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Regulation of δ-Opioid Receptor Trafficking via μ-Opioid Receptor Stimulation: Evidence from μ-Opioid Receptor Knock-Out Mice

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We recently demonstrated that prolonged treatment with morphine increases the antinociceptive potency of the δ-opioid receptor (δOR) agonist deltorphin and promotes cell surface targeting of δORs in neurons of the dorsal horn of the rat spinal cord (Cahill et al., 2001b). In the present study we examined whether these effects were mediated selectively via μOR. Using the same intermittent treatment regimen as for morphine, we found that methadone and etorphine, but not fentanyl, enhanced [D-Ala^2]-deltorphin-mediated antinociception. However, continuous delivery of fentanyl for 48 hr resulted in augmented δOR-mediated antinociception when compared with saline-infused animals. Time course studies confirmed that a 48 hr treatment with morphine was necessary for the establishment of enhanced δOR-mediated antinociception. The observed increases in δOR agonist potency and δOR plasma membrane density were reversed fully 48 hr after discontinuation of morphine injections. Wild-type C57BL/6 mice pretreated with morphine for 48 hr similarly displayed enhanced δOR-mediated antinociception in a tonic pain paradigm. Accordingly, the percentage of plasma membrane-associated δOR in the dorsal horn of the spinal cord, as assessed by immunogold electron microscopy, increased from 6.6% in naive to 12.4% in morphine-treated mice. In contrast, morphine treatment of μOR gene knock-out (KO) mice did not produce any change in δOR plasma membrane density. These results demonstrate that selective activation of μOR is critical for morphine-induced targeting of δOR to neuronal membranes, but not for basal targeting of this receptor to the cell surface.

Key words: **opiate; targeting; analgesia; narcotic; subcellular localization; electron microscopy**

Introduction

Pharmacological (Martin et al., 1976; Lord et al., 1977) and molecular cloning (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) data have demonstrated the existence of at least three subtypes (μ, δ, and κ) of opioid receptors (ORs). Several lines of anatomical and pharmacological evidence support a role for at least two of these receptors, μOR and δOR, in regulating nociceptive processes. Thus *in situ* hybridization, autoradiographic binding, and immunocytochemical studies have demonstrated the expression of δOR and μOR within dorsal root ganglion cells and their central afferent terminals in the dorsal horn of the spinal cord (Besse et al., 1990, 1991, 1992b; Dado et al., 1993; Ji et al., 1995; Minami et al., 1995; Wang and Wessendorf, 2001) (for review, see Coggeshall and Carlton, 1997). Furthermore, both μOR and δOR have been localized to postsynaptic elements throughout the dorsal horn of the rat spinal cord (Zerari et al., 1994; Arvidsson et al., 1995a,b; Cheng et al., 1995; Mansour et al., 1995; Minami et al., 1995; Cahill et al., 2001a). Accordingly, both μOR and δOR agonists were found to be involved in mediating antinociceptive responses at the spinal level. Thus injections of μOR-selective agonists, such as fentanyl, morphine, codeine, and L-methadone, into the spinal subarachnoid space produced increases in nociceptive thresholds in acute pain tests in rodents (Yaksh and Rudy, 1976, 1977). These effects were abolished in the presence of selective μOR antagonists and no longer could be elicited in μOR knock-out (KO) mice (Matthes et al., 1996). Similarly, intrathecal administration of δOR agonists induced antinociceptive responses in acute pain tests in rodents (Heyman et al., 1987; Mattia et al., 1992; Stewart and Hammond, 1993), and the percentage of mice displaying analgesia after the same treatment was reduced substantially in δOR KO mice when compared with wild-type (WT) animals (Zhu et al., 1999).

Although μOR and δOR can transduce opioid effects independently, evidence for functional interactions between them has been accumulating. Thus antinociceptive synergy between μOR and δOR agonists has been reported in a number of animal models (Heyman et al., 1989a,b; Jiang et al., 1990; Porreca et al., 1990; Abdelhamid et al., 1991; Malmberg and Yaksh, 1992). Pharmacological and autoradiographic studies have demonstrated changes in the binding of δOR-selective ligands with exposure to μ-selective ones (Rothman et al., 1986; Abdelhamid and Takemori, 1991; Besse et al., 1992a; Gouardères et al., 1993). Lately,

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direct interactions between μOR and δOR subtypes via heterodimerization have been demonstrated *in vitro* in transfected cell systems (George et al., 2000; Gomes et al., 2000).

More recently, we showed that chronic treatment with morphine *in vivo* triggered recruitment of δORs to neuronal plasma membranes in the dorsal horn of the rat spinal cord and that this effect was correlated with enhanced δOR-mediated antinociception (Cahill et al., 2001b). These results suggest that "priming" of cells by prolonged stimulation with morphine can be exploited to increase the potency of δOR agonists, a finding with potentially important clinical implications. However, the mechanisms underlying this morphine-induced increase in the density of cell surface δORs are still unclear. First, it is unknown whether this effect is specific to morphine or also may be induced by other μOR-selective agonists with different capacities to induce receptor-mediated internalization. Second, it is unclear how long μOR must be activated to elicit the observed δOR trafficking response and whether this stimulation must be sustained. Finally, the duration of this morphine-induced upregulation of cell surface δOR, which is critical for eventual clinical applications, also is unknown.

The aim of the present study was, therefore, to characterize in the rat the drug specificity and time course of δOR membrane recruitment as well as to ascertain its dependency on μOR stimulation in μOR KO mice.

Materials and Methods

Studies with rats

Animals

Experiments used adult male Sprague Dawley rats (200–250 gm; Charles River, Québec, Canada) maintained on a 12 hr light/dark cycle (7 A.M. and 7 P.M.) and allowed free access to food and water. Studies were conducted during the light phase of the cycle, between 10 A.M. and 2 P.M. Experiments were approved by the animal care committee at McGill University and complied with the policies and directives of the Canadian Council on Animal Care.

Acute antinociceptive effects of μOR agonists

To select comparable dosing for pretreatment with various μOR agonists, we first assessed the acute thermal antinociceptive effects of these drugs [morphine sulfate (MS; 1–10 mg/kg, i.p; Abbott Laboratories, Toronto, Ontario, Canada), methadone hydrochloride (0.3–10 mg/kg, i.p.; Royal Victoria Hospital, Montreal, Québec, Canada), etorphine hydrochloride (0.3–5 μg/kg, i.p.; Wildlife Pharmaceuticals, Fort Collins, CO), and fentanyl citrate (5–100 μg/kg, i.p.; Sabex, Boucherville, Québec, Canada)] by using the tail-flick test. Tail-flick latencies were determined every 10 min for up to 1 hr. Briefly, the tip of the tail was submerged in a water bath maintained at 52°C. Latency to response was determined by a vigorous flick of the tail, at which point the response time was recorded. Three baseline readings were obtained before drug injection. A cutoff of 10 sec was imposed to minimize tissue damage in the event that the rat did not respond. If the animal reached cutoff, the tail was removed from the water and the animal was assigned the maximum score. The maximum possible effect (MPE) was calculated according to the following formula:

$$\%\ \mathrm{MPE} = 100 \times [(\text{test latency}) - (\text{baseline latency})]/[(\text{cutoff}) - (\text{baseline latency})].$$

Comparative effects of various μOR agonists

Rats were injected subcutaneously every 12 hr with four increasing doses of one of the four μOR agonists (MS, methadone, etorphine, or fentanyl) diluted in 0.9% saline solution. Starting doses for chronic treatments were chosen on the basis of the antinociceptive dose–response curves described above. MS was injected at doses of 5, 8, 10 and 15 mg/kg; methadone at doses of 1, 1.6, 2, and 3 mg/kg; etorphine at doses of 1, 1.6, 2, and 3 μg/kg; and fentanyl at doses of 10, 16, 20, and 30 μg/kg. The effect of the selective δOR agonist [D-Ala^2]-deltorphin-II (DLT) (Tocris Cookson, Ellisville, MO; Sigma, St. Louis, MO) on tail-flick latencies was determined 8–12 hr subsequent to the last μOR agonist injection. To this end the animals were anesthetized with halothane and injected intrathecally (at L5–L6) with 10 μg of DLT diluted in 30 μl of saline.

Continuous pretreatment with fentanyl citrate

Because of its reported short half-life and duration of action (for review, see Gutstein and Akil, 2001), fentanyl citrate also was administered chronically via osmotic mini-pumps (Alza Pharmaceuticals, Cupertino, CA). Rats were anesthetized with 66 mg/kg ketamine hydrochloride (Vetrepharm, Belleville, Ontario, Canada) and 5.5 mg/kg of xylazine hydrochloride (Novopharm, Toronto, Ontario, Canada). Two mini-pumps, each containing 250 μl of 50 μg/ml fentanyl citrate, were inserted subcutaneously between the shoulder blades to ensure continuous delivery of 2 × 1 μl/hr over 48 hr. As a control for the surgery, another group of rats was implanted with one osmotic pump filled with 0.9% saline. At 48 hr the mini-pumps were removed under brief ketamine/xylazine anesthesia. Animals were allowed to recover for 4 hr before intrathecal DLT injection and nociceptive testing. Baseline nociceptive thresholds, as assessed by the tail-flick assay, were recorded for each rat before the pumps were removed and immediately before intrathecal DLT injection. Because these baseline thresholds were not significantly different, they were pooled for statistical analyses.

Time course studies

To determine the minimal duration of morphine pretreatment necessary to induce an increase in δOR-mediated antinociception, we treated the rats every 12 hr with increasing doses of MS for 24 hr (5 and 8 mg/kg, s.c.), 36 hr (5, 8, and 10 mg/kg, s.c.) and 48 hr (5, 8, 10, 15 mg/kg, s.c.). Tail-flick latency responses to intrathecal injections of DLT were determined 12 hr subsequent to the last morphine injection for each group. Controls were injected with 0.9% saline every 12 hr (4 injections) and were tested 12 hr after the last injection. The experimenter assessing tail-flick latencies was blinded to the animal pretreatment regimen.

Evaluation of antinociceptive tolerance to continuous morphine treatment

To determine whether our MS pretreatment regimen induced tolerance to morphine, we treated the rats, or not, with MS for 48 hr as above and challenged them 12 hr after the last injection with an intraperitoneal injection of morphine at 1, 3, 5, and 10 mg/kg; tail-flick latencies were recorded every 10 min for 50 min.

Duration of μOR-induced changes in δOR function and localization

δOR-mediated antinociception. To determine the duration of MS-induced enhancement of δOR-mediated antinociception, we treated the rats for 48 hr with MS as above and tested them for δOR-mediated antinociception 12, 24, 36, or 48 hr subsequent to the last morphine injection. Controls were treated in parallel with 0.9% saline but were tested only at 12 hr after the last injection. Here again, the experimenter assessing tail-flick latencies was blinded to the rat pretreatment regimen.

Subcellular localization of δOR. To determine δOR plasma membrane density after chronic morphine treatment, we pretreated the rats for 48 hr with MS as above and assessed the subcellular localization of δOR by electron microscopic immunocytochemistry 48 hr after the last morphine injection ($n = 3$). Results then were compared with those observed in similarly treated animals 12 hr after the last morphine injection (Cahill et al., 2001b). δOR immunolabeling was performed as previously described (Cheng et al., 1995; Cahill et al., 2001a,b). Briefly, rats were anesthetized with sodium pentobarbital and perfusion-fixed with 50 ml of 3.75% acrolein/2% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.4, followed by 400 ml of 2% PFA in 0.1 M PB, pH 7.4. Lumbar spinal cords were postfixed in 2% PFA in 0.1 M PB for 30 min and serially sectioned (40–50 μm) on a vibrating microtome. Sections were incubated for 30 min with 1% sodium borohydride in 0.1 M PB and then for a further 30 min in 25% sucrose/3% glycerol in 0.1 M PB before snap-freezing with isopentane (−70°C), liquid nitrogen, and finally thawing in 0.1 M PB. Sections were rinsed with 0.1 M TBS, blocked with 3% NGS, and immunolabeled for 36–48 hr at 4°C with a δOR antiserum (catalog

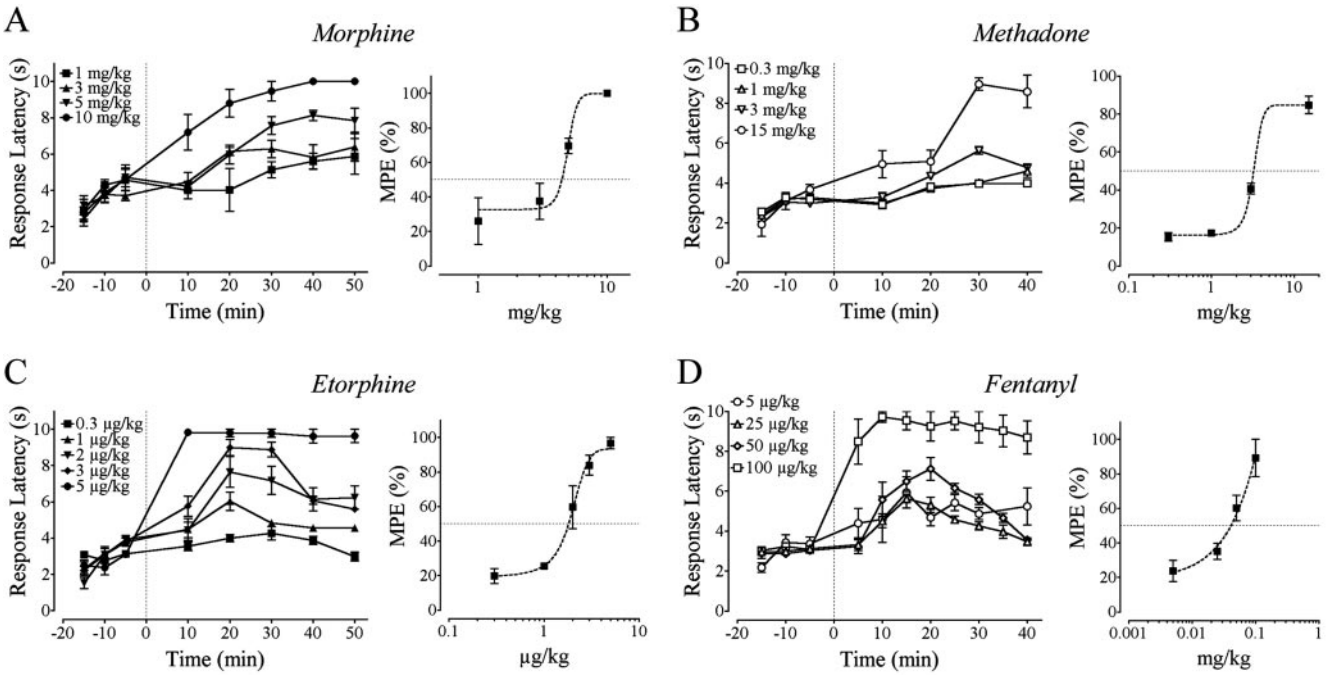

Figure 1. Comparative antinociceptive potencies of four μOR agonists in an acute pain paradigm. Left panels, Tail-flick latencies (in sec) were determined every 10 min subsequent to intraperitoneal administration (time 0, denoted by dotted line) of morphine (*A*), methadone (*B*), etorphine (*C*), and fentanyl (*D*) at various doses. Right panels, The percentage of the maximum possible effect (% MPE) was calculated at the time of peak antinociceptive response for each dose: 40 min for morphine (*A*), 30 min for methadone (*B*), 30 min for etorphine (*C*), and 20 min for fentanyl (*D*). The theoretical equation of the line (sigmoidal dose–response) is shown as a dotted curve (right panel). ED_{50} values were estimated from the theoretical curve at an MPE of 50% (dotted horizontal line, right panel). Calculations were performed with Excel 97 (Microsoft) and Prism 3.02 (Graph Pad Software). Data are presented as the average ± SEM.

number AB1560,Chemicon, Temecula, CA) diluted to 0.2–0.5 μg/ml in TBS containing 0.5% NGS. As controls the sections were processed in the absence of primary antibody. Sections then were incubated at room temperature for 2 hr with a 1:50 dilution of colloidal gold-conjugated (1 nm) goat anti-rabbit IgG (AuroProbe One GAR, Amersham Biosciences, Baie D'Urfé, Québec, Canada) diluted in 0.1 M PBS, pH 7.4, containing 2% gelatin and 8% BSA. After thorough washing the sections were fixed with 2% glutaraldehyde, and immunogold deposits were enhanced by incubation with ionic silver (IntenSE M Silver Enhancement Kit, Amersham Biosciences). Sections were postfixed with 2% OsO_4, dehydrated in graded alcohols, embedded in Epon, thin-sectioned (80 nm), counterstained with uranyl acetate and lead citrate, and observed with a JEOL 100CX transmission electron microscope (JEOL, Peabody, MA).

For quantification of the distribution of immunolabeled δOR, a minimum of 50 immunopositive dendrites from the dorsal horn (lamina III–IV) of the spinal cord was counted in each animal. Dendrites of similar size were sampled in all treatment groups. Profiles were considered to be immunolabeled if two or more gold particles were present over them. Gold particles were classified as being intracellular or plasma membrane-associated as described (Cahill et al., 2001a,b). The percentage of membrane-associated to total gold particles for each animal was calculated by dividing the sum of the number of gold particles found at the cell surface by the sum of immunogold particles detected for all sampled dendrites.

Studies with mice

Animals

Behavioral experiments used both male and female WT C57BL/6 mice (8–15 weeks of age; Charles River, Québec, Canada). Subcellular localization studies were performed on homozygous female and male mice μOR KO as well as on male and female WT C57BL/6 mice. The generation of μOR KO C57BL/6 mice has been described in detail previously (Matthes et al., 1996). Animals were maintained on a 12 hr light/dark cycle, and experiments were performed during the light phase of the cycle, between 10 A.M. and 2 P.M. Experiments were approved by the animal care committee at McGill University and complied with the policies and directives of the Canadian Council on Animal Care.

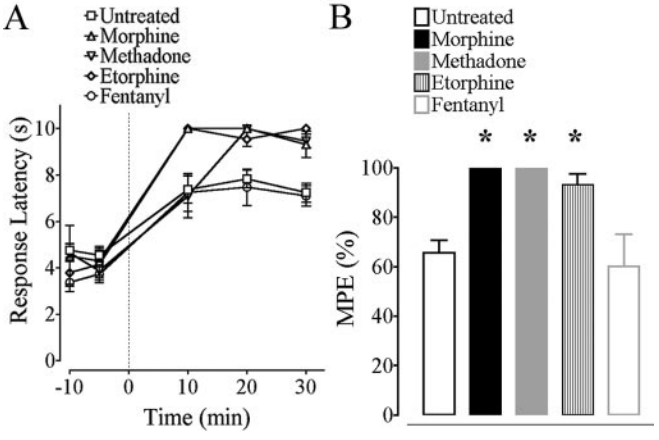

Figure 2. Effect of chronic pretreatment with μOR agonists on δOR-mediated antinociception. *A*, Morphine, methadone, etorphine, and fentanyl were administered at 12 hr intervals for 36 hr as described in the Materials and Methods. Then 12 hr subsequent to the last injection of each opioid ligand, 10 μg of DLT was administered intrathecally, and tail-flick latencies (in sec) were recorded every 10 min. *B*, Each bar in the graph represents the percentage of maximal possible effect (MPE) ± SEM 20 min after the injection of DLT. The percentages of MPE for morphine ($n = 4; p < 0.01$), methadone ($n = 6; p < 0.01$), and etorphine ($n = 6, p < 0.05$) were significantly different from the % MPE of non-pretreated rats ($n = 13$; ANOVA, Tukey's MCT; denoted by an asterisk). No significant difference was found between the % MPE for fentanyl-pretreated ($n = 5$) and non-pretreated rats ($p > 0.05$; ANOVA, Tukey's MCT) or among the percentages of MPE for morphine, methadone, and etorphine ($p > 0.05$; ANOVA, Tukey's MCT).

Behavioral studies with mice

To determine whether chronic morphine treatment induced enhanced δOR-mediated antinociception in mice as it does in the rat, we pre-

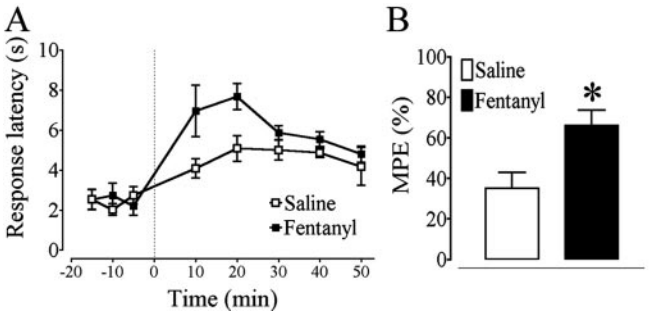

Figure 3. Continuous administration of fentanyl leads to enhanced DLT-mediated antinociception. *A*, Rats were implanted subcutaneously with osmotic mini-pumps for continuous delivery of 0.1 μg/hr fentanyl over 48 hr; saline controls were implanted osmotic pumps delivering 1 μl of 0.9% saline/hr for 48 hr. Pumps were removed 4 hr before antinociceptive testing. Baseline latencies were measured before and after the removal of the pumps and every 10 min subsequent to the intrathecal administration of DLT. *B*, Each bar in the graph represents the % MPE ± SEM calculated 20 min after the injection of DLT ($n = 5$ for each group). Statistical significance was determined by the means of an unpaired Student's *t* test (*$p < 0.05$).

treated, or not, both male and female wild-type C57BL/6 mice with morphine sulfate at 5, 8, 10, 15 mg/kg (subcutaneously every 12 hr) and tested them for δOR-mediated antinociception 8–12 hr subsequent to the last morphine injection. For this purpose the mice were anesthetized lightly with isoflurane and administered intrathecally with 5 μl of saline or 5 μg of DLT in 5 μl of saline at the L4–L5 intervertebral space. Mice then were tested for δOR-mediated antinociception by the formalin test ($n = 4–6$ female and 4–7 male mice in each group). This test was used because mice of this particular strain respond better to this tonic pain stimulus than to the acute tail-flick test (Mogil et al., 1999) and because both tests are equally sensitive for detecting morphine-induced enhancement of δOR-mediated antinociception in the rat (Cahill et al., 2001b). In C57BL/6 mice an intraplantar injection (25 μl) of formalin (2.5%) produced the biphasic nociceptive response typical of this persistent pain model. Nocifensive behaviors were assessed by a weighed score as previously described (Coderre et al., 1993). Briefly, the nocifensive behavior was assessed as 1, no favoring of the injected hind paw; 2, favoring; 3, complete elevation of the hind paw from the floor; 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)/300].

The total area under the curve (A.U.C.) (phases 1 and 2) was calculated for each animal.

Subcellular localization of δOR

Wild-type and μOR KO mice were pretreated or not with MS for 48 hr as above and perfusion-fixed 12 hr after the last morphine injection, under pentobarbital anesthesia (2.9 mg/mouse), with 35 ml of a mixture of 3.75% acrolein and 2% PFA, followed by 250 ml of 2% PFA in 0.1 M PB, pH 7.4. Lumbar spinal cords were postfixed with 2% PFA in 0.1 M PB for 30 min, sectioned on a vibratome, and processed for δOR immunogold labeling as described above. Specificity controls were obtained by replicating the experimental conditions in the absence of primary antibody.

For quantification of the distribution of immunolabeled δOR a minimum of 50 immunopositive dendrites from the dorsal horn (lamina II–V) of each mouse was counted in untreated ($n = 3$; 2 male, 1 female) and morphine-pretreated ($n = 3$; 3 female) WT mice as well as in untreated ($n = 3$; 3 female) and morphine-treated ($n = 3$; 2 female, 1 male) μOR KO mice. Profiles were considered to be immunolabeled if three or more gold particles were present over them. For each animal the density of immunoreactive δOR per unit length of membrane was calculated by dividing the total number of gold particles detected at the surface of all sampled dendrites by the sum of the respective perimeters (measured by computer-assisted morphometry; Biocom, Les Ulis, France). Similarly, for each animal the number of δOR per unit area (μm^2) was calculated by dividing the sum of the total number of gold particles detected over dendritic profiles by the sum of their surface areas. Finally, the percentage of membrane-associated to total gold particles for each animal was calculated as in the rat material.

Results

Studies with rats

Comparative analgesic potencies of μOR agonists

To select equi-effective doses of opioids for chronic pretreatments, we compared the acute antinociceptive effects of the μOR agonists morphine, methadone, etorphine, and fentanyl in an acute pain paradigm (Fig. 1). Intraperitoneal administration of morphine, methadone, and etorphine produced peak antinociceptive responses in the tail-flick test 30–40 min subsequent to injection of the μOR agonist (Fig. 1*A–C*, respectively). For fentanyl, peak antinociceptive responses were recorded 10–20 min after injection (Fig. 1*D*), consistent with the reported pharmacokinetic profile of this drug (for review, see Gutstein and Akil, 2001). To generate a dose–response curve, we calculated the percentage of the maximum possible effect (% MPE) at the time of peak antinociceptive response for each μOR agonist at each dose. ED_{50} values, estimated from the MPE of 50%, were 5 mg/kg for morphine (Fig. 1*A*), 3 mg/kg for methadone (Fig. 1*B*), and 1.5 μg/kg for etorphine (Fig. 1*C*). For fentanyl the best estimate of the ED_{50} was 40 μg/kg (Fig. 1*D*).

Effect of chronic μOR agonist administration on δOR-mediated antinociception

Then a separate group of animals was treated chronically on an intermittent dosage regimen with morphine, methadone, etorphine, or fentanyl (Fig. 2). The starting dose of each drug was based on its respective ED_{50} to use a dose equivalent to that used

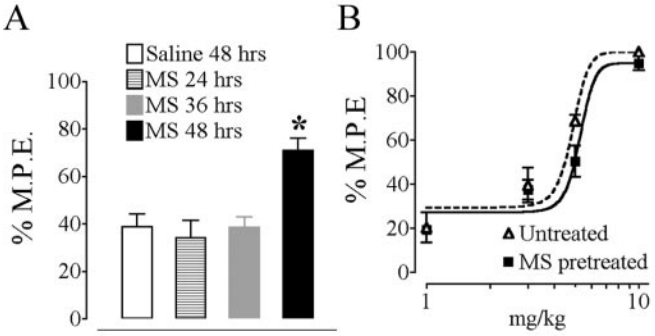

Figure 4. *A*, Time course for the establishment of enhanced δOR-mediated antinociception. Rats were treated with morphine sulfate (MS) at 5 and 8 mg/kg (subcutaneously at 12 hr intervals); 5, 8, and 10 mg/kg (subcutaneously at 12 hr intervals); or 5, 8, 10, 15 mg/kg (subcutaneously at 12 hr intervals). Tail-flick latency responses to intrathecal injections of DLT were determined 12 hr subsequent to the last morphine injection. The data were expressed as a function of the time that had elapsed between the first morphine injection and the time of testing (i.e., MS 24, 36, and 48 hr). Saline controls were injected subcutaneously with 0.9% saline every 12 hr (4 injections) and were tested 12 hr after the last injection (Saline 48 hr). Each bar represents the % MPE ± SEM 20 min after the injection of DLT. Morphine treatment for 48 hr ($p < 0.001$), but not treatment for 24 or 36 hr ($p > 0.05$ in both cases), produced DLT-mediated antinociception that was significantly greater than that observed in saline-injected animals (denoted by an asterisk). Statistical significance was determined by ANOVA, followed by Dunnett's MCT. *B*, Evaluation of antinociceptive tolerance to continuous morphine treatment. Rats were pretreated or not with morphine (MS) at 5, 8, 10, and 15 mg/kg (subcutaneously every 12 hr). At 12 hr after the last morphine administration the rats were injected intraperitoneally with morphine at 1, 3, 5, or 10 mg/kg; tail-flick latency responses were determined every 10 min for 50 min. Each data point represents the % MPE ± SEM 30 min after the morphine challenge ($n = 4–6$ for each point). No statistical difference at any of the doses was observed between untreated (dotted) and morphine-pretreated (solid line) rats (unpaired *t* tests, $p > 0.05$). Calculations were performed with Excel 97 (Microsoft) and Prism 3.02 (Graph Pad Software).

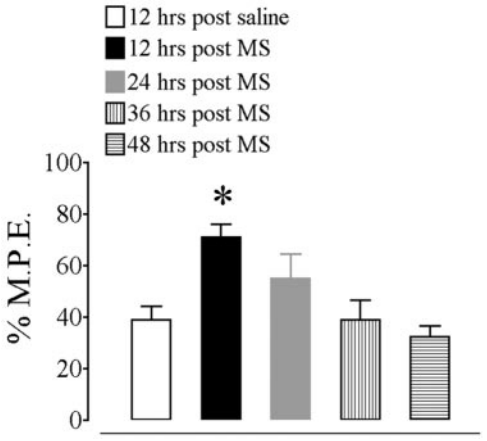

Figure 5. Duration of enhanced DLT-mediated antinociception. Rats were injected with 5, 8, 10, 15 mg/kg morphine (MS; subcutaneously at 12 hr intervals). DLT-mediated antinociception was determined 12 hr ($n = 11$), 24 hr ($n = 6$), 36 hr ($n = 5$), and 48 hr ($n = 5$) subsequent to the last morphine injection. The data were expressed as a function of the time that had elapsed between the last morphine injection and the time of testing (i.e., 12, 24, and 48 hr after MS). Saline controls were injected subcutaneously with 0.9% saline every 12 hr (4 injections) and were tested for DLT-mediated antinociception 12 hr after the last saline injection. Each bar represents the % MPE ± SEM 20 min after the injection of DLT. The asterisk denotes a statistically significant increase when compared with saline-treated controls ($p < 0.001$; ANOVA, Dunnett's MCT).

for the morphine pretreatment (Fig. 2). The antinociceptive effects of the selective δOR agonist DLT were tested 12 hr after the last injection of the μOR agonist (48 hr after the first injection). Intrathecal administration of DLT in untreated and μOR agonist-pretreated rats elicited antinociception in the tail-flick test, as exhibited by the increase in latency to response when compared with baseline values in this acute pain model (Fig. 2*A*). However, rats pretreated with morphine ($p < 0.01$), methadone ($p < 0.01$), or etorphine ($p < 0.05$) showed significantly higher DLT-induced antinociception than that produced in untreated animals (Fig. 2*B*; ANOVA, Tukey's test). When we compared % MPEs 20 min after intrathecal injection of DLT, no significant difference was found among morphine, etorphine, and methadone-pretreated rats (Fig. 2*B*; ANOVA, Tukey's test; $p > 0.05$). In contrast, rats pretreated with the highly selective μOR agonist fentanyl showed the same level of DLT-induced antinociception as untreated rats (Fig. 2*B*; ANOVA, Tukey's test; $p > 0.05$).

To test the possibility that the lack of effect of fentanyl may be attributable to the *in vivo* pharmacokinetic profile of the drug (rapid onset of action and short half-life), we treated animals at shorter time intervals (every 8 or 6 hr for 40 hr) with increasing doses of fentanyl. This treatment strategy also failed to produce an enhancement in DLT-induced antinociception in the tail-flick test 48–50 hr after the start of fentanyl treatments (results not shown). To ensure continuous exposure to fentanyl, we implanted two osmotic pumps subcutaneously for delivery of 0.1 μg/hr for 48 hr. Continuous delivery of fentanyl in this manner resulted in a significant increase in DLT-mediated antinociception (Fig. 3; $p < 0.05$) as compared with saline-infused controls.

Time course studies

To determine the time needed for the establishment of enhanced δOR-mediated antinociception via chronic μOR agonist treatment, we treated the rats with morphine for different time intervals: 24 hr (5 and 8 mg/kg, s.c., every 12 hr), 36 hr (5, 8, 10 mg/kg, s.c., every 12 hr), or 48 hr (5, 8, 10, and 15 mg/kg, s.c. every 12 hr). In all cases intrathecal injections of DLT were performed 8–12 hr after the last morphine injection. The % MPE 20 min after DLT was significantly greater after the 48 hr pretreatment when compared with saline-pretreated animals [Fig. 4*A*; ANOVA, Dunnett's multiple comparison test (MCT); $p < 0.001$]. In contrast, the % MPE for pretreatment with morphine for 24 or 36 hr was not significantly different from that of saline-injected rats (Fig. 4*A*; ANOVA, Dunnett's MCT; $p > 0.05$).

Evaluation of antinociceptive tolerance to continuous morphine treatment

To determine whether our treatment paradigm induced tolerance to morphine, we pretreated, or not, the rats with morphine at 5, 8, 10, and 15 mg/kg (subcutaneously every 12 hr), and we tested their nociceptive response after a single intraperitoneal injection of morphine 12 hr after the last dose of the pretreatment. As can be seen in Figure 4*B*, no statistically significant difference was detected in the antinociceptive effects of an acute morphine challenge at any of the doses tested between morphine-pretreated versus untreated rats (unpaired Student's *t* tests; $p > 0.05$).

Duration of enhanced δOR function and cell surface upregulation

To determine the duration of the morphine-induced increase in δOR-mediated antinociception, we pretreated the rats with 5, 8, 10, 15 mg/kg morphine (subcutaneously every 12 hr) and subsequently tested them for δOR-mediated antinociception 12, 24, 36, and 48 hr after the last morphine injection (Fig. 5). A statistically significant enhancement of δOR-mediated antinociception was observed 12 hr subsequent to the last morphine injection as compared with saline-pretreated rats (Fig. 5; $p < 0.001$). At 24 hr after the last morphine injection the % MPE for intrathecal DLT appeared elevated, albeit not significantly different from controls (Fig. 5). After 36 and 48 hr DLT-mediated antinociception had returned to the levels measured in saline-pretreated rats (Fig. 5).

Our previous studies had shown that the increase in δOR agonist potency observed 12 hr after the last injection of morphine was correlated with an upregulation of δORs at the cell

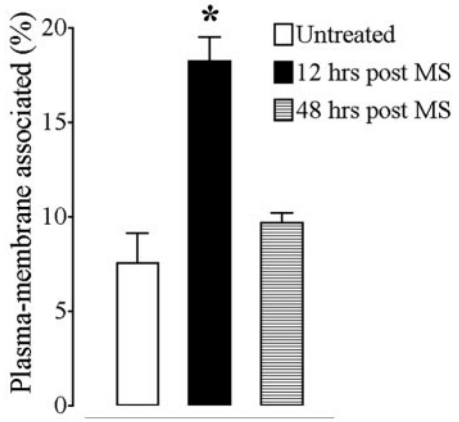

Figure 6. Subcellular distribution of δOR 48 hr after the last dose of the morphine pretreatment regimen. Rats ($n = 3$) were injected with 5, 8, 10, 15 mg/kg morphine (MS; subcutaneously at 12 hr intervals) and were processed for immunogold detection of δORs 12 and 48 hr subsequent to the last morphine injection (12 and 48 hr post-MS, respectively). Untreated rats were processed for electron microscopic detection of δORs in an analogous manner. Each bar represents the proportion of immunogold particles associated with the plasma membrane as a percentage of the total ± SEM. This percentage is not significantly different between 48 hr post-MS and untreated rats (ANOVA, Tukey's MCT; $p > 0.05$) but is significantly greater at 12 hr post-MS rats when compared with either 48 hr post-MS or untreated rats (ANOVA, Tukey's MCT; $p < 0.01$; denoted by an asterisk).

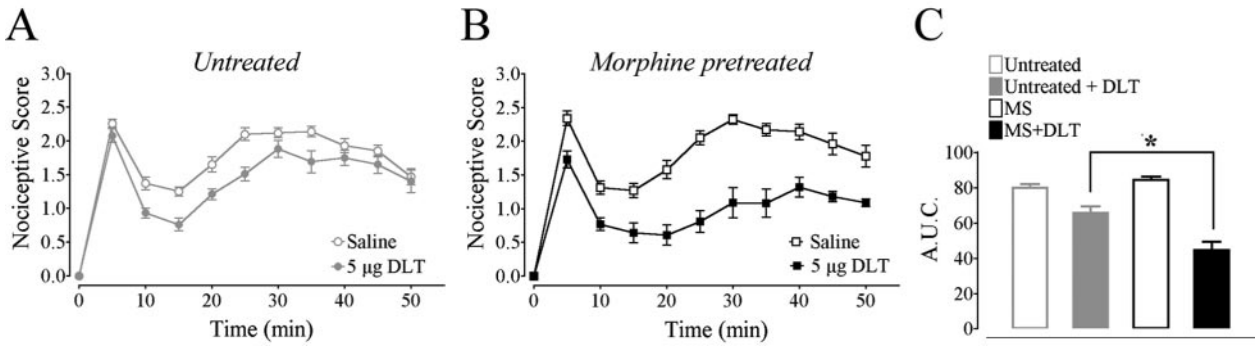

Figure 7. Prolonged treatment with morphine leads to enhanced δOR-mediated antinociception in C57BL/6 mice. WT male and female mice were pretreated (*B*) or not (*A*) with morphine sulfate at 5, 8, 10, 15 mg/kg (subcutaneously every 12 hr). At 12 hr after the last morphine injection, saline or 5 μg of DLT was injected intrathecally. In C57BL/6 mice an intraplantar injection of formalin (2.5%) produced the biphasic nociceptive response typical of this persistent pain model (*A, B*). Nocifensive behaviors in female ($n = 4–6$ per group) and male ($n = 4–7$ per group) mice were assessed in 5 min intervals with a weighted score. Because nocifensive scores were not statistically different between males and females (see Table 1), pooled male and female scores are illustrated. *C*, Pooled area under the curve (A.U.C.) for male and female mice. The A.U.C. values for intrathecal saline in morphine and untreated animals are not significantly different. However, there is a statistically significant decrease in A.U.C. between saline-treated mice and morphine-pretreated mice intrathecally injected with DLT (ANOVA, Bonferroni MCT; $p < 0.001$; denoted by an asterisk). Data are presented as the average ± SEM.

surface (Cahill et al., 2001b). To determine whether, conversely, the return of δOR antinociceptive responses to baseline levels was accompanied by a restoration of δOR membrane density to basal levels, we pretreated the rats with 5, 8, 10, 15 mg/kg morphine and assessed the subcellular localization of δOR 48 hr after the last morphine injection in dendrites from lamina III–IV of the dorsal horn of the spinal cord. As we previously reported, 12 hr after the last morphine injection a significant increase was observed in the percentage of δOR immunoreactive gold particles found in association with dendritic plasma membranes as compared with controls (Fig. 6; ANOVA, Tukey's MCT; $p < 0.01$). In contrast, 48 hr after the last morphine injection the percentage of plasma membrane-associated immunogold particles was no longer significantly different from that observed in untreated animals (Fig. 6; ANOVA, Tukey's MCT; $p > 0.05$). This result demonstrates a return of δOR plasma membrane density to premorphine treatment levels 48 hr after removal of the μOR agonist.

Studies with mice

Behavioral studies

To determine whether chronic pretreatment with μOR agonists also induced an increase in DLT-induced antinociception in mice, we injected wild-type male and female C57BL/6 mice with increasing doses of morphine (5, 8, 10, and 15 mg/kg) every 12 hr. Antinociception produced by intrathecal administration of DLT was assessed in untreated and morphine-treated male and female mice by the formalin test 8–12 hr after the last morphine dose, as previously described for rats (Cahill et al., 2001b). Intrathecal administration of DLT in mice elicited antinociception, as exhibited by the inhibition of formalin-induced nocifensive behaviors in both untreated and morphine-treated mice (Fig. 7*A,B*; compare intrathecal saline vs 5 μg of DLT). For both male and female mice a statistically significant decrease in the A.U.C. was noted between untreated and morphine-pretreated mice injected intrathecally with DLT (Table 1; Fig. 7*C*). This difference indicates a significant increase in δOR-mediated antinociception, and hence of δOR agonist effectiveness, in morphine-treated as compared with untreated mice. Because the effect was similar in male and female mice, subcellular distribution data subsequently were pooled between the two genders.

Table 1. Comparison of the A.U.C. for nocifensive behaviors elicited by intraplantar formalin injection in male and female wild-type C57BL/6 mice

		A.U.C.
WT C57BL/6 male mice	Untreated	78.68 ± 3.139
	Untreated + DLT	55.88 ± 5.060
	MS-pretreated	85.75 ± 3.182
	MS-pretreated + DLT	36.50 ± 5.556*
WT C57BL/6 female mice	Untreated	81.58 ± 3.205
	Untreated + DLT	72.16 ± 2.621
	MS-pretreated	82.82 ± 2.529
	MS-pretreated + DLT	52.75 ± 5.303**

DLT, 5 μg of Deltorphin.

*$p < 0.01$ (ANOVA, Bonferroni's MCT, untreated plus DLT vs MS-pretreated plus DLT).

**$p < 0.001$ (ANOVA, Bonferroni's MCT, untreated plus DLT vs MS-pretreated plus DLT).

Effect of morphine pretreatment on subcellular distribution of δOR in mouse spinal cord

To determine whether in mice, as in rats, pretreatment with morphine increases the cell surface density of δOR, we studied the subcellular distribution of immunoreactive δOR by electron microscopy. In the dorsal horn (lamina II–V) of untreated wild-type C57BL/6 mice, silver-intensified immunogold particles, corresponding to immunoreactive δOR, were detected in association with perikarya and dendrites (Fig. 8*A*) of small intrinsic neurons as well as with axons and axon terminals. Within these immunolabeled neurons most immunoreactive δORs were intracellular rather than on the plasma membrane (Fig. 8*A*).

Pretreatment of WT mice with morphine (5, 8, 10, 15 mg/kg, s.c., every 12 hr) did not produce any significant change in the number of immunogold particles detected per unit area of dorsal horn dendrites when compared with untreated WT mice (Figs. 8*B*, 10*A*). However, the density of immunogold particles per unit length of plasma membrane was increased significantly from 0.09 to 0.18 (Fig. 10*B*; $p < 0.01$). Furthermore, the percentage of gold particles associated with dendritic plasma membranes in the same area was increased significantly from 6.6% of total gold particles in untreated to 12.4% of total gold particles in morphine-treated WT mice (Fig. 10*C*; $n = 3$ for both groups; $p < 0.001$).

Effect of μOR gene knock-out on morphine-induced upregulation of cell surface δOR

In the dorsal horn of the spinal cord of untreated μOR KO mice, the cellular and subcellular distribution of immunoreactive δOR was similar to that observed in untreated WT mice (Figs. 8*A*, 9*A*). In fact, no significant difference was observed in the overall density of immunogold particles (per dendritic unit area) in WT as compared with μOR KO mice (ANOVA, Bonferroni's MCT; $p > 0.05$; Fig. 10*A*). In addition, both the proportion of immunogold particles associated with the plasma membrane and the density of δOR per unit length of plasma membrane were identical to those observed in WT mice (Fig. 10*B*). The lack of difference in subcellular distribution between WT and μOR KO mice suggests that, under basal conditions, the presence of μOR is not required for the trafficking of δOR to the plasma membrane.

Morphine pretreatment of μOR KO mice did not change the subcellular distribution of δOR within dendrites in the dorsal horn of the spinal cord (Fig. 9*B*). Indeed, neither the percentage of immunogold particles associated with the plasma membrane nor the density of gold particles detected per unit length of membrane was changed in morphine-pretreated as compared with untreated μOR KO mice (Fig. 10*B,C*; ANOVA, Bonferroni's MCT; $p > 0.05$). Chronic pretreatment of μOR KO mice with morphine also failed to produce any change in the density of immunogold particles per unit area of dendrites when compared with either untreated μOR KO mice or with morphine-pretreated WT mice (Fig. 10*A*; ANOVA, $p > 0.05$). These results conclusively demonstrate that morphine targets δOR to the plasma membrane via selective stimulation of μOR.

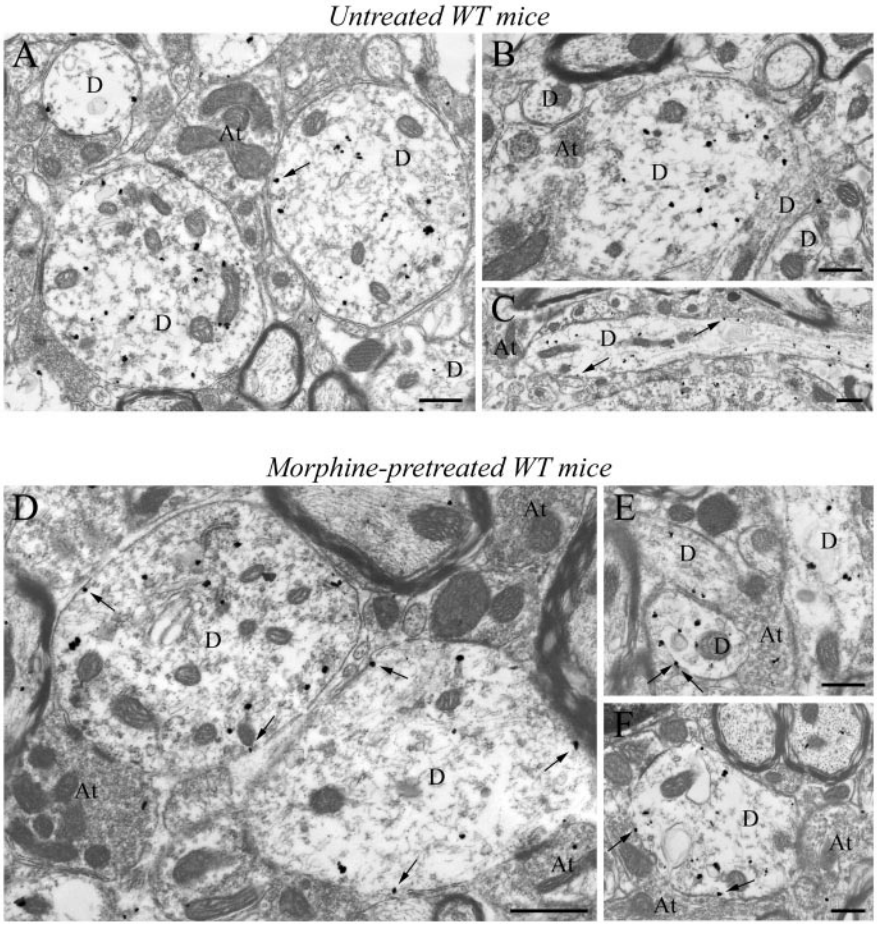

Figure 8. Subcellular localization of δOR immunoreactivity in the dorsal horn of the spinal cord of untreated versus morphine-pretreated wild-type mice. C57BL/6 WT mice were pretreated (*D–F*) or not (*A–C*) with morphine as described and were processed for immunogold detection of δORs 12 hr subsequent to the last morphine injection. In both conditions the majority of silver-intensified immunogold particles, representing δORs, is found inside dendrites (labeled D in *A–F*). In untreated mice a small proportion of gold particles is detected in association with the plasma membrane (denoted by arrows, *A–C*). However, pretreatment with morphine increases the occurrence of δORs associated with dendritic plasma membranes (arrows, *D–F*). At, Axon terminal. Scale bars: *A–F*, 0.5 μm.

Discussion

Studies with rats

We previously demonstrated that chronic treatment of rats with morphine leads to an increase in δOR-mediated antinociception (Cahill et al., 2001b). A first objective of the present study was to determine whether this effect was a property unique to morphine or characteristic of other μOR agonists. Pretreatment with methadone, etorphine, and fentanyl resulted in increases in δOR-mediated antinociception, as was observed with morphine. Because these drugs elicit acute antinociceptive effects in the tail-flick test over a wide range of ED_{50} values, the potency of the μOR agonist used as a "primer" drug does not appear to be critical in mediating the enhanced δOR response. Similarly, the capacity of these drugs to induce μOR internalization is not a determining factor. Indeed, morphine was as effective as the other μOR agonists in inducing increased δOR-mediated antinociception although it does not cause μOR internalization, in contrast to methadone, etorphine, and fentanyl (Keith et al., 1996, 1998; Whistler et al., 1999; Trafton et al., 2000; He et al., 2002). However, the *in vivo* pharmacokinetic profile of the μOR agonist is important for producing the increase in δOR-mediated antinociception. Indeed, an intermittent dosing regimen with fentanyl, in contrast to morphine, methadone, and etorphine, did not enhance δOR responsiveness. Fentanyl is characterized by a more rapid onset and duration of action than the other μOR agonists that were used, suggesting that continuous stimulation of μOR is necessary for induction of enhanced δOR responsiveness. Indeed, continuous delivery of fentanyl increased the effect of DLT when compared with saline-pretreated animals.

Continuous stimulation of μOR for up to 48 hr was necessary to induce changes in δOR responsiveness, because no increase in DLT potency was observed after 24 or 36 hr of treatment with morphine. One could argue that this enhanced responsiveness occurs only with the administration of a threshold cumulative dose of morphine. The results obtained with intermittent versus continuous delivery of fentanyl, however, do not support this interpretation. Indeed, the total amount of fentanyl delivered through the pumps over the 48 hr period was considerably lower than the cumulative amount administered during the intermittent dosage regimen (4.8 vs 17 μg, respectively, for a 225 gm rat).

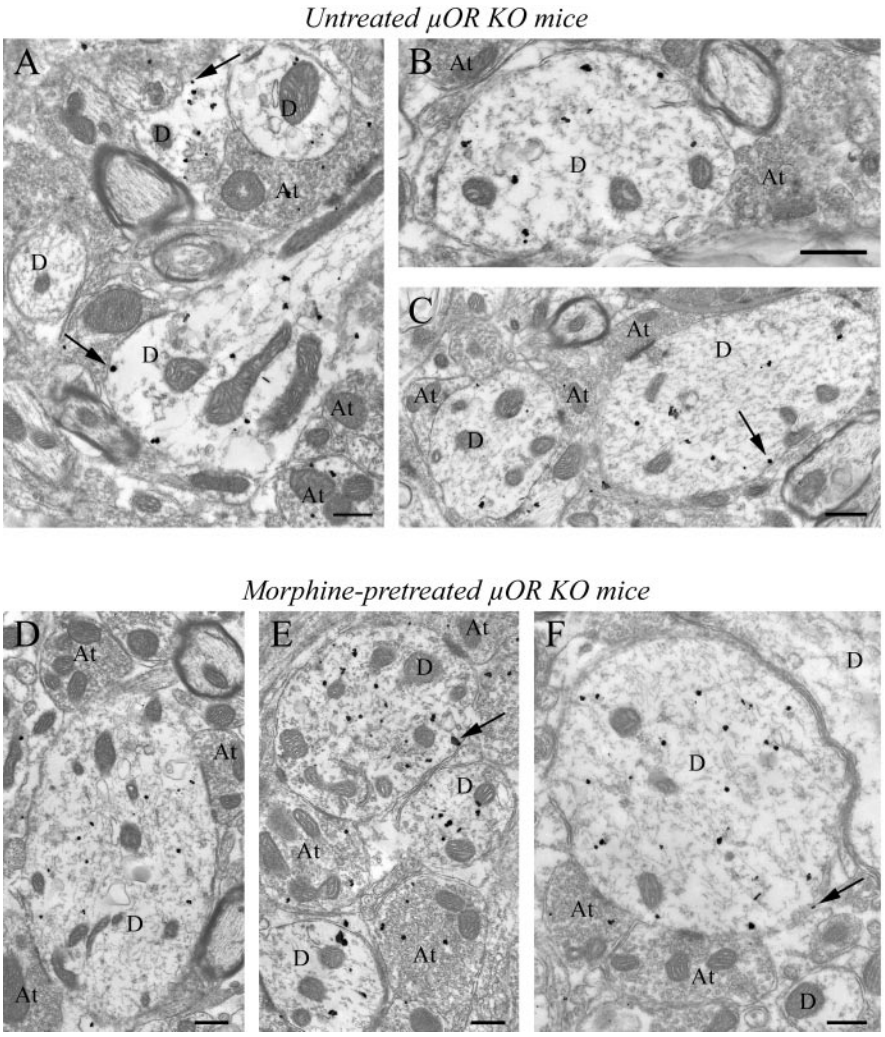

Figure 9. Immunogold electron microscopic localization of δOR in the dorsal horn of the spinal cord of untreated and morphine-pretreated μOR knock-out mice. C57BL/6 KO mice were pretreated (*D–F*) or not (*A–C*) with morphine as described and were processed for immunogold detection of δORs 12 hr subsequent to the last morphine injection. In both untreated (*A–C*) and morphine-pretreated (*D–F*) μOR KO mice the majority of immunogold particles is found within dendrites (labeled D in *A–F*). Pretreatment with morphine does not change the proportion of δORs associated with the plasma membrane. Arrows denote gold particles in association with the plasma membrane. At, Axon terminal. Scale bars: *A–F*, 0.5 μm.

Thus prolonged and sustained stimulation, rather than cumulative dosage, represents the important parameter in producing the enhancement of δOR agonist potency.

In a previous study we showed a correlation between the morphine-induced increase in δOR-mediated antinociception and enhanced recruitment of intracellular δORs to neuronal plasma membranes in the dorsal horn of the rat spinal cord (Cahill et al., 2001b). The present results imply that this cell surface targeting requires at least 48 hr of morphine pretreatment to occur. Such timing is considerably longer than previously documented for other receptor-mediated recruitment events. For example, treatment of hippocampal slices with insulin produced increased postsynaptic and dendritic plasma membrane density of $GABA_A$ receptors in the CA1 region in <10 min (Wan et al., 1997). Moreover, dopamine D_1 receptor density was increased after a 1 min pretreatment with atrial natriuretic peptide (ANP) in both LLCPK and kidney cells, whereas neuropeptide Y-induced recruitment of the α_{1A}-adrenergic receptor to the plasma membrane in LLPCK cells was observed after only 10 sec of stimulation (Holtbäck et al., 1999). The mechanisms governing the morphine-induced recruitment of δOR therefore must differ from those implicated in the recruitment of these other receptor types and likely involve novel protein synthesis. A number of accessory proteins have been linked to cell surface recruitment of G-protein-coupled receptors (GPCRs; for review, see Bouvier, 2001; Brady and Limbird, 2002). For example, chaperone molecules, such as ninaA for the rhodopsin 1 receptor (Colley et al., 1991), calnexin for the vasopressin V_2 receptor (Morello et al., 2001), or RAMP-1 (receptor activity modifying protein 1) for the calcitonin receptor-like receptor (CRLR; McLatchie et al., 1998), can assist in the proper folding of proteins and/or trafficking of GPCRs to the plasma membrane. Similarly, the trafficking to the plasma membrane of type I metabotropic glutamate receptors (mGluR) appears to be regulated by the interplay of constitutively expressed and inducible members of the Homer family (Xiao et al., 1998; Roche et al., 1999; Ango et al., 2000, 2002; Sato et al., 2001). In the case of δOR it was proposed recently that intracellular chaperoning of the receptor from the endoplasmic reticulum to the cell surface by δOR agonists and antagonists may rescue improperly folded receptor proteins, leading to increased agonist binding (Petaja-Repo et al., 2002). Hence prolonged stimulation with a μOR agonist could induce the expression of a gene product involved in the trafficking/folding of δORs to the plasma membrane.

A number of studies have provided evidence for the involvement of δOR in the development and/or maintenance of morphine-induced tolerance (Abdelhamid et al., 1991; Fundytus et al., 1995; Kest et al., 1996; Hepburn et al., 1997; Zhao et al., 2002). Moreover, tolerance to repeated administration of morphine does not develop in δOR KO mice (Zhu et al., 1999). Therefore, it was tempting to speculate that the μOR-induced increase in δOR membrane density documented here might be implicated in producing the tolerance to μOR agonists observed after repeated stimulation with these agonists. However, the results of our acute morphine challenge experiments demonstrated that the enhanced recruitment of δORs to the plasma membrane observed after chronic morphine pretreatment was not correlated directly with tolerance to morphine.

The morphine-induced enhancement of δOR-mediated antinociception was no longer measurable 24 hr after the last dose of the pretreatment, indicating that this effect is reversible. This reversibility is likely attributable to restoration of membrane-targeted δORs to intracellular stores after cessation of morphine administration, because the decrease in δOR-mediated antinociception was correlated with a return of immunoreactive δOR plasma membrane levels to basal values, as determined by electron microscopic immunocytochemistry. Because of the prolonged time course, constitutive internalization of δOR is the

most likely explanation to account for this restoration of cell surface receptors to baseline; indeed, agonist-independent internalization recently has been shown for δOR in CHO cells transfected with this receptor (Trapaidze et al., 2000). Nonetheless, we cannot exclude the possibility that the cell surface density of δOR also decreases in response to steady-state endogenous release of opioid peptides. Indeed, δORs have been documented in neuro2A and HEK293 cells to be endocytosed efficiently after exposure to peptide agonists and to be targeted to lysosomes for degradation (Ko et al., 1999; Tsao and von Zastrow, 2000; Whistler et al., 2002), which would account for their lack of reappearance at the cell surface.

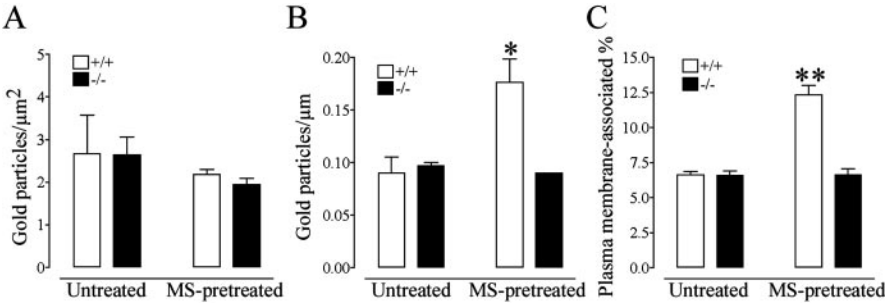

Figure 10. The presence of μOR is necessary for morphine-induced targeting of δOR to plasma membranes. *A*, Each bar represents the density of immunogold particles (particles/μm^2; $n = 3$ for each group). No statistically significant differences were observed (ANOVA; $p > 0.05$). *B*, Each bar in the graph represents the density of immunogold particles per unit length of plasma membrane. Morphine pretreatment markedly increases the density (from 0.09 to 0.18) in WT mice as compared with untreated controls (ANOVA, Bonferroni's MCT; $^*p < 0.01$). No statistical difference was found between the density of immunogold particles per unit length of membrane in morphine-pretreated as compared with untreated μOR KO mice (ANOVA, Bonferroni's MCT; $p > 0.05$). *C*, The percentage of gold particles associated with the plasma membrane was increased significantly in WT mice pretreated with morphine when compared with untreated WT mice (ANOVA, Bonferroni's MCT; $^{**}p < 0.001$). No change in the percentage of δOR associated with the plasma membrane was observed between untreated and morphine-pretreated μOR KO mice (ANOVA; $p > 0.05$). In all panels +/+ denotes the WT, whereas −/− represents μOR KO C57BL/6 mice. Data are presented as the average ± SEM.

Studies with mice

To determine whether stimulation of μOR was necessary for targeting of δORs to neuronal plasma membranes, we investigated the functionality and cellular distribution of δOR in mice with or lacking μORs.

Wild-type mice pretreated with morphine displayed the same enhancement of δOR-mediated antinociception in a persistent pain paradigm as previously documented in rats (Cahill et al., 2001b). Also as observed in the rat, this change in δOR responsiveness was correlated with alterations in the subcellular distribution of δORs, as assessed by electron microscopic immunocytochemistry. Whereas in untreated animals the bulk of δOR immunoreactivity detected within layers II–V of the dorsal horn of the spinal cord was predominantly intracellular, as previously described in the rat (Cheng et al., 1995, 1997; Cahill et al., 2001a,b), in mice pretreated with morphine a significantly higher proportion of δOR was found in association with neuronal plasma membranes. This change in the subcellular distribution of δORs was not accompanied by an augmentation in total δOR protein levels, as assessed by electronic microscopic localization of δORs. These observations support the hypothesis that the increase in DLT-mediated antinociception is a consequence of mobilization of δOR from intracellular stores to the plasma membrane rather than of augmented receptor neosynthesis.

The overall density of δORs in layers II–V of the dorsal horn of the spinal cord was the same in WT and μOR KO mice. These results are consistent with those of earlier radioligand binding studies with DPDPE and deltorphin-I, which also detected no major compensatory changes in total δOR expression in the brain of μOR KO mice (Matthes et al., 1996; Kitchen et al., 1997; Sora et al., 1997; Loh et al., 1998; Chen et al., 2000) (for review, see Kieffer and Gavériaux-Ruff, 2002). The subcellular distribution (cell surface vs intracellular) of δOR was also similar between WT and μOR KO mice, suggesting that the presence of μOR is not a prerequisite for the maintenance of δOR cell surface density at steady state and thus of the integrity of basal δOR responsiveness. Congruent with this interpretation, previous studies have reported similar levels of [^{35}S]GTPγS binding in the brain of WT and μOR KO mice in response to stimulation with deltorphin-I (Matthes et al., 1998; Hosohata et al., 2000). Furthermore, the antinociceptive potency of intrathecally administered deltorphin-I was identical between μOR KO and WT mice (Hosohata et al., 2000), in accord with the present subcellular distribution results.

Treatment of μOR KO mice with morphine did not promote recruitment of δOR to the plasma membrane from intracellular stores. This result provides important confirmation that the morphine-induced upregulation of cell surface δOR was, in fact, the result of morphine acting via selective stimulation of μOR and not via interaction with δORs or other targets. Nonetheless, it remains possible that events independent from μOR stimulation may be implicated in the upregulation of cell surface δOR and/or enhanced δOR responsiveness in μOR KO mice. Indeed, δOR agonists were found to produce significantly greater antihyperalgesia in μOR KO mice when compared with WT mice injected with Complete Freund's Adjuvant (Qiu et al., 2000). Nonetheless, the present results demonstrate that KO animals may exhibit discrete phenotypic traits that can be evidenced only after pathophysiological or pharmacological challenge.

In conclusion, we have demonstrated that sustained pretreatment with different μOR agonists leads to a reversible increase in δOR-mediated antinociception in rats and mice and have established, via studies in μOR KO mice, that this effect is entirely dependent on activation of μOR. These results have important clinical ramifications because they suggest that "priming" of subjects with μOR agonists such as morphine could be exploited to increase the potency of δOR agonists.

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