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# MORPHINE PRIMING IN RATS WITH CHRONIC INFLAMMATION REVEALS A DICHOTOMY BETWEEN ANTIHYPERALGESIC AND ANTINOCICEPTIVE PROPERTIES OF DELTORPHIN

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**Abstract—We previously showed that prolonged morphine treatment and chronic inflammation both enhanced delta opioid receptor ($\delta$OR) cell surface density in lumbar spinal cord neurons. Here, we sought to determine whether administration of morphine to rats with chronic inflammation would further increase the bio-availability of $\delta$OR, and thereby the analgesic properties of the $\delta$OR agonist deltorphin, over that produced by inflammation alone. We found that chronic inflammation produced by injection of complete Freund's adjuvant (CFA) into the hind paw resulted in a bilateral increase in the binding and internalization of fluorescent deltorphin in neurons of the lumbar spinal cord as did prolonged morphine treatment [Morinville A, Cahill CM, Aibak H, Rymar VV, Pradhan A, Hoffert C, Mennicken F, Stroh T, Sadikot AF, O'Donnell D, Clarke PB, Collier B, Henry JL, Vincent JP, Beaudet A (2004a) Morphine-induced changes in delta opioid receptor trafficking are linked to somatosensory processing in the rat spinal cord. J Neurosci 24:5549–5559]. This effect was accompanied by an increase in the antinociceptive efficacy of intrathecal deltorphin as measured using the tail-flick test. Treatment of CFA-injected rats with morphine decreased the cell surface availability of $\delta$OR in neurons of the dorsal horn of the lumbar spinal cord as compared with treatment with CFA alone. Behaviorally, it significantly enhanced the antihyperalgesic effects of deltorphin (plantar test; % maximum possible antihyperalgesic effect (MPAHE)=113.5%±32.4% versus 26.1%±11.6% in rats injected with CFA alone) but strongly reduced the antinociceptive efficacy of the drug (tail-flick test; % maximum possible antinociceptive effect (MPE)=29.6%±3.6% versus 66.6%±6.3% in rats injected with CFA alone) suggesting that the latter, but not the former, is linked to the $\delta$OR trafficking events observed neuroanatomically. These results demonstrate that in chronic inflammation, the antihyperalgesic effects of $\delta$OR agonists may be enhanced by morphine pre-treatment. They also reveal a dichotomy between mechanisms underlying antihyperalgesic and antinociceptive effects of $\delta$OR agonists. © 2006 IBRO. Published by Elsevier Ltd. All rights reserved.**

**Key words: delta opioid receptor, deltorphin, chronic inflammation, antihyperalgesia, antinociception, tolerance.**

Agonists acting through the mu opioid receptor ($\mu$OR), such as morphine and its derivatives, induce potent analgesic effects (Bodnar and Klein, 2004). However, they also give rise to undesirable side-effects such as nausea, constipation, and respiratory depression (Colpaert, 1996; Kreek, 1996). In addition, chronic stimulation of $\mu$OR induces tolerance and physical dependence (Cowan et al., 1988). By contrast, drugs acting on $\delta$OR produce more limited analgesia, but also give rise to considerably less undesirable side-effects and induce virtually no tolerance (Porreca et al., 1984; May et al., 1989; Sheldon et al., 1990; Szeto et al., 1999; Gallantine and Meert, 2005). For these reasons, $\delta$OR agonists have been proposed as possible alternatives to $\mu$OR agonists for the treatment of chronic pain, including neuropathic (Mika et al., 2001; Petrillo et al., 2003; Morinville et al., 2004a) and chronic inflammatory pain (Desmeules et al., 1993; Stewart and Hammond, 1994; Fraser et al., 2000; Hurley and Hammond, 2000; Qiu et al., 2000; Cahill et al., 2003; Petrillo et al., 2003).

One of the reasons for the relatively poor analgesic efficiency of $\delta$OR agonists may be that only a small proportion of $\delta$OR is actually present on neuronal plasma membranes under baseline conditions (Cheng et al., 1995, 1997; Elde et al., 1995; Zhang et al., 1998; Cahill et al., 2001a). However, under conditions of chronic inflammation, such as produced in rodents by injection of complete Freund's adjuvant (CFA) in the hind paw, we observed a massive recruitment of $\delta$OR from intracellular stores to the plasma membrane in neurons of the dorsal horn of the spinal cord (Cahill et al., 2003; Morinville et al., 2004b). This increase in the pharmacological availability of $\delta$OR was postulated to account for the enhanced antihyperalgesic efficacy of the centrally administered $\delta$OR agonists reported in conditions of chronic inflammation (Hylden et al., 1991; Stewart and Hammond, 1994; Fraser et al.,

2000; Hurley and Hammond, 2000; Cahill et al., 2003; Morinville et al., 2004b). The CFA-induced up-regulation of cell surface $\delta$OR in spinal cord neurons involves the participation of $\mu$OR as it can no longer be elicited in $\mu$OR knockout mice (Morinville et al., 2004b). The effect of chronic inflammation on $\delta$OR trafficking is therefore akin to that of prolonged treatment with $\mu$OR-preferring agonists (including morphine, fentanyl, methadone and etorphine), which was shown to induce a selective membrane recruitment of $\delta$OR in neurons of layers I–VI of the rat lumbar spinal cord (Cahill et al., 2001b; Morinville et al., 2003). As in animals with chronic inflammation, this increased density of cell surface $\delta$OR translates into a potentiation of the antinociceptive properties of deltorphin II injected intrathecally (i.t.) (Cahill et al., 2001b; Morinville et al., 2003). However, it is unclear whether chronic inflammation and sustained morphine treatment affect the same neuronal populations in the lumbar spinal cord and whether their effects are independent or cumulative.

The aim of the present study was therefore to investigate the distribution of neurons which up-regulate cell surface $\delta$OR in the lumbar spinal cord in response to chronic inflammation of the hind paw and to determine whether this up-regulation is affected by a morphine treatment in an attempt to determine if pre-treatment of animals with morphine (to heighten the cell surface density of $\delta$OR) would further enhance the analgesic efficacy of $\delta$OR agonists in the treatment of chronic pain.

## EXPERIMENTAL PROCEDURES

### Animals

All experiments were carried out in adult male Sprague–Dawley rats (220–280 g; Charles River, St-Constant, Quebec, Canada), maintained on a 12-h light/dark cycle. All experiments were approved by the local animal care committees of McGill University, Queen's University, or AstraZeneca R&D Montreal and conformed to Canadian Council on Animal Care guidelines on the ethical use of animals. All experiments were designed to minimize the number of animals used and their suffering.

### Induction of chronic inflammation

Chronic inflammation was induced by a s.c. injection of 100 $\mu$l CFA (Calbiochem, San Diego, CA, USA) in the plantar surface of the left hind paw of rats under ketamine/xylazine (i.p. 20/4 mg per 100 g of body weight) or isoflurane anesthesia (3%, 1 L/min). Control rats were left untreated (naïve). Behavioral testing or *in vivo* $\delta$OR internalization assays were carried out 72 h after CFA injection as described below. To evaluate the level of inflammation, following behavioral testing the hind paws were cut at the level of the ankle joint and weighed.

### Prolonged morphine treatment

Naïve rats and rats injected with intraplantar CFA 24 h earlier received s.c. injections of increasing doses of morphine sulfate (MS; Sabex Inc., Boucherville, Quebec, Canada) every 12 hours for 48 h (5, 8, 10 and 15 mg/kg; standard dose), as described elsewhere (Cahill et al., 2001b). The drug was diluted in aqueous 0.9% NaCl solution (saline) from a 50 mg/ml stock solution in saline. Control rats (saline) received corresponding volumes of saline. Behavioral testing or *in vivo* $\delta$OR internalization assays

were carried out 12 h after the last morphine or saline injection. To determine the dose-response of morphine pre-treatment on the antinociceptive efficacy of deltorphin, additional groups of naïve rats were treated: (1) for 48 h with half (1, 3, 5, and 8 mg/kg; low dose) or twice (10, 15, 20 and 30 mg/kg; high dose) the standard morphine dose; or (2) for 96 h with a modified standard morphine dose (5, 8, 10, 10, 10, 10, 10, 15 mg/kg).

### In vivo $\delta$OR internalization assay

To assess the cell surface availability of $\delta$OR in neurons of the lumbar spinal cord (L4-5), naïve rats ($n=3$), rats treated with CFA 72 h earlier ($n=3$), and rats treated with CFA and subjected to prolonged treatment with morphine (standard dose; $n=3$) were injected i.t. with 30 $\mu$l of $\omega$-Bodipy 576/589 deltorphin-I 5-aminopentylamide (Fluo-DLT) as described (Morinville et al., 2004a). Briefly, animals were anesthetized with sodium pentobarbital (Somnotol; MTC Pharmaceuticals, Cambridge, Ontario, Canada, 6.5 mg/100 g of body weight) and injected i.t. via a lumbar puncture with 0.8 nmol Fluo-DLT diluted in 30 $\mu$l saline at the L5–L6 intervertebral space. Appropriate placement of the needle was validated by the observation of a little flick of the tail. Twenty minutes after injection of the fluorescent ligand, rats were killed by intra-aortic arch perfusion of, in succession, 4% paraformaldehyde (PFA) in 500 ml 0.1 M phosphate buffer (PB, pH 7.4) at 4 °C and 100 ml each of 10%, 20%, and 30% sucrose in 0.2 M PB (pH 7.4). The lumbar segment of the spinal cord was snap-frozen in isopentane at −45 °C and stored at −80 °C until sectioning. Tissues were sectioned on a cryostat at a thickness of 20 $\mu$m and thaw-mounted onto chrome alum/gelatin-coated slides (without coverslips).

Neurons having specifically bound and internalized Fluo-DLT (characterized by the presence of intra-cytoplasmic fluorescent puncta) were visualized using a Zeiss confocal laser scanning microscope LSM510 (Carl Zeiss Canada Ltd., Toronto, Ontario, Canada) equipped with an inverted microscope (oil-immersion objectives, 25×, 40× and 63×) and a He/Ne laser with an excitation wavelength of 543 nm. For each animal, five representative images were acquired on each side of the lumbar spinal cord, contralateral and ipsilateral to the inflamed paw, in each of laminae III, V–VI, and IX. The measurement of internalized Fluo-DLT in laminae I–II was excluded because distinct neuronal profiles were difficult to identify. Indeed, in these superficial layers, the labeling is mainly in processes and the level of background fluorescence is higher than in the deeper layers. Images were converted to a gray scale and an internal background value defined. The fluorescence-labeling density above the background was measured (by an experimenter blinded to treatments) for each single cell profile using NIH Image J software. Only cells in which the nucleus could be clearly discerned were analyzed (three to seven cells per image). Fluorescence intensity values were averaged for each lamina sampled in each animal. Means were then determined for each experimental group ($n=3$ per group) and expressed as relative fluorescence intensity (in arbitrary units)$\pm$S.E.M. Calculations and statistical analyses were carried out using Microsoft Excel 2000, Prism GraphPad 3.0 and Sigma Plot 2001.

### Real-time PCR analysis of $\delta$OR expression

To determine whether prolonged treatment with morphine or intraplantar injection of CFA affected the expression of $\delta$OR in the lumbar spinal cord, rats left untreated (naïve animals), rats treated either with saline or morphine (standard dose: 5, 8, 10 and 15 mg/kg), and rats injected with CFA (100 $\mu$l in the left hind paw) were anesthetized with sodium pentobarbital (Somnotol; 6.5 mg/100 g of body weight) and humanely killed by decapitation. The spinal cord was removed by pressure extrusion and the L4-5 lumbar segments were dissected. In CFA-treated animals, these lumbar

segments were further divided in four sub-sections: dorsal and ventral horns of ipsi- and contralateral sides.

Tissue samples were then processed for RNA extraction using the SV total RNA isolation system (Promega, Madison, WI, USA). Amplification of δOR mRNA was achieved using the one-step QuantiTect® SYBR® Green RT-PCR kit (Qiagen, Mississauga, Ontario, Canada) as described by the supplier. Briefly, 120 ng of template RNA was mixed on ice with 12.5 pmol of both sense (position 306 in exon 1: 5′-TGCTCGTCATGTTTG-GAATCGTC-3′) and antisense (position 386 in exon 2: 5′-GC-CAAGGCCAGATTGAAGATGTAG-3′) primers (amplicon's length is 79 bp, with a melting temperature of 81.3 °C), 12.5 $\mu$l of the 2X QuantiTect® SYBR® Green RT-PCR Master Mix, and 0.25 $\mu$l of QuantiTect® RT mix in a final reaction volume of 25 $\mu$l. A primer pair was also specifically designed for the amplification of rat GAPDH (5′-TGGTGCCAAAAGGGTCATC-3′ and 5′-CTTCCAC-GATGCCAAAGTTG-3′ for sense and antisense primers, positions 366 and 541 of exons 6 and 7, respectively), used as internal standard (the amplicon's length is 176 bp, with a melting temperature of 85.2 °C). The one-step, real-time RT-PCR analysis was performed on a Rotor-Gene RG-3000 and data were analyzed with RotorGene 4.6 software (Corbett Research, MBI, Montreal, Quebec, Canada). The following parameters were used: Reverse transcriptase reaction was performed at 50 °C for 30 min, after which the enzymes (Omniscript® and Sensiscript® reverse transcriptases) were inactivated at 95 °C for 15 min. The latter step also served in activating the HotStartTaq® DNA polymerase. Amplification was then achieved with 35 cycles of denaturation (15 s at 94 °C), annealing (30 s at 54 °C), and extension (30 s at 72 °C; fluorescence intensity was read at the end of this step). Comparative concentration of δOR mRNA in each sample was determined with RotorGene 4.6 software and Excel 2000 (Microsoft Corporation, Seattle, USA), using GAPDH as the housekeeping gene, i.e. the ratio of δOR/GAPDH mRNA fluorescence signal levels.

## Behavioral testing

*Plantar test.* To test for thermal withdrawal thresholds, rats treated with 0.9% NaCl (saline; $n=6$ animals), rats treated with prolonged morphine (standard dose; $n=5$), rats injected with CFA 72 h prior to testing ($n=6$), and rats injected with CFA and subjected to prolonged treatment with morphine (standard dose; $n=6$) were placed in Plexiglas® boxes positioned on a glass surface (IITC Life Science Inc., Woodland Hills, CA, USA). After a 15 min habituation period, the heat source was positioned under the plantar surface of the hind paw and the paw withdrawal latency in response to radiant heat was measured. For animals injected with CFA and with CFA and morphine, the paw withdrawal latency in response to radiant heat was measured prior to, and every 10 min after, deltorphin injection (10 $\mu$g in 30 $\mu$l of 0.9% saline, i.t.) over a period of 30 min. Appropriate placement of the needle was validated by the observation of a little flick of the tail. The intensity of the light beam was adjusted so that baseline latencies were 6–8 s in naïve rats. A cutoff time of 3× baseline was imposed to prevent tissue damage. The cutoff time was calculated independently for each paw. If an animal reached the cutoff, the light beam was turned off and the animal was assigned the maximum score. The maximum possible antihyperalgesic effect (MPAHE, return to baseline pre-injury withdrawal thresholds) of deltorphin in CFA rats was calculated, for the inflamed hind paw (i.e. the paw injected with CFA), according to the following formula:

$$\%MPAHE=100\times$$

$$\frac{(\text{test latency}) - (\text{baseline latency following CFA})}{(\text{baseline prior to CFA}) - (\text{baseline latency following CFA})}$$

From the latter calculation, a MPAHE of 0% represents no antihyperalgesic effect of the drug while a MPAHE of 100% cor-

responds to a complete relief of the hyperalgesia, i.e. to a response latency to radiant heat identical to baseline (prior to CFA injection).

The maximum possible antinociceptive effect (MPE; contralateral hind paw) of deltorphin in rats injected with CFA and treated or not with morphine (5, 8, 10, and 15 mg/kg) was calculated according to the following formula:

$$\%MPE=100\times\frac{(\text{test latency}) - (\text{baseline latency})}{(\text{cutoff}) - (\text{baseline latency})}$$

*Tail-flick test.* To test for deltorphin's antinociceptive effects, tail-flick latencies were measured in rats injected with saline ($n=6$) and in rats treated with CFA for 72 h ($n=6$) and subjected or not to prolonged treatment with morphine (low doses; $n=6$, standard doses; $n=8$), before and every 10 min after deltorphin injection (10 $\mu$g in 30 $\mu$l of 0.9% saline, i.t.) over a period of 50 min. Appropriate placement of the needle was validated by the observation of a little flick of the tail. In an additional set of experiments, deltorphin challenge was carried out in rats treated for 48 h with standard (5, 8, 10 and 15 mg/kg; standard doses, $n=7$), half (1, 3, 5, and 8 mg/kg; low doses, $n=7$), or double (10, 15, 20 and 30 mg/kg; high doses, $n=9$) doses of morphine, or for 96 h with modified standard doses (5, 8, 10, 10, 10, 10, 10, 15 mg/kg, $n=4$). Five centimeters of the tail was immersed in a waterbath maintained at 52 °C. Latency to response was determined by a vigorous tail-flick. A cutoff time of 10 s was imposed to avoid tissue damage. If an animal reached cutoff, the tail was removed from the water and the animal was assigned the maximum score. The MPE of deltorphin was calculated according to the following formula:

$$\%MPE=100\times\frac{(\text{test latency}) - (\text{baseline latency})}{(\text{cutoff}) - (\text{baseline latency})}$$

## Met-enkephalin measurements

To determine whether injection of CFA induced the release of met-enkephalin in the spinal cord, untreated rats (naïve; $n=3$) or rats treated with CFA for 72 h ($n=4$) were anesthetized with sodium pentobarbital (Somnotol; 6.5 mg/100 g of body weight) and killed by decapitation. The spinal cord was rapidly removed by pressure extrusion and the L4-5 lumbar segments were dissected out. These lumbar segments were further divided in four subsections: dorsal and ventral horns of ipsi- and contralateral sides, and immediately frozen on dry ice. Tissue samples were then kept at −80 °C until assayed. The spinal cord tissue was homogenized in 1 ml of 50 mM Tris–HCl (pH 7.5) containing 10% sucrose and protease inhibitors (cocktail containing peptstatin A, E-64, bestatin, leupeptin, and aprotinin and 4-(2-aminoethyl)benzenesulfonyl fluoride; Sigma). An aliquot was removed for protein estimation. The homogenate was incubated at 90 °C for 30 min to inactivate proteases and clarified by centrifugation at 13,000×$g$ for 15 min. Approximately 200 $\mu$l of sample supernatant was analyzed for ir-Met-Enk using the Met-enkephalin radioimmunoassay (RIA). For this, the samples were incubated with a 1:750 dilution of Met-enkephalin antiserum (Bachem Bioscience Inc, Philadelphia, PA, USA) in a RIA buffer (10 mM Tris–HCl buffer, pH 7.5, containing 0.1% gelatin (Bio-Rad, Hercules, CA, USA), 0.1% bovine serum albumin (protease-free; Sigma), 0.1% Triton X-100, and 0.02% sodium azide). On the following day, ^{125}I-Met-enkephalin (∼10,000 cpm/tube; Bachem Bioscience Inc.) was added and the tubes were incubated overnight at 4 °C. To terminate the reaction, 100 $\mu$l of goat anti-rabbit globulin and 100 $\mu$l of normal rabbit serum were added. The antigen-antibody complex was separated from the unbound radioligand according to the manufacturer's protocol (Peninsula Laboratories Inc, San Carlos, CA, USA). Since there was no significant difference between them, data from contra- and ipsilateral sides were pooled.

## RESULTS

### Effect of CFA injection, followed or not by a 48 h treatment with morphine, on the cell surface availability of δOR in rat lumbar spinal cord

To determine the distribution of neurons showing increased cell surface recruitment of δOR in rats subjected to chronic inflammation and to assess whether this recruitment would be affected by treating the animals with morphine for 48 h, we used an *in vivo* internalization assay based on the i.t. injection of the fluorescent deltorphin analog, Fluo-DLT. As previously described elsewhere (Morinville et al., 2004a), this assay is based on the principle that the amount of Fluo-DLT internalized over a 20 min period, as quantified by densitometry in confocal images of the spinal cord, reflects the density of cell surface receptors available to bind and internalize the fluorescent ligand during this period.

In sections from the lumbar spinal cord of saline-treated, CFA-injected, and CFA-injected plus morphine-treated rats, numerous fluorescently-labeled neurons were detected by confocal microscopy throughout the gray matter of the spinal cord. Labeled cells were filled with small fluorescent puncta, typical of endocytosed Fluo-DLT (Fig. 1). Densitometric quantification of intracellular fluorescence levels revealed that Fluo-DLT internalization, and by extension cell surface availability of δOR, was higher in rats subjected to CFA injection than in naïve rats in lamina III ($1.31\pm0.06$ fold; $P<0.01$) and in laminae V–VI ($1.53\pm0.05$ fold; $P<0.01$) on the side ipsilateral to the inflammation (Fig. 1A, B and 1F, G, respectively; Fig. 2A, B). This effect was selective for neurons of the dorsal horn, as no significant difference between naïve and CFA-injected rats was observed in motor neurons of lamina IX (Fig. 1K, L; Fig. 2C; $P>0.05$, ANOVA, Tukey's multiple comparison test). A significant increase in Fluo-DLT internalization was also observed on the side contralateral to the inflammation (lamina III: $P<0.01$; laminae V–VI: $P<0.05$ as compared with saline; ANOVA, Tukey's multiple comparison test).

The increase in Fluo-DLT internalization observed here following CFA injection occurred in the same layers of the lumbar spinal cord as those previously reported following prolonged treatment with morphine (Morinville et al., 2004a). However, when morphine was administered to CFA-treated animals, we observed a bilateral reduction in fluorescence intensity levels, and hence in δOR availability, in neurons from lamina III ($P<0.001$) and laminae V–VI ($P<0.01$ and $P<0.05$ for ipsi- and contralateral sides, respectively) as compared with rats treated with CFA alone (Fig. 1D, E and I, J, versus Fig. 1B, C and G, H respectively; Fig. 2) (ANOVA, Tukey's multiple comparison test).

### Effect of prolonged morphine treatment and of chronic inflammation on the expression of δOR in the lumbar spinal cord

To determine whether the increase in δOR cell surface availability observed after CFA injection and the decrease observed after combined CFA/morphine were pure traffick-

ing events or could be attributed, at least in part, to modified levels of expression of the receptor, we quantified by real-time PCR δOR mRNA levels in the spinal cord of morphine-treated, CFA-, or CFA/MS-injected rats and compared them to those in saline-injected (s.c.) and untreated controls (naïve), respectively.

As shown in Table 1, no significant differences in δOR mRNA levels were apparent between the lumbar spinal cords of CFA-, CFA/MS, and naïve animals ($P>0.05$, ANOVA, Tukey's multiple comparison test) or between ipsi- and contralateral dorsal or ventral horns in CFA- and CFA/MS-treated rats ($P>0.05$, ANOVA, Tukey's multiple comparison test). Likewise, there was no statistically significant difference in lumbar spinal cord δOR mRNA levels between morphine-treated and saline-injected rats ($P=0.745$, two-tailed unpaired $t$-test; Table 1).

### Effect of prolonged morphine treatment on the antihyperalgesic effects of deltorphin II in CFA-injected rats

We previously showed that deltorphin had a greater analgesic potency in CFA-treated rats than in control animals (Cahill et al., 2003). In the present study, we sought to characterize the antihyperalgesic effects of deltorphin in this model of chronic inflammation and to determine whether these effects could be further enhanced by subjecting the animals to prolonged morphine exposure, since prolonged morphine treatment on its own had also been shown to augment δOR-mediated antinociception (Cahill et al., 2001b). To address this issue, we compared plantar thermal withdrawal latencies of the inflamed paw following i.t. injection of deltorphin in rats: (1) injected with CFA alone; and (2) injected with CFA and treated with morphine (CFA/MS).

To ensure that all of our animals had comparable levels of inflammation and hyperalgesia, we first assessed the gain in weight (edema) and the plantar thermal withdrawal latency of the inflamed paw in rats injected with CFA alone and in rats injected with CFA and then treated with morphine. Three days post-CFA injection, there was no significant difference ($P=0.177$, two-tailed unpaired $t$-test) in the weight of the inflamed hind paw between CFA-injected rats pre-treated ($137\%\pm2\%$ that of naïve rats, $n=9$) or not ($133\%\pm2\%$ that of naïve rats, $n=9$) with morphine. At that time, both groups of animals had developed comparable unilateral hyperalgesia (i.e. decreased latency to response in the plantar test 72 h post-CFA injection compared with naïve animals; Table 2). No hyperalgesic symptoms were apparent on the contralateral side in either group of animals (Table 2). Thus, using our parameters for the plantar test, morphine treatment did not affect CFA-induced hyperalgesia (as tested 12 h after the last morphine injection) when administered after the pro-inflammatory drug.

As illustrated in Fig. 3A, i.t. administration of deltorphin produced a slight antihyperalgesic effect (%MPAHE= $26.1\%\pm11.6\%$) in rats injected with CFA alone but a complete relief of hyperalgesia (i.e. response latency to radiant heat identical to baseline prior to CFA injection) in CFA-injected rats submitted to prolonged morphine exposure (%MPAHE=$113.5\%\pm32.4\%$, $P=0.035$ as compared with
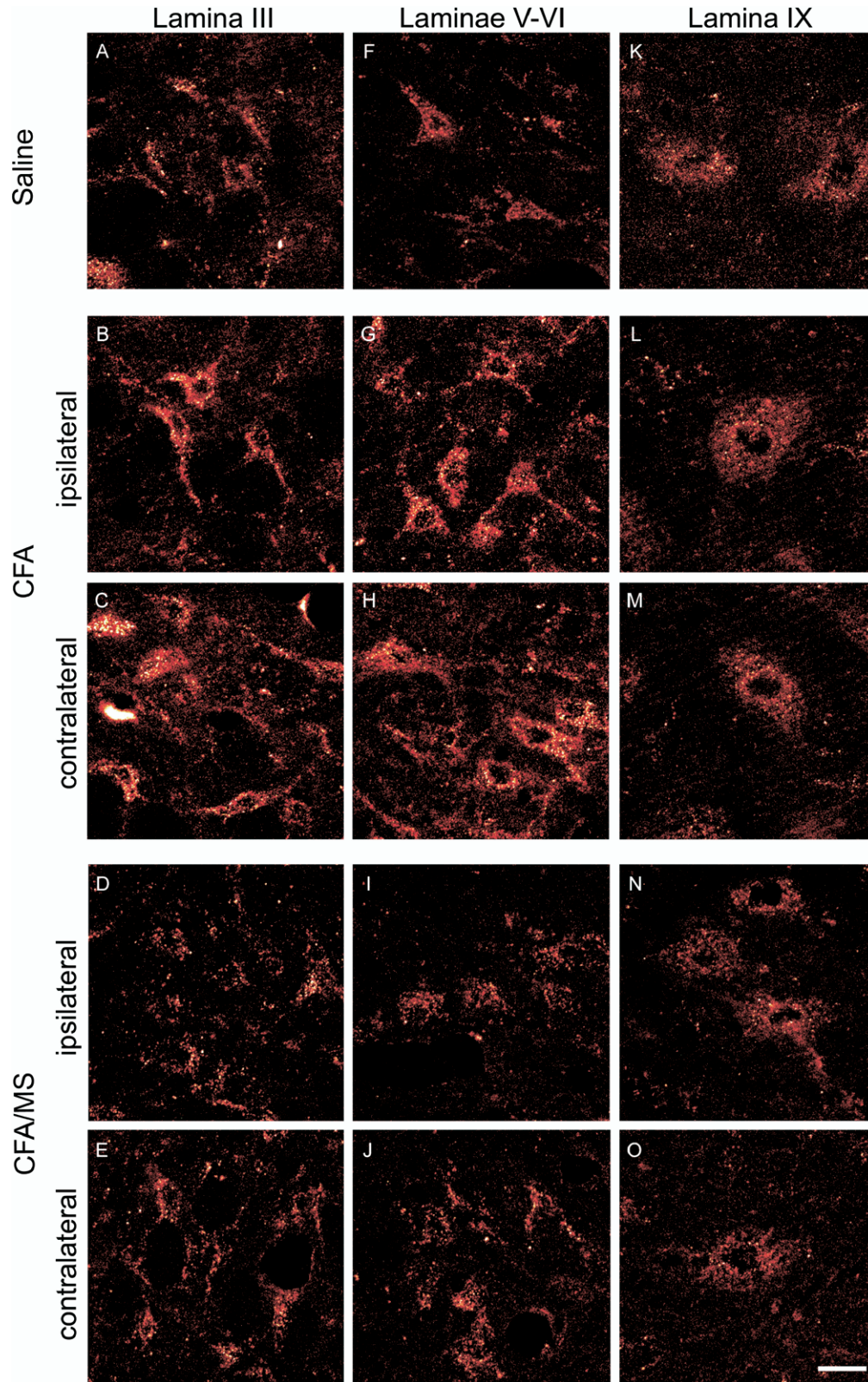
**Fig. 1.** Effect of CFA and CFA followed by a MS treatment on Fluo-DLT internalization in rat spinal cord. Naïve (A, F and K), CFA- (B–C, G–H and L–M), and CFA/MS-treated animals (D–E, I–J and N–O) were injected i.t. with 0.8 nmol of Fluo-DLT and processed for confocal microscopic analysis as described in Experimental Procedures. Red–white glow-scale images (scale bar=20 $\mu$m) of neurons from lamina III (A–E), laminae V–VI (F–J) and lamina IX (K–O).
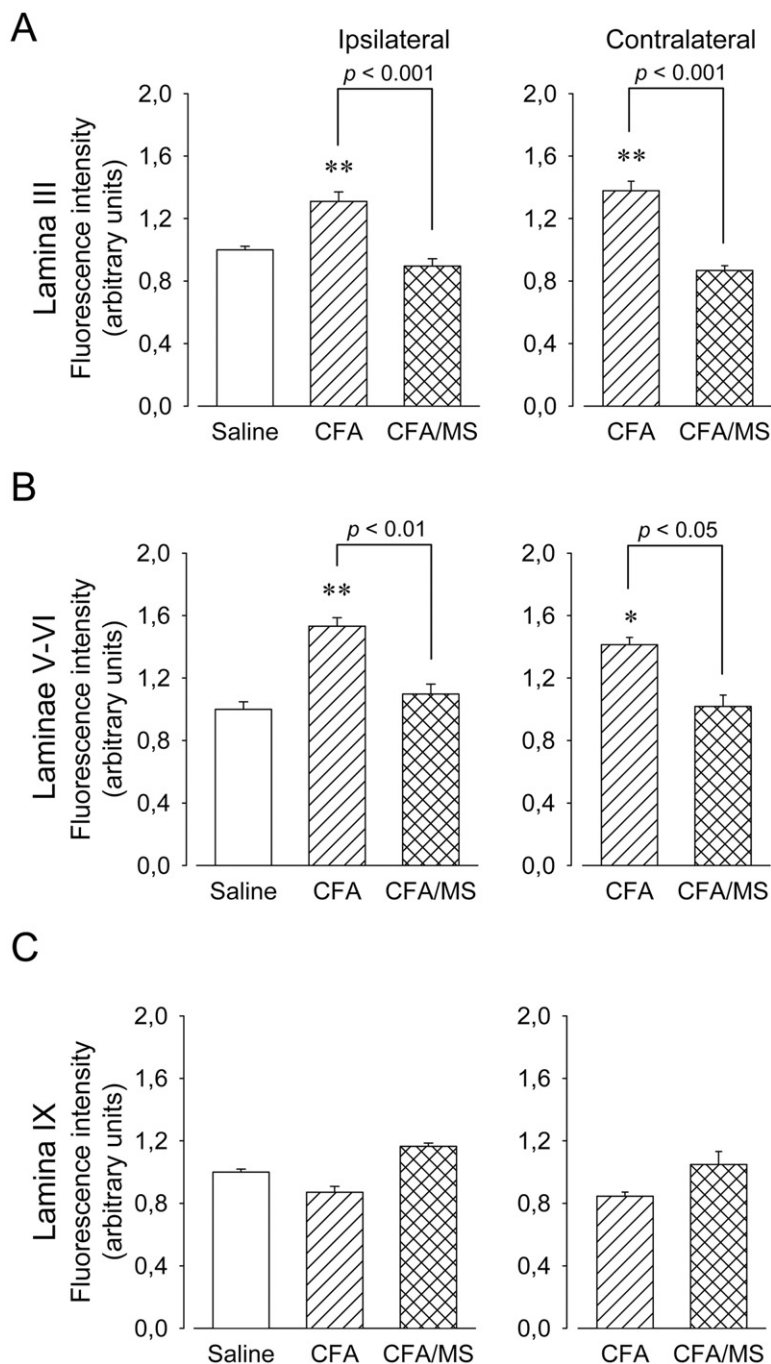
**Fig. 2.** Densitometric analysis of the effect of CFA, MS, and CFA+MS on Fluo-DLT internalization in rat spinal cord. Quantitative analysis of Fluo-DLT internalization in neurons of lamina III (A), laminae V–VI (B), and lamina IX (C). Fluorescence intensities (corresponding to Fluo-DLT internalization levels) are the mean±S.E.M. of three rats in each condition. CFA treatment selectively enhances the mean fluorescence intensity in neurons from the dorsal horn (lamina III and laminae V–VI) of the rat lumbar spinal cord, bilaterally. In rats administered CFA+MS, fluorescence intensity levels are significantly lower than in rats injected with CFA alone. Note the absence of effect in neurons of lamina IX in all conditions. (* $P<0.05$ and ** $P<0.01$ versus naïve animals; ANOVA, Tukey's multiple comparison test).

animals injected with CFA alone; two-tailed unpaired *t*-test; $n=5$). Deltorphin injection did not modify the latency to paw withdrawal in the contralateral, uninflamed hind paw (Fig. 3 B; %MPE=1.6%±0.7% and 8.1%±3.0% respectively for CFA and CFA/MS groups). Note that %MPE=0% means absence of analgesic properties.

**Effect of prolonged morphine treatment on the antinociceptive effects of deltorphin II in CFA-injected rats**

To determine whether the increase in the antihyperalgesic effects of deltorphin observed after morphine treatment on

**Table 1.** Ratio of δOR/GAPDH mRNA expression in the rat lumbar spinal cord

| Group | Dorsal horn | Ventral horn |
|---|---|---|
| Naïve | 0.152±0.004 (5) | 0.188±0.017 (5) |
| CFA Contra | 0.160±0.017 (4) | 0.164±0.037 (4) |
| CFA Ipsi | 0.169±0.026 (4) | 0.198±0.043 (4) |
| CFA/MS Contra | 0.166±0.011 (5) | 0.175±0.014 (5) |
| CFA/MS Ipsi | 0.145±0.006 (5) | 0.178±0.006 (5) |

Data are the mean±SEM of the ratio of δOR/GAPDH mRNA expression in the rat lumbar spinal cord. Significance was calculated using a one-way ANOVA followed by a Tukey's multiple comparison test. No significant difference ($P>0.05$) was found between any groups. Numbers in parentheses represent the number of animals tested for each group. MS standard doses, morphine 48 h (5, 8, 10 and 15 mg/kg); CFA/MS, CFA+morphine 48 h (5, 8, 10 and 15 mg/kg); Contra, side contralateral to CFA injection; Ipsi, side ipsilateral to CFA injection. Saline-injected, 0.194±0.055 (4); MS standard doses, 0.220±0.053 (4).

the side ipsilateral to the injection of CFA was accompanied by an overall augmentation of the antinociceptive properties of deltorphin, heat-induced tail-flick latencies were measured in rats: (1) treated with standard morphine doses alone (48 h: 5, 8, 10 and 15 mg/kg); (2) injected with CFA alone; (3) injected with CFA and then treated with low (CFA/MS Low) or standard doses of morphine (CFA/MS Standard); (4) treated with saline (saline). As illustrated in Fig. 4A, i.t.-injected deltorphin produced peak antinociceptive responses in the tail-flick test 20 min after injection in all groups of animals with the exception of the saline-treated group. Comparison of the %MPE at 20 min post-injection indicated that when administered independently, MS and CFA both strongly increased the antinociceptive effects of deltorphin as compared with naïve animals treated with saline (80.6%±5.1% and 66.6%±6.3%, respectively, as compared with 11.3%±3.8% in saline-treated rats; $P<0.001$; Fig. 4B). By contrast, when standard morphine doses and CFA treatments were administered jointly, the antinociceptive effects of deltorphin were reduced (29.6%±3.6% for CFA/MS standard doses versus 80.6%±5.1% and 66.6%±6.3% for MS and CFA groups,

**Table 2.** Baseline paw withdrawal latencies

| Group | Thermal latency (s) | ANOVA |
|---|---|---|
| Naïve | 6.8±0.3 (6) | |
| CFA Contra | 5.6±0.4 (6) | |
| CFA Ipsi | 3.3±0.2 (5) | $P<0.001$ vs. naïve |
| CFA/MS Contra | 6.6±0.7 (6) | |
| CFA/MS Ipsi | 2.9±0.4 (5) | $P<0.001$ vs. naïve |

Data are the mean±SEM of the basal thermal latencies expressed in seconds (s). Numbers in parentheses represent the number of animals tested for each group. Significance was calculated using a one-way ANOVA followed by a Tukey's multiple comparison test. No hyperalgesia was apparent on the contralateral side in either group of CFA-injected animals. Moreover, morphine did not decrease the basal thermal latencies when administered to CFA-injected animals (as compared with CFA-injected animals). Contra, side contralateral to CFA injection; Ipsi, side ipsilateral to CFA injection; MS, morphine 48 h (5, 8, 10 and 15 mg/kg).
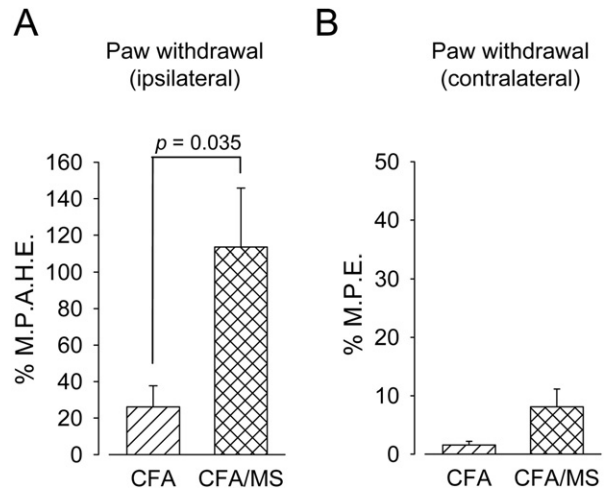


**Fig. 3.** Effect of prolonged MS treatment on δOR-mediated antihyperalgesia in CFA-injected rats. Thermal latencies (in s) to noxious heat (plantar test) were recorded every 10 min following i.t. administration of deltorphin II (10 μg). Percentages of maximal possible antihyperalgesic effects (MPAHE)±S.E.M. (A) and percentages of maximal possible antinociceptive effects (MPE)±S.E.M. (B) were determined 20 min after injection of deltorphin II. I.t. deltorphin has greater antihyperalgesic effects in CFA animals treated with morphine (5, 8, 10 and 15 mg/kg) than in animals treated with CFA alone ($P=0.035$, two-tailed unpaired $t$-test). No antinociceptive effects of deltorphin was observed on the contralateral hind paw.

respectively; $P<0.001$), as if the two treatments were antagonistic to each other (Fig. 4A, B). Administration of lower doses of morphine to animals injected with CFA had no effect on the CFA-induced antinociceptive effects of deltorphin (61.3%±5.9% in CFA+MS low versus 66.6%± 6.3% in CFA alone; $P>0.05$, ANOVA, Tukey's multiple comparison test; Fig. 4A, B).

## Dose-response of morphine pre-treatment on the antinociceptive effects of deltorphin II

Based on our earlier observations that the increased targeting of δOR in neurons of the lumbar spinal cord following CFA injection could no longer be elicited in μOR-KO mice (Morinville et al., 2004b), we postulated that this effect was due to stimulation of μOR by endogenously released opioids and that, consequently, the loss of potentiation of deltorphin antinociceptive effects observed here in rats treated with CFA and then with morphine reflected a cross-tolerance-like mechanism due to overstimulation of μOR. To test this hypothesis, we investigated whether rats treated with doses of morphine twice as high as our standard doses would likewise exhibit cross-tolerance to deltorphin.

As seen in Fig. 5A, in animals pre-treated with high doses of morphine, i.t.-injected deltorphin produced peak antinociceptive responses in the tail-flick test 20 min after injection, as it did in animals pre-treated with the standard or lower doses of morphine. However, in contrast to the standard morphine pre-treatment which increased the %MPE of deltorphin over sevenfold as compared with saline-treated rats (80.6%±5.1% versus 11.3%±3.8%), pre-treatment with low or high doses of morphine as well
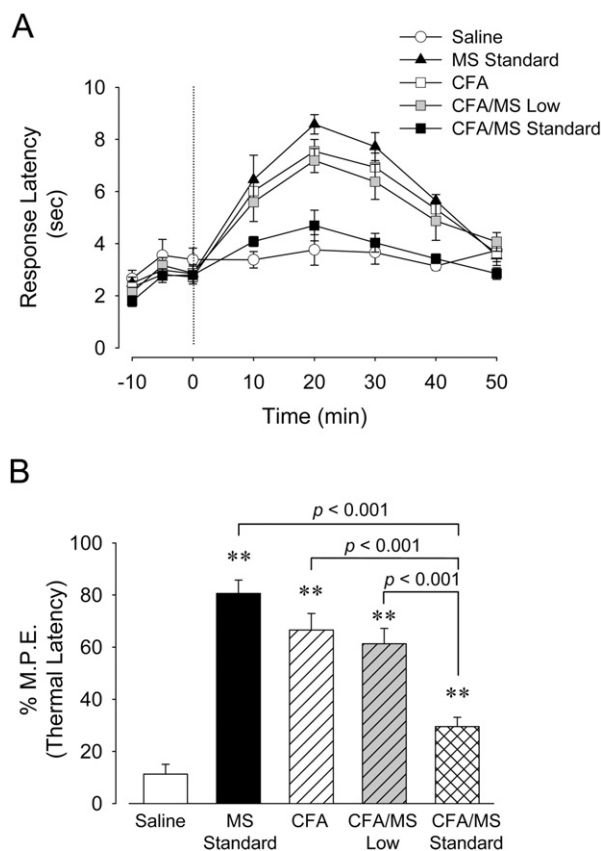
A



B



**Fig. 4.** Comparative analgesic effects of deltorphin II in animals treated with CFA and/or with MS. (A) Tail-flick latencies (in s) were determined every 10 min following i.t. administration of deltorphin II (10 μg) to rats treated with: (1) saline but absence of CFA ($n=6$); (2) standard doses of morphine but absence of CFA (MS, 48 h: 5, 8, 10 and 15 mg/kg, $n=7$); (3) CFA (injected in the left hind paw; 72 h postinjection; $n=6$); (4) CFA together with either low (MS, 48 h: 1, 3, 5 and 8 mg/kg, $n=6$) or standard doses of morphine ($n=7$). Data correspond to the mean±S.E.M. (B) Percentage of maximal possible antinociceptive effect (MPE)±S.E.M. determined 20 min after injection of deltorphin II. Percent MPEs for MS, CFA, CFA/MS (low doses) and CFA/MS (standard doses) are significantly different from %MPE in saline-treated rats (** $P<0.001$). Percent MPEs are also significantly different between MS- and CFA/MS- (standard doses) treated rats ($P<0.001$) and between CFA- and CFA/MS- (standard doses) treated animals ($P<0.001$). Statistical significance determined using one-way ANOVA followed by Tukey's multiple comparison test.

as longer treatment (up to 96 h; modified standard doses) only increased the antinociceptive effects of deltorphin by two- to threefold compared with saline-treated rats (respectively 30.8%±5.3%, 23.0%±4.5% and 31.2%±9.0% for low, high doses and modified standard doses versus 11.3%±3.8% in saline-treated rats) (Fig. 5A, B).

**Effect of chronic inflammation on met-enkephalin levels in the lumbar spinal cord**

The similarity between the loss of potentiation of deltorphin antinociceptive effects observed after treatment with high doses of morphine versus after CFA followed by standard doses of morphine suggested to us that the chronic inflammation induced by CFA injection caused the release of

endogenous opioid agonists. Indeed, increased enkephalin immunoreactivity was reported in chronic inflammatory states (Faccini et al., 1984; Millan et al., 1986, 1988; Hurley and Hammond, 2001). We sought to determine whether in the present chronic inflammation model, endogenous met-enkephalin levels were modified in the rat lumbar spinal cord. As illustrated in Fig. 6, we found that 72 h post-CFA injection, the levels of met-enkephalin in both dorsal and ventral horns of CFA animals were not significantly different from those measured in naïve animals ($P>0.05$, two-tailed unpaired $t$-test). Note that because no
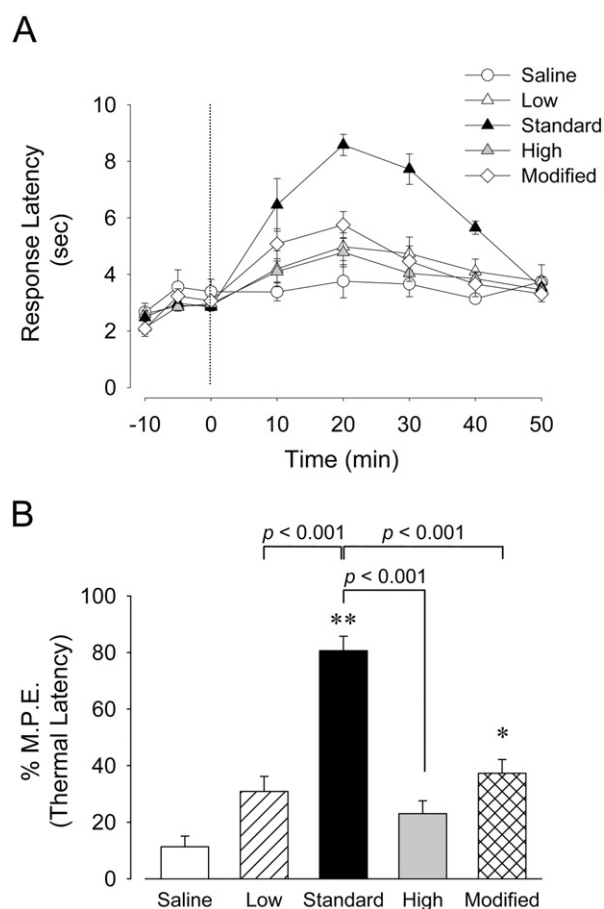
A



B



**Fig. 5.** Analgesic effects of deltorphin II following different morphine pre-treatment paradigms. (A) Tail-flick latencies (in s) were determined every 10 min following i.t. administration of deltorphin II (10 μg) to saline-treated animals ($n=6$) or to animals pre-treated with low doses (1, 3, 5 and 8 mg/kg; $n=7$), standard doses (5, 8, 10 and 15 mg/kg; $n=7$), or high doses (10, 15, 20 and 30 mg/kg; $n=9$) of morphine, or with a modified standard morphine doses paradigm (96 h: 5, 8, 10, 10, 10, 10, 10 and 15 mg/kg; $n=4$). Note that these experiments were conducted in the absence of CFA. Data correspond to the mean±S.E.M. (B) Percentages of maximal possible antinociceptive effects (MPE)±S.E.M. were determined 20 min after injection of deltorphin II. Percent MPE for morphine (standard doses) and modified standard morphine doses are significantly different from the %MPE in saline-treated rats (** $P<0.001$ and * $P<0.05$ respectively). Percent MPEs in animals treated with high, low, or modified standard doses of morphine are significantly lower than in rats treated with standard doses of morphine ($P<0.001$). Statistical significance determined using one-way ANOVA followed by Tukey's multiple comparison test.
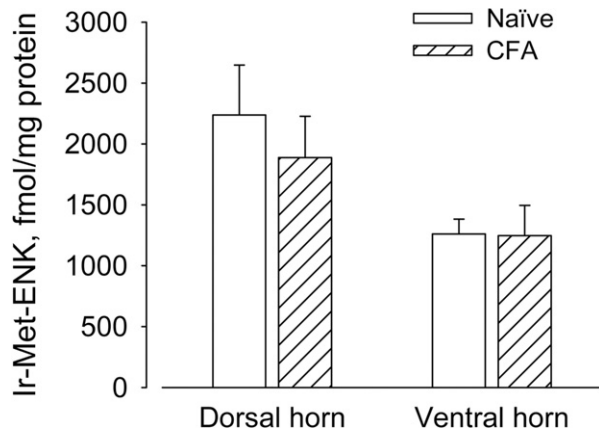
**Fig. 6.** Met-enkephalin levels in dorsal and ventral horn of lumbar spinal cord of rats injected with CFA. Immunoreactive levels of met-enkephalin in dorsal and ventral horns of rat lumbar spinal cord were determined by RIA in naïve animals ($n=3$) and in animals injected with CFA in the left hind paw 72 h prior to kill ($n=4$). Results are expressed as fmol of met-enkephalin/mg of protein ±S.E.M. No significant difference is observed between CFA-treated and naïve animals ($P>0.05$). Statistical significance determined using a two-tailed unpaired $t$-test. Because no difference was observed between them, data from contra- and ipsilateral sides were pooled.

difference was observed between them, data from contra- and ipsilateral sides were pooled.

## DISCUSSION

The present study investigated the effects of morphine priming on the analgesic properties of deltorphin II, a selective delta opioid receptor ($\delta$OR) agonist, in the CFA model of chronic inflammation. Our results reveal that whereas antihyperalgesic effects (plantar test) of i.t. administered deltorphin are potentiated by a pre-treatment with morphine, antinociceptive effects (tail flick test) are markedly reduced when morphine is administered to CFA-treated rats.

We previously demonstrated that prolonged treatment of rodents with $\mu$OR agonists including morphine induced a selective increase in cell surface density of $\delta$OR (Cahill et al., 2001b; Morinville et al., 2003) and internalization of $\delta$OR agonists in neurons of layers I–VI of the lumbar spinal cord (Morinville et al., 2004a). We also showed that chronic inflammation resulting from the injection of CFA into the rat hind paw similarly enhanced, bilaterally, the membrane density of $\delta$OR in neurons of the dorsal horn (Cahill et al., 2003). However, it was still unknown if the distribution of neurons affected by CFA injection was the same as the one showing up-regulation of cell surface $\delta$OR after morphine treatment.

We demonstrate here that the effect of chronic inflammation on the cell surface availability of $\delta$OR selectively occurs in neurons of the dorsal horn as was found to be the case for prolonged morphine treatment (Morinville et al., 2004a). Furthermore, this effect is bilateral, as was the effect of sustained morphine (Morinville et al., 2004a). This is not to say, however, that both regimens affect the same neuronal populations. Indeed, in dorsal root ganglia

(DRG), morphine treatment increases cell surface $\delta$OR in neurons of all types and sizes, whereas CFA injection selectively up-regulates cell surface $\delta$OR in small- and medium-sized ganglion cells (Gendron et al., 2006).

The enhanced availability of cell surface $\delta$OR observed in CFA-injected rats is likely due to an increase in the membrane recruitment of intracellular reserve receptors rather than to the neosynthesis of $\delta$OR since no change in the expression of $\delta$OR mRNA was observed in the spinal cord of these animals using quantitative PCR on whole spinal cord quadrants. Likewise, CFA injection in the hind-limb selectively affected trafficking as opposed to expression of $\delta$OR in rat lumbar DRGs (Gendron et al., 2006). However, we previously observed a bilateral increase in $\delta$OR mRNA in a discrete subpopulation of the dorsal horn neurons of CFA-treated animals using quantitative *in situ* hybridization (Cahill et al., 2003), suggesting that a small number of neurons exhibiting enhanced $\delta$OR availability, diluted in the sample assayed here, might be up-regulating $\delta$OR.

To determine whether prolonged morphine treatment would affect the increase in $\delta$OR availability observed in the lumbar spinal cord following chronic inflammation, we repeated our *in vivo* Fluo-DLT internalization assay in CFA-injected animals treated for 48 h with our standard morphine regimen. Surprisingly, not only did this morphine treatment fail to further increase cell surface availability of $\delta$OR as compared with animals treated with CFA alone, but it totally abolished the effect produced by injection of CFA.

To correlate our anatomical findings with the pharmacological effects of i.t.-administered deltorphin, we measured the antihyperalgesic effects of deltorphin in CFA-injected rats treated or not with morphine, using the plantar test. As previously reported (Cahill et al., 2003), deltorphin induced antihyperalgesic effects when administered i.t. to CFA-injected rats. Surprisingly, treatment of CFA-injected animals with morphine further enhanced the antihyperalgesic effect of i.t. deltorphin as compared with animals treated with CFA alone, despite the fact that it abolished the CFA-induced increase in the binding and internalization of Fluo-DLT in the lumbar spinal cord. These results suggest that the antihyperalgesic effects of deltorphin in the CFA model are unrelated to the increase in the cell surface availability of $\delta$OR in neurons of the dorsal horn. Alternatively, they might be related to the increase in $\delta$OR cell surface density observed in small- and medium-sized DRG neurons following CFA injection (Gendron et al., 2006). The enhancement of $\delta$OR's functional competence recently reported in trigeminal nociceptors following activation of bradykinin B2 receptors supports this interpretation (Patwardhan et al., 2005). Admittedly, membrane $\delta$OR were also shown to be increased in peripheral neurons (Patwardhan et al., 2005), suggesting that the enhanced antihyperalgesic effects of deltorphin could result from stimulation of peripheral receptors. This interpretation appears unlikely, however, since deltorphin was injected i.t. and does not cross the blood–brain barrier. Whatever the underlying mechanism involved, the present study indi-

cates that the antihyperalgesic efficiency of δOR agonists in chronic inflammation may be further enhanced by treating the animals with morphine.

Very different results were obtained when the antinociceptive effects of i.t. deltorphin were assessed using the tail-flick test in CFA-injected rats. Injection of CFA alone resulted in an augmentation of the antinociceptive effects of i.t. deltorphin comparable to those observed after prolonged morphine treatment. This result was surprising in itself since CFA-induced inflammation affects lumbar somatosensory inputs while the tail-flick test measures a sacral reflex. However, primary afferent nociceptors have been shown to project both rostrally and caudally in the cervical spinal cord (Abbadie et al., 2002) and L5–L6 nerve injury was found to induce extrasegmental changes extending downward into S1–S2 (Ossipov et al., 1995; Malan et al., 2000; Wang et al., 2003). Furthermore, electrical stimulation of various acupuncture points in the limbs was reported to elicit an inhibition of the tail nociceptive withdrawal reflexes (Romita and Henry, 1996), further supporting the existence of neuronal connections between lumbar and sacral segments. In animals co-treated with CFA and morphine, the antinociceptive effects of i.t. deltorphin were no longer different from those recorded in saline-injected rats. Therefore, antinociceptive (unlike antihyperalgesic) behavior correlated with the return to baseline cell surface δOR availability levels measured in the lumbar spinal cord using our *in vivo* deltorphin internalization assay.

The question arises as to why, when administered together, CFA and morphine treatments neutralize both the anatomical and behavioral effects that they exert individually. One possibility is that systemic morphine acts on peripheral nerves to induce nerve block, thereby reducing the CFA-induced afferent drive. This interpretation could account for the observed behavioral, but not for δOR trafficking events. Another possibility is that both effects have a joint central origin. Peripheral inflammation is known to increase the release of endogenous opioid peptides, both in the periphery (Cabot et al., 1997) and in the CNS (Millan et al., 1986, 1988; Iadarola et al., 1988; Hurley and Hammond, 2001; Parra et al., 2002). Specifically, unilateral inflammation of the hindlimb was reported to rapidly increase both the mRNA levels of dynorphin and enkephalin precursors (Iadarola et al., 1988; Noguchi et al., 1992) as well as the level of immunoreactive dynorphin (Iadarola et al., 1988; Millan et al., 1988; Parra et al., 2002). Bilateral changes in the level of met- and leu-enkephalin peptide content were also observed in the superficial dorsal horn of the rat spinal cord 5 weeks after CFA injection (Millan et al., 1988). Thus, although our own RIA showed no increase in enkephalin levels 72 h after CFA injection, the bilateral augmentation in δOR bio-availability observed in neurons of the lumbar spinal cord could be due to enhanced release of endogenous opioid peptides acting on μOR, which would be coherent with the fact that it is not present in μOR knockout animals (Morinville et al., 2004b). Because prolonged morphine treatment can induce spinal changes and central sensitization (Ossipov et al., 2005; Trang et al., 2005), we figured that the addition of an

exogenous μOR agonist (morphine) to the endogenous opioid stimulus would result in an overstimulation of μOR and, through a opioid tolerance mechanism, reduce both δOR trafficking and the antinociceptive effects of deltorphin.

To determine whether an overstimulation of μOR would indeed reduce the antinociceptive effects of deltorphin, we repeated the tail-flick experiments in animals pre-treated with high doses of morphine alone. We found that this heightened morphine regimen significantly decreased the antinociceptive effects of i.t. deltorphin, in much the same way as did our standard doses of morphine in CFA-treated rats. Furthermore, we also observed that low doses of morphine administered to CFA-treated rats did not reduce the antinociception induced by deltorphin. Taken together, these results suggest that these priming effects of morphine operate within a narrow dose range and that too high a dose of morphine may reduce the responsiveness to δOR agonists.

## CONCLUSION

In sum, the present findings demonstrate that the antihyperalgesic effects of δOR agonists may be enhanced by prolonged exposure to morphine in a model of chronic inflammation and that under such priming conditions, δOR agonists able to cross the blood–brain barrier could provide effective analgesia in chronic inflammation states. Our results also show, however, that δOR targeting to the plasma membrane of dorsal horn neurons, and by way of consequence the pharmacological sensitivity of spinal δOR, are highly sensitive to primer doses of μOR agonists, i.e. to the intensity of μOR stimulation. Finally, the present study demonstrates that different cellular mechanisms underlie antihyperalgesic and antinociceptive efficacy of i.t. deltorphin in CFA-treated rats. A better understanding of the mechanisms underlying the morphine-induced potentiation of the antihyperalgesic effects of δOR agonists in chronic inflammation will be pivotal in the development of δOR-targeted medication for the treatment of chronic pain.

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