig. 5*D, first two columns*; $p < 0.0001$). Furthermore, the mean distance separating intracellular immunogold particles from the plasma membrane was significantly shorter in morphine-treated (326 ± 18.3 nm) than in saline-treated animals (514 ± 32.4 nm), indicating that morphine treatment resulted in a mobilization of intracellular δORs toward the plasmalemnal region (Fig. 5*D* and *inset*).

One possible pharmacological corollary of the increase in spinal cell surface δORs produced by chronic morphine treatment is an augmentation in the pharmacological potency of δOR agonists. To test this possibility, antinociception produced by intrathecal administration of a selective δOR agonist, [D-Ala²]deltorphin II (DELT), was assessed in paired saline- (control) and morphine-treated rats subjected to either one of the following pain paradigms: (1) phasic pain thresholds using the hot plate test and (2) the formalin test as a model of tonic pain. Rats were injected with morphine or saline subcutaneously every 12 hr for 48 hr. At least 8–12 hr elapsed between the last morphine or saline injection and the evaluation of the effects of the δ agonist (Fig. 6*A*). Baseline thermal threshold latencies were the same in naïve, saline-, or morphine-treated rats. Thermal threshold latencies elicited by 10 μg DELT were significantly increased ($p < 0.05$) in morphine-treated rats compared with controls (Fig. 6*B*). These effects were completely abolished in either morphine- or saline-treated groups by the application of naltrindole, a δOR antagonist, confirming the selectivity of DELT for the δORs (data not shown). At lower doses of DELT (3 μg), no significant difference was observed between morphine-treated and control rats, although increased thermal latency was suggested. At higher doses of DELT, no change in hot plate latency was observed, which may be attributable to the limitations imposed to prevent physical injury to the animal.

Intrathecal DELT administration also elicited antinociception in a tonic pain model as exhibited by the inhibition of formalin-induced nocifensive behaviors (Fig. 6*C*). Intrathecal DELT induced a dose-dependent antinociceptive effect in both control and morphine-treated rats (Fig. 6*C*). Here again, these effects were completely abolished by concomitant administration of the δOR antagonist naltrindole, indicating that these effects were δOR-mediated (data not shown). A more prominent augmentation in the antinociceptive effects of DELT was evident in the group pretreated with morphine compared with control rats (Fig. 6*C,D*). The dose–response curve for DELT-induced antinociception was significantly shifted to the left in morphine-treated rats compared with controls for both phases of the formalin test. Indeed, the ED₅₀ values for the first phase of the formalin test were 3.19 and 7.71 μg, whereas for the second phase of the formalin response, the ED₅₀ values were 4.9 and 32.4 μg for morphine-treated and control rats, respectively (Fig. 6*D*).

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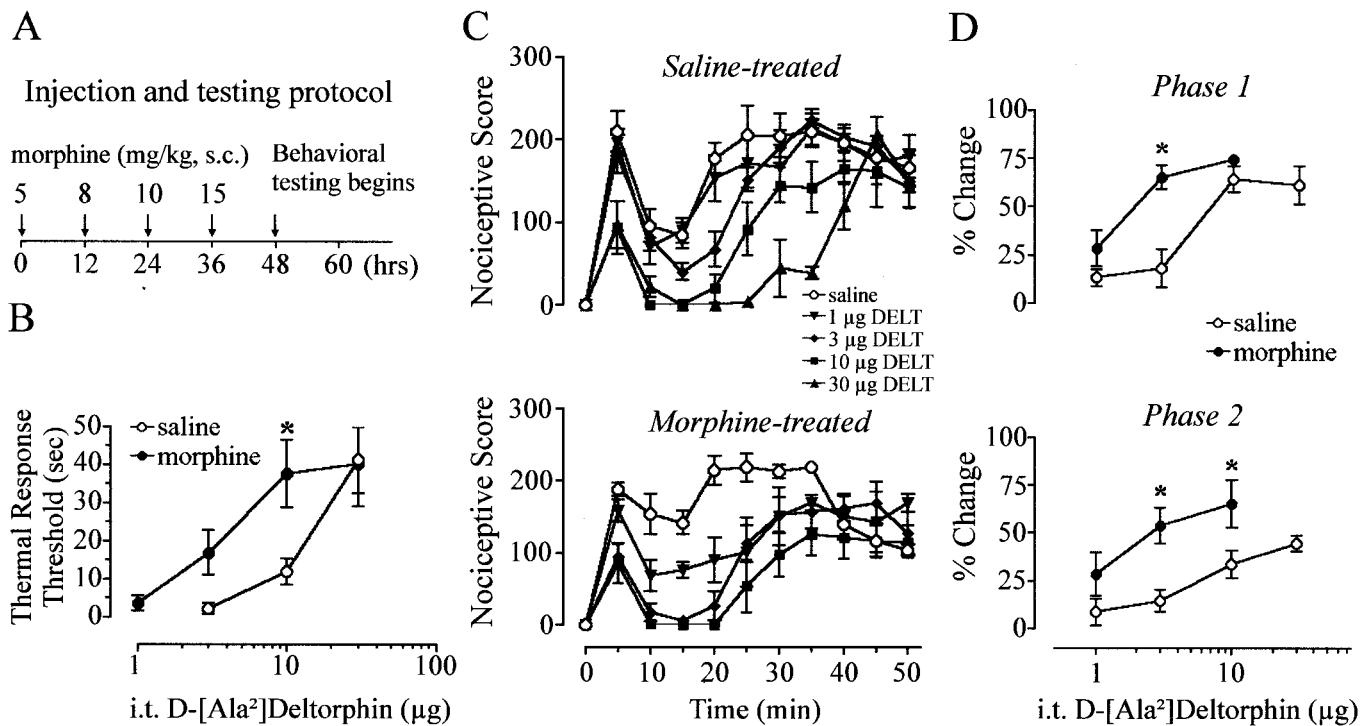
Figure 6. Rats chronically treated with morphine exhibit enhanced antinociceptive effects of intrathecal δOR agonist in both thermal acute pain response (*B*) and tonic pain (*C, D*) compared with control rats. *A,* Diagram illustrating the testing and treatment regimen for morphine and saline injections of rats. The dose administered is indicated by *arrows* at each specified time point (hours). *B,* Response threshold to intrathecal administration of D-[Ala²]Deltorphin II (*DELT*) in the hot plate test ($n = 6$ per group). Statistical analysis using a two-tailed unpaired *t* test revealed a significant difference between groups ($p < 0.05$), as denoted by the *asterisk*. *C,* Nocifensive behaviors assessed using a weighed score produced by intraplantar injection of formalin ($n = 5$–6 per group). All testing was performed 8–20 hr after the final injection of morphine. *D,* Area under the curve values for phase 1 (time 0–10 min) and phase 2 (time 15–40 min) of the formalin test were converted to percentage change from control in respective groups to obtain antinociceptive dose–response curves. ED_{50} values for the first phase of the formalin test are 3.19 μg for morphine-treated and 7.71 μg for control rats. ED_{50} values for the second phase of the formalin response are 4.9 and 32.4 μg for morphine-treated and control rats, respectively.

DISCUSSION

In the present study, we have demonstrated a novel type of interaction between μ and δ opioid receptors whereby prolonged *in vitro* stimulation of μOR enhances cell surface targeting, and hence bioavailability, of δORs. We also showed that this phenomenon could be elicited *in vivo* and that it was correlated with enhanced antinociceptive effects of δOR agonists.

In vitro studies

The selective fluorescent agonist, Fluo-DLT, used in the current study has previously been shown to internalize both in COS-7 cells transfected with cDNA encoding the δORs (Gaudriault et al., 1997) and in cortical neurons in culture (Cahill et al., 1999; Morinville et al., 2000; Lee et al., 2002). As confirmed by the present results, this internalization is receptor-dependent because it is completely abolished in the presence of the selective δOR antagonist ICI-174,864. Such receptor dependency is consistent with the earlier demonstration of agonist-induced δOR internalization in neuronal-derived neuro2A cells stably transfected with HA-δOR cDNA (Ko et al., 1999).

A major finding of the present study was that prolonged treatment of neuronal cultures with the μ-preferring opioid agonist, morphine, markedly enhanced the amount of internalized Fluo-DLT. This increase in Fluo-DLT internalization could have resulted from either (1) an increased rate of receptor turnover whereby internalized receptors would be recycled more rapidly back to the cell surface for internalization of additional ligand molecules or (2) an increase in the density of the receptors at the

plasma membrane at the onset of the internalization assay leading to a greater number of ligand molecules being internalized, as illustrated in Figure 7. To discriminate between these two possibilities, quantitative analysis of δOR cell surface density was performed using immunogold microscopy. Our results demonstrated a low number of cell surface δORs in untreated cells, in agreement with previous reports to the effect that this receptor subtype is localized primarily intracellularly *in vivo* (Arvidsson et al., 1995a; Cheng et al., 1995, 1997; Zhang et al., 1998; Cahill et al., 2001). Pretreatment of the cells with morphine significantly increased δOR cell surface density, indicating that the observed increase in the Fluo-DLT internalization elicited by this drug was caused by an increase in the number of δORs accessible for activation and sequestration.

Three lines of evidence suggest that the morphine-induced increase in cell surface δORs is attributable to an indirect effect via stimulation of μORs rather than to a direct effect of the drug on δORs, to which morphine has been shown to bind in the range of concentrations used in the present study (Goldstein, 1987). First, direct stimulation of δORs by morphine would have been expected to result in a decrease, rather than an increase, in cell surface δOR density, because stimulation with δOR-selective agonists has been shown to result in a downregulation of this receptor (Zadina et al., 1994). Second, the increase in Fluo-DLT internalization was blocked by the addition of the selective μ antagonist CTOP, suggesting that morphine produced its effects through interaction with the μORs rather than with δORs. Third,

Low density

High density
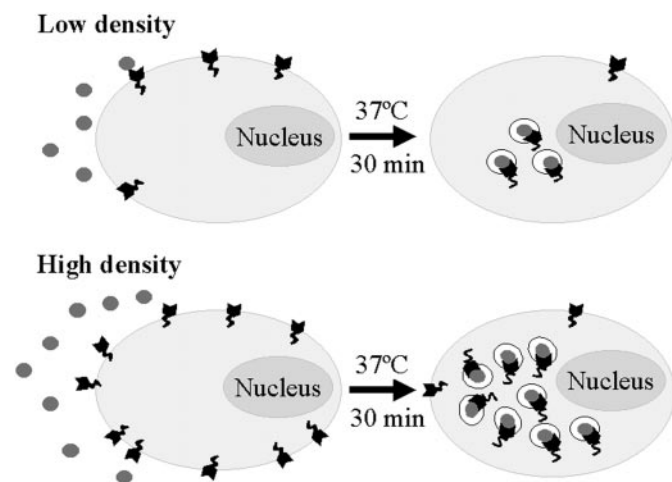
Figure 7. Schematic representation of the ligand-induced receptor internalization assay used to assess the density of plasma membrane-associated δOR receptors. At low receptor density on the plasma membrane, a small number of receptors can bind and internalize their cognate ligand at the permissive temperature of 37°C. If, on the other hand, the receptor density on the plasma membrane is high at the start of the internalization assay, a greater number of receptors can undergo ligand-induced receptor internalization. Surface-bound ligand is removed by hypertonic acid-wash after ligand incubation to ensure that only intracellular ligand is detected. Chronic stimulation with morphine leads to μOR-induced increase in bioavailable δORs at the plasma membrane.

stimulation with the highly selective μOR agonist fentanyl citrate elicited an increase in Fluo-DLT internalization comparable to that produced by morphine.

The most obvious interpretation for the morphine-induced increase in the density of cell surface δORs was that stimulation of μORs by morphine triggered an upregulation of δOR expression/synthesis. To test this possibility, we examined whether prolonged exposure to morphine altered δOR immunoreactive protein levels, as detected by either Western blotting or immunocytochemistry. Both experimental approaches concurred in demonstrating that total δOR protein levels remained unchanged after morphine treatment compared with controls. These results could not be attributed to a lack of sensitivity of our protein detection assays, because prolonged exposure to naloxone, a treatment documented to result in an upregulation of δORs (Zadina et al., 1994), produced a measurable increase in δOR protein content using either technique. The present results therefore suggest that, unlike naloxone, morphine induces its effect on δORs by increasing the recruitment to the plasma membrane of pre-existing intracellular reserve receptors. This interpretation was validated by quantitative immunogold electron microscopy demonstrating an upward shift in cell surface to intracellular receptor ratio.

In vivo studies

To determine whether the morphine-induced increase in δOR membrane targeting evidenced *in vitro* could also be elicited *in vivo*, animals were exposed to systemic morphine for an equivalent period of time, and the distribution of δORs was examined by electron microscopic immunohistochemistry in the dorsal horn of the spinal cord.

As demonstrated previously by us (Cahill et al., 2001) and others (Cheng et al., 1995), the bulk of δOR immunoreactivity in the dorsal horn was detected in association with the perikarya and dendrites of intrinsic lamina II–III neurons. Also as described

previously, only a small proportion of immunolabeled δORs were associated with the plasma membrane of either perikarya or dendrites in these regions (Arvidsson et al., 1995a; Cheng et al., 1995; Cahill et al., 2001). By contrast, in animals treated with morphine, a significantly higher plasma membrane to intracellular receptor ratio was observed, as in our *in vitro* model. Furthermore, this shift from the intracellular to the plasma membrane compartment was accompanied by a decrease in the mean segmental distance separating intracellular δORs from the plasma membrane, as expected from an outward movement of intracellular receptors from the core of the cell to the plasma membrane.

It has been well established that δOR agonists elicit antinociception in both acute and tonic pain models (Stewart and Hammond, 1993; Hammond et al., 1998). In the current study, we demonstrate that targeting of δORs after morphine treatment translates into enhanced antinociceptive effects of δOR agonists in these two distinct pain paradigms. These findings indicate that the δORs newly recruited to the plasma membrane are functional and suggest that morphine could potentially be used as a primer to enhance the antinociceptive effects of δOR agonists.

Antinociceptive synergy between μOR and δOR agonists has been reported previously in various pain models (Heyman et al., 1989; Jiang et al., 1990; Porecca et al., 1990; Malmberg and Yaksh, 1992). However, in the present study, the enhanced antinociceptive effects of DELT are not caused by a synergistic interaction with morphine in so far as synergism is taken to reflect the simultaneous activation of different receptors or of their downstream effectors (Solomon and Gebhart, 1994). Indeed, all of our behavioral experiments were performed a minimum of 8 hr after the last injection of morphine. Furthermore, no alterations in baseline latencies were evident between saline- and morphine-treated groups, nor was there any change in δOR-induced antinociceptive effects after a single injection of morphine (data not shown). It is therefore more appropriate to interpret this increase in δOR-elicited effects as being an adaptive response subsequent to chronic μOR stimulation. Although the enhanced δOR membrane labeling was not directly proven to be causative of the increased antinociceptive potency of the δOR agonist, its correlation with the augmented antinociceptive effectiveness of DELT strongly suggests that these two events are in fact related.

Concluding remarks

Earlier *in vitro* studies have proposed homologous or heterologous cell surface recruitment for regulating receptor responsiveness. For instance, somatostatin was reported to upregulate cell surface somatostatin type 5 receptors in transfected COS-7 cells (Stroh et al., 2000), insulin was reported to enhance recruitment of functional GABA$_A$ receptors in human embryonic kidney 293 cells expressing the $\alpha 1$, $\beta 2$, and $\gamma 2$ subunits of rat GABA$_A$ receptors (Wan et al., 1997), and neuropeptide Y and atrial natriuretic peptide were reported to induce membrane recruitment of α_{1A} receptors and dopamine D1 receptors, respectively, in a renal epithelial cell line (Holtbäck et al., 1999). Nonetheless, the present study is the first to demonstrate that membrane recruitment of heterologous receptors may be induced *in vivo* and that this mechanism may be harnessed for pharmacological purposes. It remains to be determined whether the μOR-induced targeting of δORs occurs in the same neuron or whether μOR stimulation of one neuron targets receptors to the cell surface of another. The cortical cultures used in the present experiments express both μORs and δORs (Lee et al., 2002), and a large proportion of neurons co-express both receptors (A. Morinville,

unpublished observations). In the dorsal horn of the spinal cord, subcellular localization of μORs and δORs likewise suggests that a proportion of spinal intrinsic neurons express both receptors (Cheng et al., 1997). If these μOR–δOR interactions occur in the same cells, they might also involve μOR–δOR heterodimerization, a phenomenon that was recently demonstrated to occur in transfected cell systems (George et al., 2000; Gomes et al., 2000).

Earlier electron microscopic studies have reported an extensive association of immunoreactive δORs with large dense-core vesicles in axon terminals from the rat dorsal horn and have proposed neuropeptide exocytosis as a possible mechanism for δOR membrane targeting (Elde et al., 1995; Zhang et al., 1998). However, such a mechanism is unlikely to account for the μOR-induced targeting of δORs observed in the present study because the bulk of upregulated δORs was associated with dendrites, in which these receptors are rarely, if ever, associated with large dense-core vesicles (Cheng et al., 1997; Cahill et al., 2001; this study).

The physiological consequence of the μ-induced membrane targeting of δORs demonstrated in the present study is intriguing in that both opioid receptor subtypes can bind the same endogenous ligands, namely the enkephalins, albeit with different affinities (for review see Goldstein, 1987). Heterologous targeting of one receptor subtype by the other could therefore provide for a functional shift by which endogenously released enkephalins could exert a first set of physiological effects through interaction with μORs, followed by a second set of effects mediated by the newly recruited δORs. Pharmacologically, this mechanism might be exploited, as demonstrated here, to manipulate the subcellular distribution of δORs for enhanced agonist potency toward a desired clinical end-point. These results have exciting ramifications for the development of clinical therapeutics, including pain therapy.

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