

# Treatment with Carbon Monoxide-releasing Molecules and an HO-1 Inducer Enhances the Effects and Expression of $\mu$ -Opioid Receptors during Neuropathic Pain

Arnau Hervera, B.Sc., M.Sc.,\* Sergi Leáñez, B.S.,† Roberto Motterlini, Ph.D.,‡ Olga Pol, Ph.D.§

## ABSTRACT

**Background:** The administration of  $\mu$ -opioid receptors (MOR) and  $\delta$ -opioid receptors (DOR) as well as cannabinoid-2 receptor (CB2R) agonists attenuates neuropathic pain. We investigated if treatment with two carbon monoxide-releasing molecules (CORM-2 and CORM-3) or an inducible heme oxygenase inducer (cobalt protoporphyrin IX, CoPP) could modulate the local and systemic effects and expression of MOR, DOR, and CB2R during neuropathic pain.

**Methods:** In C57BL/6 mice, at 10 days after the chronic constriction of sciatic nerve, we evaluated the effects of the intraperitoneal administration of 10 mg/kg of CORM-2, CORM-3, or CoPP on the antiallodynic and antihyperalgesic actions of a locally or systemically administered MOR (morphine), DOR ([d-Pen(2),d-Pen(5)]-enkephalin) or CB2R ((2-methyl-1-propyl-1H-indol-3-yl)-1-naphthalenyl-methanone) agonist. The effects of CORM-2 and CoPP treatments on the expression of MOR, DOR, CB2R, inducible and constitutive heme oxygenases, microglia activation marker (CD11b/c), and neuronal and inducible nitric oxide synthases were also assessed.

**Results:** Treatments with CO-RMs and CoPP reduced the mechanical and thermal hypersensitivity induced by sciatic nerve injury, increased the local, but not systemic,

## What We Already Know about This Topic

- Administration of  $\mu$ -opioid receptor and  $\delta$ -opioid receptors as well as cannabinoid 2 receptor agonists attenuates neuropathic pain; receptor modulation by carbon monoxide-releasing molecules or inducible heme oxygenase inducers, e.g., cobalt protoporphyrin IX, could be leveraged for therapeutic purposes

## What This Article Tells Us That Is New

- In a mouse model of sciatic nerve injury, carbon monoxide-releasing molecules and cobalt protoporphyrin IX treatments increased local antinociceptive effects of morphine through enhancing  $\mu$ -opioid receptor peripheral expression and inhibiting spinal microglial activation and neuronal/inducible nitric oxide synthases overexpressions

antinociceptive effects of morphine, and decreased those produced by DPDPE and JWH-015. Both CORM-2 and CoPP treatments enhanced MOR and inducible heme oxygenase expression, unaltered DOR and constitutive heme oxygenase expression, and decreased the overexpression of CB2R, CD11b/c, and neuronal and inducible nitric oxide synthases induced by sciatic nerve injury.

**Conclusions:** This study shows that CO-RMs and CoPP treatments increase the local antinociceptive effects of morphine through enhancing MOR peripheral expression and inhibiting spinal microglial activation and overexpression of neuronal/inducible nitric oxide synthases.

NEUROPATHIC pain is a disease state characterized by the presence of allodynia and hyperalgesia, and it is difficult to treat with the systemic administration of morphine and other classic opioids.<sup>1-2</sup> In contrast, the local administration of  $\mu$ -opioid receptors (MOR) and  $\delta$ -opioid receptor (DOR) as well as cannabinoid 2 receptor (CB2R) agonists elicits antiallodynic and antihyperalgesic effects during neuropathic pain.<sup>3-8</sup> However, whereas the local antinociceptive effects of morphine are produced by activation of the peripheral nitric oxide–cyclic guanosine monophosphate–protein kinase G (PKG)–adenosine triphosphate–sensitive potassium channels signaling pathway,<sup>6</sup> the activation of this pathway is implicated as a mechanism limiting the local antiallodynic and antihyperalgesic efficiency of DOR and CB2R agonists

\* Ph.D. Student, † Technician, § Chief, Grup de Neurofarmacologia Molecular, Institut d'Investigació Biomèdica Sant Pau and Institut de Neurociències, Universitat Autònoma de Barcelona, Barcelona, Spain. ‡ Senior Researcher, INSERM U955, Equipe 3, Faculty of Medicine, University Paris-Est, Creteil, France.

Received from Grup de Neurofarmacologia Molecular, Institut d'Investigació Biomèdica Sant Pau and Institut de Neurociències, Universitat Autònoma de Barcelona, Barcelona, Spain. Submitted for publication March 13, 2012. Accepted for publication January 2, 2013. This work was supported by the Fundació La Marató de TV3 Barcelona (Grant: 070810) and Fondo de Investigación Sanitaria, Madrid (Grant: PS0900968), Spain.

Address correspondence to Dr. Pol: Grup de Neurofarmacologia Molecular, Institut de Neurociències, Facultat de Medicina, Edifici M2-115, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain. opol@santpau.es. Information on purchasing reprints may be found at [www.anesthesiology.org](http://www.anesthesiology.org) or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

Copyright © 2013, the American Society of Anesthesiologists, Inc. Lippincott Williams & Wilkins. Anesthesiology 2013; 118:1180-97

under neuropathic pain conditions.<sup>5</sup> Accordingly, while the local antiallodynic effects of morphine were significantly reduced by their local coadministration with selective neuronal (NOS1) or inducible (NOS2) nitric oxide synthases, L-guanylate cyclase, or PKG inhibitors,<sup>6</sup> the local antinociceptive effects of DOR and CB2R agonists were significantly increased.<sup>5</sup> Moreover, nitric oxide is also implicated in the dorsal root ganglia down- (MOR and DOR) and upregulation (CB2R) of these receptors after sciatic nerve injury.<sup>5–6</sup>

Carbon monoxide, another gaseous neurotransmitter, synthesized by inducible (HO-1) and constitutive (HO-2) heme oxygenases, also activates the cyclic guanosine monophosphate–PKG pathway.<sup>9,10</sup> The overexpression of HO-2 isoform exerts a pronociceptive effect after nerve injury.<sup>11–13</sup> In contrast, the enhanced expression of HO-1 produces potent antiinflammatory and antinociceptive effects.<sup>14–17</sup> Indeed, the administration of HO-1-inducing compounds, such as cobalt protoporphyrin IX (CoPP), or carbon monoxide-releasing molecules (CO-RMs), a new class of chemical agents able to reproduce several biological effects of HO-1-derived carbon monoxide, inhibits inflammation and/or acute nociception.<sup>15,18–21</sup> However, the exact contribution of carbon monoxide synthesized by HO-1 in the modulation of main symptoms of neuropathic pain induced by sciatic nerve injury remains unknown.

It is well known that HO-2 modulates the effects of morphine under neuropathic pain conditions,<sup>22,23</sup> but the role played by CO-RMs or CoPP in the effects and expression of MOR, DOR, and CB2R as well as in the possible mechanisms implicated in these actions still remains unknown.

Therefore, in sciatic nerve injury-induced neuropathic pain, we evaluated the following: (1) the antiallodynic and antihyperalgesic effects of the subplantar and subcutaneous administration of specific MOR (morphine), DOR ([d-Pen(2),d-Pen(5)]-enkephalin; DPDPE), or CB2R ((2-methyl-1-propyl-1H-indol-3-yl)-1-naphthalenylmethanone; JWH-015) agonists alone or combined with two CO-RMs, tricarbonyldichloro ruthenium (II) dimer (CORM-2) and tricarbonyl-chloro (glycinato) ruthenium (II) (CORM-3), or a classical inducer of HO-1, CoPP intraperitoneally administered; (2) the antinociceptive effects of morphine, DPDPE, or JWH-015 subplantar or subcutaneously administered, alone or combined, with the HO-1 inhibitor, tin protoporphyrin IX (SnPP); (3) the reversibility of the effects of morphine, DPDPE and JWH-015 by their coadministration with specific antagonists; and (4) the effect of CORM-2 and CoPP treatments on the expression of MOR, DOR, CB2R, HO-1, HO-2, CD11b/c (as a marker of microglial activation), NOS1, and NOS2 in the dorsal root ganglia or spinal cord from sciatic nerve-injured mice.

## Materials and Methods

### Animals

The experiments were performed in male C57BL/6 mice acquired from Harlan Laboratories (Barcelona, Spain). All mice weighing 21–25 g were housed under 12-h/12-h

light/ dark conditions in a room with controlled temperature (22°C) and humidity (66%). Animals had free access to food and water and were used after a minimum of 6 days acclimatization to the housing conditions. All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health and approved by the local Committee of Animal Use and Care of the Autonomous University of Barcelona.

### Induction of Neuropathic Pain

Neuropathic pain was induced by the chronic constriction of the sciatic nerve.<sup>5</sup> Briefly, sciatic nerve ligation was performed under isoflurane anesthesia (3% induction, 2% maintenance). The biceps femoris and the gluteus superficialis were separated by blunt dissection, and the right sciatic nerve was exposed. The injury was produced by tying three ligatures around the sciatic nerve as described by Bennett and Xie.<sup>24</sup> The ligatures (4/0 silk) were tied loosely around the nerve with 1 mm spacing, until they elicited a brief twitch in the respective hindlimb, which prevented overtightening of the ligations, taking care to preserve epineural circulation. Sham-operated mice that underwent exposure of the right sciatic nerve without ligature were used as a surgery control.

The development of mechanical and thermal allodynia as well as thermal hyperalgesia was evaluated by using the von Frey filaments, cold plate, and plantar tests, respectively. All animals were tested in each paradigm before surgery and at 10 days after surgery.

### Nociceptive Behavioral Tests

*Mechanical allodynia* was quantified by measuring the hind paw withdrawal response to von Frey filament stimulation. Animals were placed in methacrylate cylinders (20 cm high, 9 cm diameter; Servei Estació, Barcelona, Spain) with a wire grid bottom through which the von Frey filaments (North Coast Medical, Inc., San Jose, CA) with a bending force in the range of 0.008–3.5 g were applied by using a modified version of the up–down paradigm, as previously reported by Chaplan *et al.*<sup>25</sup> The filament of 0.4 g was used first and the 3.5-g filament was used as a cut-off. Then, the strength of the next filament was decreased or increased according to the response. The threshold of response was calculated from the sequence of filament strength used during the up–down procedure by using an Excel program (Microsoft Iberia SRL, Barcelona, Spain) that includes curve fitting of the data. Clear paw withdrawal, shaking, or licking of the paw was considered as a nociceptive-like response. Both ipsilateral and contralateral hind paws were tested. Animals were allowed to habituate for 1 h before testing in order to allow an appropriate behavioral immobility.

Thermal hyperalgesia was assessed as previously reported by Hargreaves *et al.*<sup>26</sup> Paw withdrawal latency in response to radiant heat was measured using the plantar test apparatus (Ugo Basile, Varese, Italy). Briefly, the mice were placed in methacrylate cylinders (20 cm high × 9 cm diameter)

positioned on a glass surface. The heat source was positioned under the plantar surface of the hind paw and activated with a light beam intensity, chosen in preliminary studies to give baseline latencies from 8 to 9 s in control mice. A cut-off time of 12 s was used to prevent tissue damage in the absence of response. The mean paw withdrawal latencies from the ipsilateral and contralateral hind paws were determined from the average of three separate trials, taken at 5-min intervals to prevent thermal sensitization and behavioral disturbances. Animals were habituated to the environment for 1 h before the experiment to become quiet and to allow testing.

Thermal allodynia to cold stimulus was assessed by using the hot/cold-plate analgesia meter (Ugo Basile), previously described by Bennett and Xie.<sup>24</sup> The number of elevations of each hind paw was recorded in the mice exposed to the cold plate ( $4 \pm 0.5$  °C) for 5 min.

### Western Blot Analysis

Sham-operated and sciatic nerve-injured mice were killed at 10 days after surgery by cervical dislocation. Tissues from the ipsilateral lumbar section of the spinal cord and dorsal root ganglia (L3 to L5) were removed immediately after killing, frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  until assay. Samples from the spinal cord and dorsal root ganglia from three to five animals were pooled into one experimental sample to obtain enough protein levels for performing the Western blot analysis. The MOR, DOR, CB2R, HO-1, HO-2, CD11b/c, NOS1, and NOS2 protein levels were analyzed by Western blot. Tissues were homogenized in ice-cold lysis buffer (50 mM Tris-Base, 150 mM NaCl, 1% NP-40, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.5 Triton X-100, 0.1% sodium dodecyl sulfate, 1 mM  $\text{Na}_3\text{VO}_4$ , 25 mM NaF, 0.5 % protease inhibitor cocktail, and 1% phosphatase inhibitor cocktail). All reagents were purchased at Sigma (St. Louis, MO) with the exception of NP-40 from Calbiochem (Darmstadt, Germany). The crude homogenate was solubilized for 1 h at  $4^{\circ}\text{C}$ , sonicated for 10 s, and centrifuged at  $4^{\circ}\text{C}$  for 15 min at 700 g. The supernatant (50 or 100  $\mu\text{g}$  of total protein) was mixed with 4  $\times$  laemmli loading buffer and then loaded onto 4% stacking/10% separating sodium dodecyl sulfate polyacrylamide gels.

The proteins were electrophoretically transferred onto polyvinylidene difluoride membrane for 120 minutes for MOR, DOR, CB2R, HO-1, and HO-2 or overnight for NOS1, NOS2, and CD11b/c detection; blocked with PBST + 5% nonfat dry milk; and subsequently incubated overnight at  $4^{\circ}\text{C}$  with polyclonal rabbit anti-MOR (1:1000, Chemicon-Millipore, Billerica, MA), anti-DOR (1:2500, Chemicon-Millipore), anti-CB2R (1:500, Abcam, Cambridge, United Kingdom), anti-HO-1 (1:300, Stressgen, Ann Arbor, MI), anti-HO-2 (1:1000, Stressgen), and anti-CD11b/c (1:300, Novus Biologicals, Littleton, CO) antibody against the type 3 complement receptor to detect activated microglial cells,<sup>27</sup> anti-NOS1 (1:100, BD Transduction Laboratories, San Diego, CA), or anti-NOS2 antibodies (1:200, Chemicon-Millipore). The proteins were detected by a horseradish peroxidase-conjugated anti-rabbit

secondary antibody (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) and visualized with chemiluminescence reagents (ECL kit; GE Healthcare) and by exposure onto hyperfilm (GE Healthcare). The intensity of blots was quantified by densitometry. The membranes were stripped and reprobed with a monoclonal rabbit anti- $\beta$ -actin antibody (1:10,000, Sigma) used as a loading control.

### Experimental Protocol

In a first set of experiments, we assessed the expression of neuropathic pain by using the mouse model of chronic constriction of sciatic nerve previously used by us.<sup>6</sup> After the habituation period, baseline responses were established in the following sequence: von Frey filaments, plantar, and cold-plate tests. After baseline measurements, neuropathic pain was induced and animals were again tested in each paradigm at day 10 after surgery by using the same sequence as for baseline responses. Sham-operated mice were used as controls ( $n = 6$  animals per group).

In a second set of experiments, we investigated the mechanical antiallodynic, thermal antihyperalgesic and thermal antiallodynic effects produced by the intraperitoneal administration of different doses of two CO-RMs (CORM-2 and CORM-3),<sup>17,28,29</sup> their inactive forms (iCORM-2 and iCORM-3), an HO-1 inducer (CoPP),<sup>30</sup> or an HO-1 inhibitor (SnPP)<sup>16</sup> in sciatic nerve-injured or sham-operated animals on day 10 after surgery ( $n = 6$  animals per group).

In a third set of experiments, we evaluated the mechanical antiallodynic, thermal antihyperalgesic, and thermal antiallodynic effects of the subcutaneous administration of different doses of a specific MOR (morphine), DOR (DPDPE), or CB2R (JWH-015) agonist and their respective vehicles in sciatic nerve-injured or sham-operated animals on day 10 after surgery ( $n = 6$  animals per group).

In another set of experiments, we investigated the mechanical antiallodynic, thermal antihyperalgesic, and thermal antiallodynic effects produced by the intraperitoneal administration of 10 mg/kg of CORM-2, CORM-3, or CoPP alone or combined with the subplantar or subcutaneous administration of a low dose of morphine (50  $\mu\text{g}$  or 1 mg/kg) or high doses of DPDPE (100  $\mu\text{g}$  or 5 mg/kg) or JWH-015 (30  $\mu\text{g}$  or 3 mg/kg)<sup>5,6</sup> in sciatic nerve-injured or sham-operated animals on day 10 after surgery ( $n = 6$  animals per group).

In another set of experiments, we evaluated the mechanical antiallodynic, thermal antihyperalgesic, and thermal antiallodynic effects produced by the subplantar or subcutaneous administration of 290  $\mu\text{g}$  or 10 mg/kg of SnPP alone or combined with the subplantar or subcutaneous administration of high doses of morphine (100  $\mu\text{g}$  or 5 mg/kg) or low doses of DPDPE (25  $\mu\text{g}$  or 0.5 mg/kg) or JWH-015 (5  $\mu\text{g}$  or 0.15 mg/kg)<sup>5,6</sup> in sciatic nerve-injured or sham-operated animals on day 10 after surgery ( $n = 6$  animals per group).

The doses of CORM-2, CORM-3, CoPP, and SnPP combined with morphine, DPDPE, or JWH-015 were selected in accordance to other studies<sup>15,17,20,30-32</sup> and to the dose-response performed in this study, as the ones that produce a relevant effect. The doses of all tested opioid and



cannabinoid receptor agonists subplantarly administered were selected according to our previous works,<sup>5,6,33</sup> while the doses for their subcutaneous administration were chosen from the dose–response curves performed in this study, as the ones that produced a minimal or a maximal antinociceptive effect in sciatic nerve injury-induced neuropathic pain.

The reversibility of the antinociceptive effects produced by the subplantar or subcutaneous administration of morphine (100 µg or 5 mg/kg), DPDPE (100 µg or 5 mg/kg), or JWH-015 (30 µg or 3 mg/kg), as doses that produce the maximal antiallodynic and antihyperalgesic effects after sciatic nerve injury,<sup>5,6</sup> by their subplantar or subcutaneous coadministration with specific (H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH<sub>2</sub> [CTAP]; 120 µg or 4 mg/kg, naltrindole; 50 µg or 2 mg/kg; AM630; 30 µg or 1 mg/kg) and an unspecific peripheral opioid antagonist (naloxone methiodide, NX-ME; 20 µg or 1 mg/kg) or a cannabinoid 1 receptor (CB1R) antagonist (AM251; 150 µg or 5 mg/kg),<sup>5,6</sup> at 10 days after surgery, was also evaluated (n = 6 animals per group). The doses of all tested opioid and cannabinoid receptor antagonists were selected according to our previous data obtained in sciatic nerve-injured mice.<sup>5,6,33</sup>

Finally, and considering the analogous behavioral responses produced by CORM-2 and CORM-3 treatments, in another set of experiments we evaluated the effects of CORM-2 and CoPP treatments in the expression of MOR, DOR, CB2R, HO-1, HO-2, CD11b/c, NOS1, and NOS2 in the ipsilateral site of the spinal cord and/or dorsal root ganglia from sciatic nerve-injured mice, at 10 days after surgery, by using Western blot assay. In these experiments, sham-operated mice treated with vehicle have been used as controls (n = 5 samples per group).

## Drugs

CORM-2 was purchased from Sigma, CoPP and SnPP from Frontier scientific (Livchem GmbH & Co, Frankfurt, Germany), and CORM-3 was synthesized as previously described by Clark *et al.* (2003).<sup>29</sup> Morphine hydrochloride was obtained from Alcaiber S.A. (Madrid, Spain); DPDPE, CTAP, naltrindole, and NX-ME were acquired from Sigma. JWH-015, AM630, and AM251 were purchased from Tocris (Ellisville, MI).

CORM-2, CoPP, and SnPP were dissolved in dimethyl sulfoxide (1% solution in saline). JWH-015, AM630, and AM251 were dissolved in DMSO (50% solution in saline). CORM-3, morphine-HCl, DPDPE, CTAP, NX-ME, and naltrindole were dissolved in saline solution (0.9% NaCl). As negative controls for CO-RMs, inactive CORM-2 (iCORM-2) or CORM-3 (iCORM-3) was prepared by leaving solutions of CORM-2 or CORM-3 in dimethyl sulfoxide or saline solution, at room temperature for 2 days, respectively. The iCORM-2 and iCORM-3 solutions were finally bubbled with nitrogen to remove any residual carbon monoxide present in the solutions.

All drugs were freshly prepared before use. CORM-2, CORM-3, and CoPP were intraperitoneally administered, 3–4 h before testing, in a final volume of 10 ml/kg. SnPP, morphine, DPDPE, JWH-015, CTAP, NX-ME, naltrindole, AM630, and AM251 were administered into the plantar side

of the right paw or subcutaneously, 30 min before behavioral testing, in a final volume of 30 µl or 10 ml/kg, respectively. For each group treated with a drug, the respective control group received the same volume of vehicle.

## Statistical Analysis

Data are expressed as mean ± SEM. The statistical analysis was performed by using the SPSS (version 17 for Windows, IBM España, Madrid, Spain). All comparisons were run as two-tailed testing.

The comparison of the mechanical and thermal responses induced by sciatic nerve injury *versus* surgery (sham) in the contralateral and ipsilateral paws of mice was evaluated by using a two-way ANOVA repeated measures (paw and surgery as between factors of variation) followed by the corresponding unpaired Student *t* test. The comparison of the mechanical allodynia, thermal hyperalgesia, and thermal allodynia induced by sciatic nerve injury *versus* surgery (sham) was evaluated by using an unpaired Student *t* test.

For each test assessed, the comparison of the effects produced by the intraperitoneal administration of several doses of CORM-2, iCORM-2, CORM-3, iCORM-3, CoPP, or SnPP *versus* the effects produced by their corresponding vehicle was evaluated by using a one-way ANOVA followed by the Student Newman–Keuls test.

For each test and drug evaluated, the comparison of the effects produced by the subcutaneous administration of different doses of morphine, DPDPE, JWH-015, or their corresponding vehicle was evaluated by using a two-way ANOVA (dose and treatment as between factors of variation) followed by the corresponding one-way ANOVA and Student Newman–Keuls test.

For each behavioral test, the comparison of the effects produced by the intraperitoneal administration of CORM-2, CORM-3, or CoPP on the local or systemic antinociceptive effects produced by morphine, DPDPE, or JWH-015 was evaluated by using a three-way ANOVA (treatment, drug, and route of administration as between factors of variation) followed by the corresponding one-way ANOVA and Student Newman–Keuls test.

For each behavioral test, the comparison of the effects produced by the subplantar or subcutaneous administration of SnPP on the local or systemic antinociceptive effects produced by morphine, DPDPE, or JWH-015 was evaluated by using a three-way ANOVA (treatment, drug, and route of administration as between factors of variation) followed by the corresponding one-way ANOVA and Student Newman–Keuls test.

In these experiments, antinociception in von Frey filaments and plantar test are expressed as the percentage of maximal possible effect, where the test latencies pre- (baseline) and postdrug administration are compared and calculated according to the following equation:

$$\text{Maximal possible effect (\%)} = ([\text{drug} - \text{baseline}] / [\text{cut-off} - \text{baseline}]) \times 100$$

In the cold-plate test, the inhibitory effects were calculated according to the following equation:

Inhibition (%) = [(paw elevations number at baseline–paw elevations number after drug)/paw elevations number at baseline] × 100

For each test, the reversal of the local and systemic antinociceptive effects produced by morphine, DPDPE, or JWH-015 with their respective antagonists and the effects produced by these antagonists administered alone were analyzed by using a one-way ANOVA followed by the Student Newman–Keuls test.

Changes in the expression of MOR, DOR, CB2R, HO-1, HO-2, CD11b/c, NOS1, and NOS2 in the dorsal root ganglia and/or spinal cord from sciatic nerve-injured mice treated with vehicle, CORM-2, or CoPP were also analyzed by using a one-way ANOVA followed by Student Newman–Keuls test. A value of  $P < 0.05$  was considered as a significant.

## Results

### Induction of Neuropathic Pain

In accordance to our previous findings, sciatic nerve ligation produced unilateral mechanical allodynia, thermal hyperalgesia, and thermal allodynia at 10 days after surgery (table 1). For each test evaluated, the two-way ANOVA showed a significant effect of the paw ( $P < 0.001$ ) and surgery ( $P < 0.001$ ) as well as their interaction ( $P < 0.001$ ). Indeed, sciatic nerve injury led to a significant decrease in the threshold for evoking paw withdrawal to a mechanical stimulus, a decrease in paw withdrawal latency to thermal stimulus, and an increase in the number of paw elevations to cold thermal stimulus in the ipsilateral paw of these animals compared with the ipsilateral paw of sham-operated mice ( $P < 0.01$ ; unpaired Student  $t$  test). In all tests, non-significant changes were observed in the contralateral paw when compared sciatic nerve-injured *versus* sham-operated mice.

### Effects of CORM-2, CORM-3, CoPP, and SnPP on the Mechanical Allodynia, Thermal Hyperalgesia, and Thermal Allodynia Induced by Sciatic Nerve Injury in Mice

The effects of the intraperitoneal administration of different doses of CORM-2 (5 and 10 mg/kg), iCORM-2 (10 mg/kg), CORM-3 (5 and 10 mg/kg), iCORM-3 (10 mg/kg), CoPP (5 and 10 mg/kg), and SnPP (10 and 20 mg/kg) on the mechanical allodynia, thermal hyperalgesia, and thermal allodynia induced by sciatic nerve injury at 10 days after surgery were investigated.

Our results show that the intraperitoneal administration of 5 and 10 mg/kg of CORM-2 or CORM-3 similarly inhibited the mechanical allodynia, thermal hyperalgesia, and thermal allodynia induced by sciatic nerve injury ( $P < 0.001$ ; one-way ANOVA *vs.* their respective vehicle treated mice, table 2). Our results also demonstrate that the intraperitoneal administration of 10 mg/kg of iCORM-2 or iCORM-3 did not have any significant effect in the principal symptoms of neuropathic pain evaluated in this study. In contrast, the intraperitoneal administration of 10 mg/kg, but not 5 mg/kg, of CoPP also inhibited the mechanical allodynia, thermal hyperalgesia, and thermal allodynia induced by sciatic nerve injury ( $P < 0.001$ ; one-way ANOVA *vs.* their respective vehicle treated mice). However, the intraperitoneal administration of 10 or 20 mg/kg of SnPP did not alter the principal symptoms of neuropathic pain.

Taking into account that 5 or 10 mg/kg of CORM-2 and CORM-3 produce a similar inhibitory effect, in the following experiments we used a dose of 10 mg/kg for both CORMs in order to maintain the same dosage tested for CoPP (10 mg/kg, a dose that produces an inhibitory effect) or SnPP (10 mg/kg). In addition and because the intraperitoneal administration of 10 mg/kg of iCORM-2 or iCORM-3 did not produce any significant inhibitory effect, we did not test their effects in the subsequent experiments.

The administration of CORM-2, iCORM-2, CORM-3, iCORM-3, CoPP, or SnPP did not have any significant effect neither on the ipsilateral paw of sham-operated mice nor on the contralateral paw of sciatic nerve-injured or sham-operated animals (data not shown).

### Effects of the Subcutaneous Administration of Morphine, DPDPE, and JWH-015 on the Mechanical Allodynia, Thermal Hyperalgesia, and Thermal Allodynia Induced by Sciatic Nerve Injury in Mice

The subcutaneous administration of morphine (1–10 mg/kg), DPDPE (0.5–10 mg/kg), or JWH-015 (0.15–3 mg/kg) dose dependently inhibited the mechanical allodynia (fig. 1A), thermal hyperalgesia (fig. 1B), and thermal allodynia (fig. 1C) induced by sciatic nerve injury in mice. For each drug and test evaluated, the two-way ANOVA revealed a significant effect of the dose ( $P < 0.003$ ), treatment ( $P < 0.001$ ), and their interaction ( $P < 0.003$ ). Indeed, the mechanical antiallodynic, thermal

**Table 1.** Mechanical Response (Von Frey Filaments Strength, g), Thermal Heat Response (Withdrawal Latency, s) and Thermal Cold Response (Paw Lifts, Number) in the Contralateral and Ipsilateral Paw of Sham-Operated and Sciatic Nerve-Injured Mice at 10 Days after Surgery

Paw	Surgery	Mechanical Response Von Frey Filaments Strength (g)	Thermal Heat Response Withdrawal Latency (s)	Thermal Cold Response Paw Lifts (No.)
Contralateral	sham	2.6 ± 0.1	8.4 ± 0.2	0.0 ± 0.0
	CCI	2.6 ± 0.1	8.4 ± 0.2	0.2 ± 0.1
Ipsilateral	sham	2.6 ± 0.1	8.1 ± 0.2	0.2 ± 0.2
	CCI	1.3 ± 0.1*	4.3 ± 0.1*	4.2 ± 0.7*

Results are shown as mean values ± SEM; n = 6 animals per experimental group. For each test and paw.

\* $P < 0.01$  denotes significant differences between sciatic nerve-injured (CCI) and sham-operated (sham) mice (unpaired Student  $t$  test).

**Table 2.** Mechanical Antiallodynic, Thermal Antihyperalgesic, and Thermal Antiallodynic Effects of the Intraperitoneal Administration of Different Doses of CORM-2, CORM-3, CoPP, or SnPP as Well as the Inactive CO-RMs (iCORM-2 and iCORM-3) in the Ipsilateral Paw of Sciatic Nerve-injured Animals

Treatments	Dose, mg/kg	Mechanical Antiallodynic Maximal Possible Effect, %	Thermal Antihyperalgesic Maximal Possible Effect, %	Thermal Antiallodynic Inhibition, %
Vehicle	—	0.9 ± 0.9	4.7 ± 2.7	1.7 ± 1.5
CORM-2	5	27.6 ± 9.5*	38.3 ± 2.8*	31.3 ± 7.6*
CORM-2	10	29.2 ± 4.9*	40.7 ± 1.4*	32.6 ± 8.5*
iCORM-2	10	4.3 ± 2.2	6.3 ± 3.2	3.3 ± 3.3
vehicle	—	2.9 ± 2.9	5.3 ± 3.8	1.6 ± 1.4
CORM-3	5	35.4 ± 8.0*	37.3 ± 2.6*	38.7 ± 7.0*
CORM-3	10	34.2 ± 4.5*	37.3 ± 3.2*	36.1 ± 5.1*
iCORM-3	10	5.2 ± 2.5	7.9 ± 2.5	2.5 ± 2.5
Vehicle	—	3.0 ± 3.0	6.8 ± 4.0	1.5 ± 1.2
CoPP	5	5.3 ± 2.2	7.8 ± 1.9	0.0 ± 0.0
CoPP	10	28.9 ± 7.3*	25.8 ± 3.7*	24.4 ± 2.8*
Vehicle	—	3.7 ± 3.7	4.4 ± 3.0	3.0 ± 1.0
SnPP	10	8.2 ± 3.8	5.3 ± 2.4	8.0 ± 4.9
SnPP	20	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

For each test and drug tested.

\*  $P < 0.05$  indicates significant differences vs. their respective vehicle treated group (one-way ANOVA, followed by the Student Newman-Keuls test). Results are shown as mean values ± SEM;  $n = 6$  animals per experimental group.

CoPP = cobalt protoporphyrin IX; CO-RMs = carbon monoxide-releasing molecules; CORM-2 = tricarbonyldichloro ruthenium (II) dimer; iCORM-2 = inactive tricarbonyldichloro ruthenium (II) dimer; CORM-3 = tricarbonylchloro (glycinato)ruthenium (II); iCORM-3 = inactive tricarbonylchloro (glycinato)ruthenium (II); SnPP = tin protoporphyrin IX.

antihyperalgesic, and thermal antiallodynic effects produced by high doses of morphine, DPDPE, or JWH-015 in the ipsilateral paw of sciatic nerve-injured mice were significantly higher than those produced by low doses of the same drug or their corresponding vehicle treated animals ( $P < 0.001$ , one-way ANOVA followed by the Student Newman-Keuls test).

The subcutaneous administration of morphine, DPDPE, JWH-015, or vehicle did not elicit any antinociceptive effect neither in the ipsilateral paw of sham-operated mice nor in the contralateral paw of sciatic nerve-injured or sham-operated animals (data not shown).

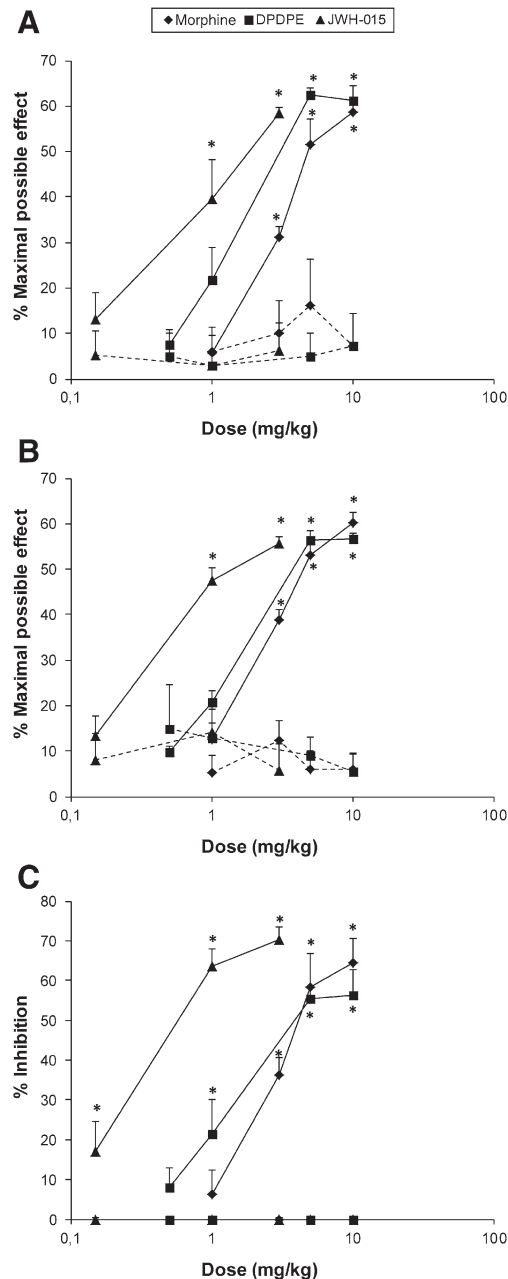
#### **Effects of CORM-2, CORM-3, and CoPP on the Antiallodynic and Antihyperalgesic Responses to Morphine, DPDPE, and JWH-015 in Sciatic Nerve-Injured Mice**

The effects of the intraperitoneal administration of 10 mg/kg of CORM-2, CORM-3, and CoPP on the mechanical antiallodynic, thermal antihyperalgesic, and thermal antiallodynic effects produced by the subplantar or subcutaneous administration of morphine (50 µg or 1 mg/kg), DPDPE (100 µg or 5 mg/kg), JWH-015 (30 µg or 3 mg/kg), or vehicle in sciatic nerve-injured mice at 10 days after surgery were investigated.

For morphine and each test evaluated, the three-way ANOVA revealed a significant effect of the treatment ( $P < 0.025$ ), drug ( $P < 0.002$ ), and route of drug administration ( $P < 0.025$ ). In addition, a significant interaction between drug and their administration route ( $P < 0.027$ ) was also demonstrated. Indeed, our results showed that the intraperitoneal administration of CORM-2, CORM-3, or CoPP

alone significantly attenuated the mechanical allodynia (fig. 2, A and B), thermal hyperalgesia (fig. 2, C and D), and thermal allodynia (fig. 2, E and F) induced by sciatic nerve injury ( $P < 0.001$ ; one-way ANOVA *vs.* control vehicle treated mice). Our results also demonstrate that treatment with CORM-2, CORM-3, or CoPP significantly increased the local mechanical antiallodynic (fig. 2A), thermal antihyperalgesic (fig. 2C), and thermal antiallodynic (fig. 2E) effects produced by the subplantar administration of morphine in the ipsilateral paw of sciatic nerve-injured mice ( $P < 0.001$ , one-way ANOVA *vs.* their respective control group treated with morphine, CORM-2, CORM-3, or CoPP plus vehicle). Treatment with CORM-2, CORM-3, or CoPP only enhanced the mechanical antiallodynic (fig. 2B), thermal antihyperalgesic (fig. 2D), and thermal antiallodynic (fig. 2F) effects produced by the subcutaneous administration of morphine compared to their respective control group treated with morphine plus vehicle ( $P < 0.05$ , one-way ANOVA), but not to those produced by CORM-2, CORM-3, or CoPP plus vehicle in the ipsilateral paw of sciatic nerve-injured mice.

Regarding DPDPE, the three-way ANOVA revealed a significant effect of the treatment ( $P < 0.035$ ), drug ( $P < 0.002$ ), and route of drug administration ( $P < 0.002$ ) as well as a significant interaction between treatment and drug ( $P < 0.001$ ) and treatment with the route of drug administration ( $P < 0.025$ ), for each test evaluated. Indeed, treatment with CORM-2, CORM-3, or CoPP significantly attenuated the mechanical allodynia (fig. 3, A and B), thermal hyperalgesia (fig. 3, C and D), and thermal allodynia (fig. 3, E and F)



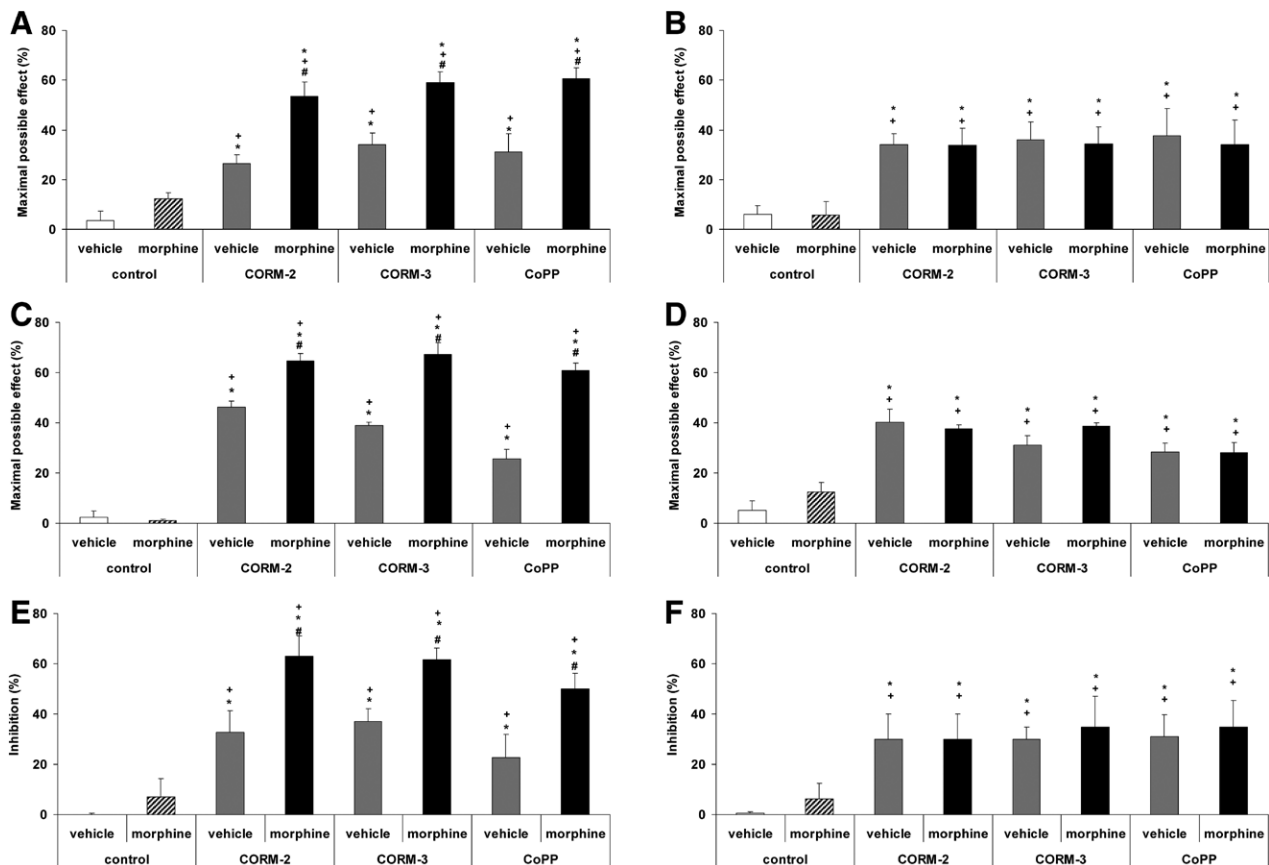
**Fig. 1.** Effects of the subcutaneous administration of morphine, [d-Pen(2),d-Pen(5)]-enkephalin (DPDPE) and (2-methyl-1-propyl-1H-indol-3-yl)-1-naphthalenylmethanone (JWH-015) on the mechanical allodynia, thermal hyperalgesia, and thermal allodynia induced by sciatic nerve injury in mice. Mechanical antiallodynic (A), thermal antihyperalgesic (B), and thermal antiallodynic (C) effects of the subcutaneous administration of different doses (logarithmic axis) of morphine, DPDPE, JWH-015 (continuous lines), or their respective vehicles (discontinuous lines) in the ipsilateral paw of sciatic nerve-injured mice at 10 days after surgery. Data are expressed as mean values of maximal possible effect (%) for mechanical allodynia and thermal hyperalgesia or inhibition (%) for thermal allodynia  $\pm$  SEM (six animals for dose). For each test, drug, and dose, \* denotes significant differences versus other doses of the same drug or their respective vehicle treated animals ( $P < 0.05$ ; one-way ANOVA followed by the Student Newman-Keuls test).

induced by injury in the ipsilateral paw of sciatic nerve-injured mice ( $P < 0.001$ ; one-way ANOVA *vs.* their respective control vehicle treated mice). Moreover, and in contrast to morphine, treatments with CORM-2, CORM-3, or CoPP significantly reduced the mechanical antiallodynic (fig. 3, A and B), thermal antihyperalgesic (fig. 3, C and D), and thermal antiallodynic (fig. 3, E and F) effects produced by the subplantar or subcutaneous administration of DPDPE in the ipsilateral paw of sciatic nerve-injured mice ( $P < 0.02$ , one-way ANOVA *vs.* their respective control group treated with DPDPE). The interaction between treatment and route of drug administration ( $P < 0.025$ ) could be explained by the fact that while the antinociceptive effects produced by the subcutaneous administration of DPDPE were diminished by CoPP treatment ( $P < 0.02$ ; one-way ANOVA *vs.* their respective control vehicle or DPDPE treated mice), the antinociceptive effects produced by the subplantar administration of DPDPE were completely blocked by CoPP treatment ( $P < 0.001$ ; one-way ANOVA *vs.* their respective control DPDPE, but not vehicle, treated mice).

Regarding JWH-015, the three-way ANOVA also revealed a significant effect of the treatment ( $P < 0.001$ ), drug ( $P < 0.001$ ), and route of drug administration ( $P < 0.021$ ) as well as a significant interaction between treatment and drug ( $P < 0.001$ ) and treatment with the route of drug administration ( $P < 0.050$ ), for each test evaluated. Thus, and similar to that occurred with DPDPE, while treatment with CORM-2, CORM-3, or CoPP significantly attenuated the mechanical allodynia (fig. 4, A and B), thermal hyperalgesia (fig. 4, C and D), and thermal allodynia (fig. 4, E and F) induced by injury in the ipsilateral paw of sciatic nerve-injured mice ( $P < 0.001$ ; one-way ANOVA *vs.* their respective control vehicle treated mice), all of these treatments significantly reduced the mechanical antiallodynic (fig. 4, A and B), thermal antihyperalgesic (fig. 4, C and D), and thermal antiallodynic (fig. 4, E and F) effects produced by the subplantar or subcutaneous administration of JWH-015 in the ipsilateral paw of sciatic nerve-injured mice ( $P < 0.001$ , one-way ANOVA *vs.* their respective control group treated with JWH-015). The interaction between treatment and route of drug administration ( $P < 0.025$ ) could be explained by the fact that while the antinociceptive effects produced by the subcutaneous administration of JWH-015 were diminished by CoPP treatment ( $P < 0.001$ ; one-way ANOVA *vs.* their respective control vehicle or JWH-015 treated mice), this treatment completely blocked the antinociceptive effects produced by the subplantar administration of JWH-015 ( $P < 0.001$ ; one-way ANOVA *vs.* their respective control JWH-015, but not vehicle, treated mice).

The three-way ANOVA did not reveal any significant effect of the treatment (CORM-2, CORM-3, or CoPP), drug (morphine, DPDPE, or JWH-015), and route of drug administration (subplantar or subcutaneous), and non-significant interaction between them was demonstrated neither in the ipsilateral paw of sham-operated mice nor in the





**Fig. 2.** Effects of tricarboxyldichloro ruthenium (II) dimer (CORM-2), tricarboxylchloro (glycinato)ruthenium (II) (CORM-3), and cobalt protoporphyrin IX (CoPP) on the antiallodynic and antihyperalgesic responses to morphine. Mechanical antiallodynic (A, B), thermal antihyperalgesic (C, D), and thermal antiallodynic (E, F) effects of the subplantar (50  $\mu$ g; A, C, E) or subcutaneous (1 mg/kg; B, D, F) administration of morphine or vehicle in the ipsilateral paw of sciatic nerve-injured mice pretreated with 10 mg/kg of CORM-2, CORM-3, or CoPP at 10 days after surgery. The effects of the intraperitoneal administration of CORM-2, CORM-3, or CoPP alone are also shown. Data are expressed as mean values of the maximal possible effect (%) for mechanical allodynia and thermal hyperalgesia and as inhibition (%) for thermal allodynia  $\pm$  SEM (six animals per group). For each behavioral test, \* denotes significant differences *versus* control group treated with vehicle ( $P < 0.05$ , one-way ANOVA followed by Student Newman–Keuls test), + denotes significant differences *versus* control group treated with morphine ( $P < 0.05$ , one-way ANOVA followed by the Student Newman–Keuls test) and # denotes significant differences *versus* group treated with CORM-2, CORM-3, or CoPP plus vehicle ( $P < 0.05$ ; one-way ANOVA followed by the Student Newman–Keuls test).

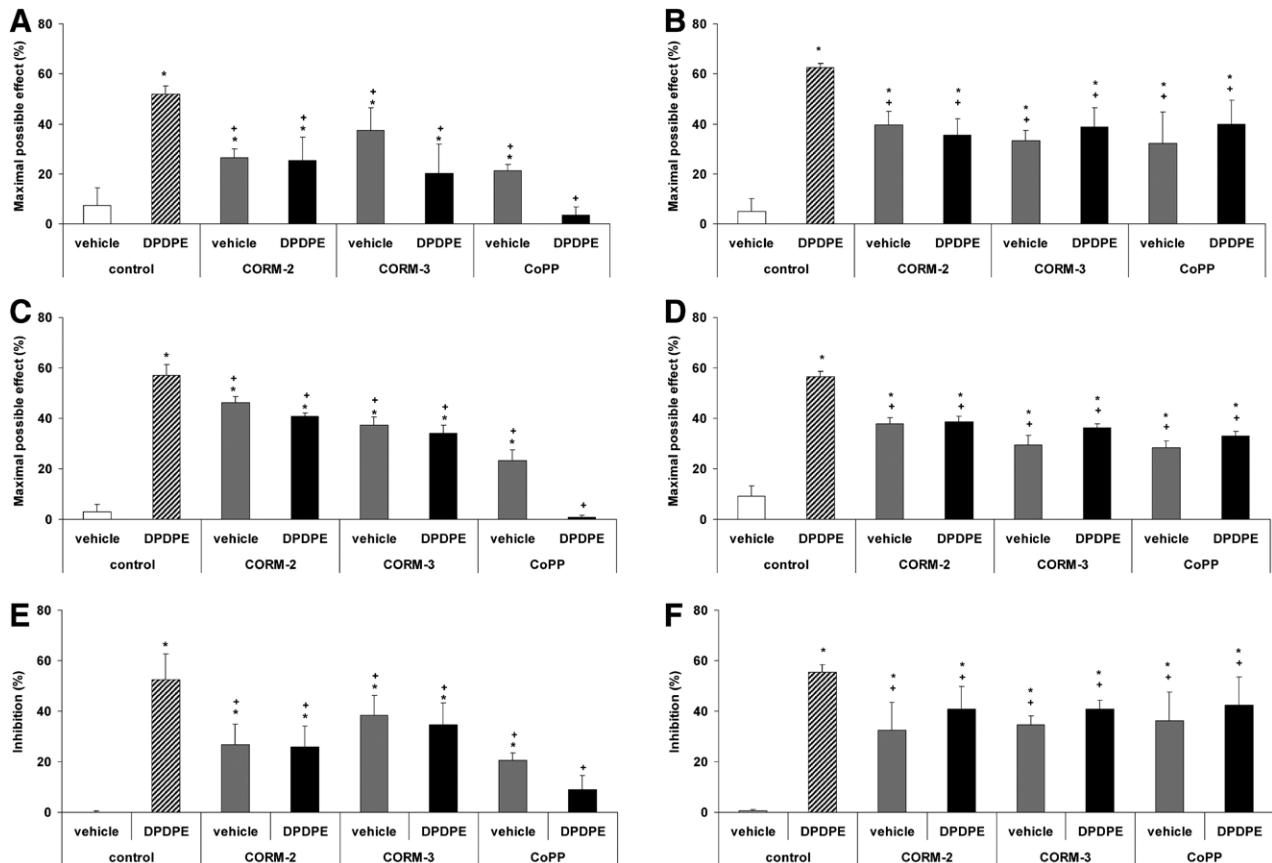
contralateral paw of sciatic nerve-injured or sham-operated animals. That is, the subplantar or subcutaneous administration of morphine, DPDPE, or JWH-015 alone or combined with CORM-2, CORM-3, or CoPP did not have any significant effect neither on the ipsilateral paw of sham-operated mice nor on the contralateral paw of sciatic nerve-injured or sham-operated animals (data not shown).

#### Effects of the HO-1 Inhibitor, SnPP, on the Antinociceptive Response to Morphine, DPDPE, and JWH-015 in Sciatic Nerve-Injured Mice

The effects of the subplantar (290  $\mu$ g) or subcutaneous (10 mg/kg) administration of SnPP on the mechanical antiallodynic, thermal antihyperalgesic, and thermal antiallodynic effects produced by the subplantar or subcutaneous administration of morphine (100  $\mu$ g or 5 mg/kg), DPDPE (25  $\mu$ g or 0.5 mg/kg), or JWH-015 (5  $\mu$ g or 0.15 mg/kg) in sciatic nerve-injured mice at 10 days after surgery were assessed.

For morphine and each test evaluated, the three-way ANOVA revealed a significant effect of the treatment ( $P < 0.004$ ), drug ( $P < 0.001$ ), and route of administration ( $P < 0.045$ ). In addition, a significant interaction between drug and administration route ( $P < 0.029$ ), treatment and drug ( $P < 0.004$ ), treatment and administration route ( $P < 0.047$ ), and a triple interaction between treatment, drug, and the administration route ( $P < 0.009$ ) were also demonstrated. Indeed, our results show that while the subplantar administration of SnPP alone did not alter the mechanical allodynia (fig. 5A), thermal hyperalgesia (fig. 5B), and thermal allodynia (fig. 5C) induced by sciatic nerve injury, their local coadministration with a high dose of morphine (100  $\mu$ g) significantly decreased the local mechanical antiallodynic (fig. 5A), thermal antihyperalgesic (fig. 5B), and thermal antiallodynic (fig. 5C) effects produced by morphine in the ipsilateral paw of sciatic nerve-injured mice





**Fig. 3.** Effects of tricarboxyldichloro ruthenium (II) dimer (CORM-2), tricarboxyldichloro (glycinato)ruthenium (II) (CORM-3), and cobalt protoporphyrin IX (CoPP) on the antiallodynic and antihyperalgesic responses to [d-Pen(2),d-Pen(5)]-enkephalin (DPDPE). Mechanical antiallodynic (A, B), thermal antihyperalgesic (C, D), and thermal antiallodynic (E, F) effects of the subplantar (100  $\mu$ g; A, C, E) or subcutaneous (5 mg/kg; B, D, F) administration of DPDPE or vehicle in the ipsilateral paw of sciatic nerve-injured mice pretreated with 10 mg/kg of CORM-2, CORM-3, or CoPP at 10 days after surgery. The effects of the intraperitoneal administration CORM-2, CORM-3, CoPP, or vehicle alone are also shown. Data are expressed as mean values of the maximal possible effect (%) for mechanical allodynia and thermal hyperalgesia and as inhibition (%) for thermal allodynia  $\pm$  SEM (six animals per group). For each behavioral test, \* denotes significant differences *versus* control group treated with vehicle ( $P < 0.05$ , one-way ANOVA followed by Student Newman–Keuls test) and + denotes significant differences *versus* control group treated with DPDPE ( $P < 0.05$ , one-way ANOVA followed by the Student Newman–Keuls test).

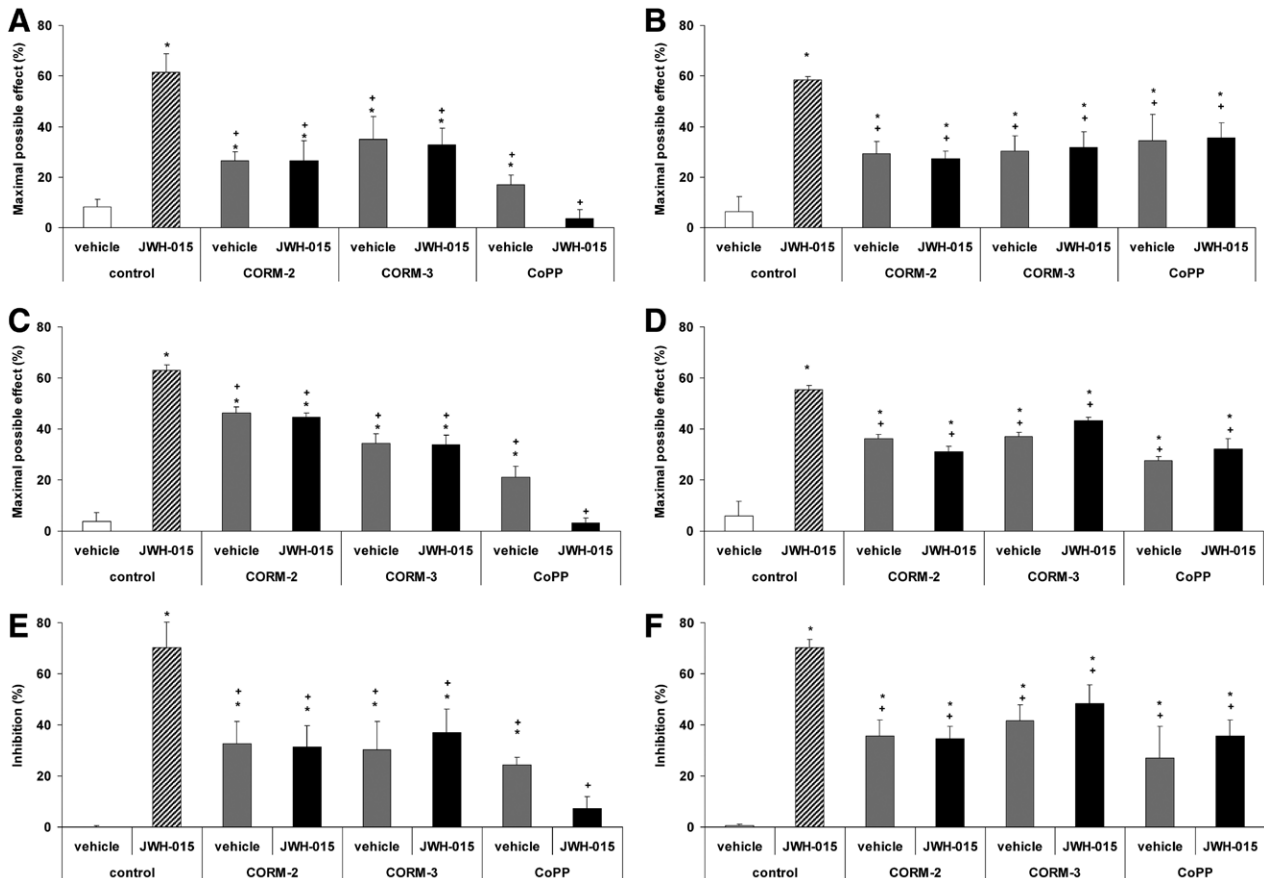
( $P < 0.001$ , one-way ANOVA *vs.* vehicle group treated with morphine). In contrast, the subcutaneous administration of SnPP alone did not alter the mechanical allodynia (fig. 5D), thermal hyperalgesia (fig. 5E), and thermal allodynia (fig. 5F) induced by sciatic nerve injury as well as the mechanical antiallodynic (fig. 5D), thermal antihyperalgesic (fig. 5E), and thermal antiallodynic (fig. 5F) effects produced by the subcutaneous administration of a high dose of morphine (5 mg/kg) in the ipsilateral paw of sciatic nerve-injured mice.

For DPDPE and JWH-015, the three-way ANOVA did not reveal any significant effect of the treatment, drug, and route of administration as well as non-significant interaction between them was demonstrated, in the three tests evaluated. That is, the subplantar or subcutaneous coadministration of SnPP with a low dose of DPDPE or JWH-015 did not alter the antiallodynic and antihyperalgesic responses produced by these drugs administered alone in the ipsilateral paw of sciatic nerve-injured mice (fig. 5).

For morphine, DPDPE, and JWH-015, the three-way ANOVA did not reveal any significant effect of the treatment, drug, and route of administration as well as non-significant interaction between them, neither on the ipsilateral paw of sham-operated mice nor on the contralateral paw of sciatic nerve-injured or sham-operated animals. That is, the subplantar or subcutaneous administration of morphine, DPDPE, or JWH-015 alone or combined with SnPP did not have any significant effect neither on the ipsilateral paw of sham-operated mice nor on the contralateral paw of sciatic nerve-injured or sham-operated animals (data not shown).

#### **Reversal of the Antinociceptive Effects of Morphine, DPDPE, and JWH-015 by Specific Antagonists after Sciatic Nerve Injury**

The mechanical and thermal antiallodynic as well as the antihyperalgesic effects produced by the subplantar administration of 100  $\mu$ g of morphine in the ipsilateral paw of sciatic

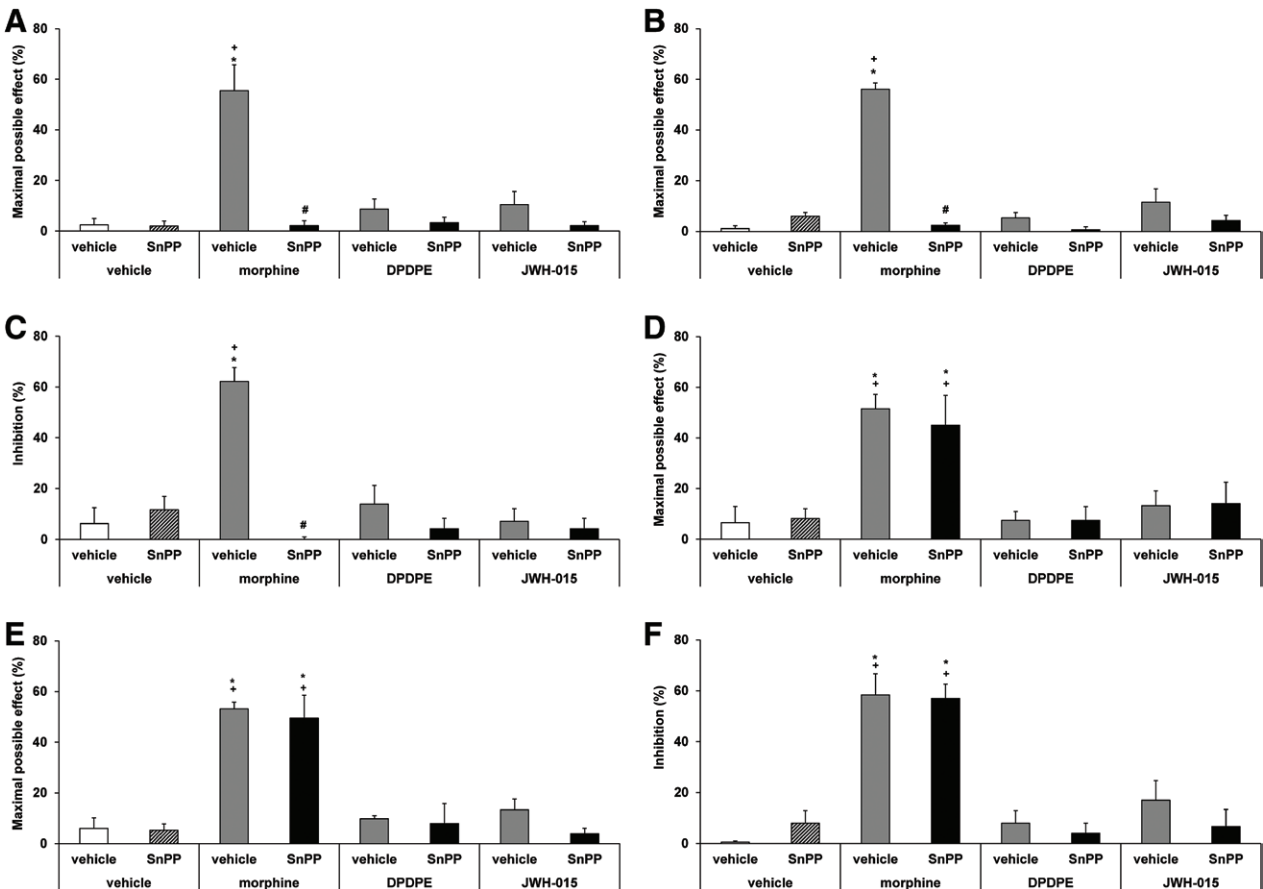


**Fig. 4.** Effects of tricarboxyldichloro ruthenium (II) dimer (CORM-2), tricarboxylchloro (glycinato)ruthenium (II) (CORM-3), and cobalt protoporphyrin IX (CoPP) on the antiallodynic and antihyperalgesic responses to (2-methyl-1-propyl-1H-indol-3-yl)-1-naphthalenylmethanone (JWH-015). Mechanical antiallodynic (A, B), thermal antihyperalgesia (C, D), and thermal antiallodynic (E, F) effects of the subplantar (30  $\mu$ g; A, C, E) or subcutaneous (3 mg/kg; B, D, F) administration of JWH-015 or vehicle in the ipsilateral paw of sciatic nerve-injured mice pretreated with 10 mg/kg of CORM-2, CORM-3, or CoPP at 10 days after surgery. The effects of the intraperitoneal administration CORM-2, CORM-3, CoPP, or vehicle alone are also shown. Data are expressed as mean values of the maximal possible effect (%) for mechanical allodynia and thermal hyperalgesia and as inhibition (%) for thermal allodynia  $\pm$  SEM (six animals per group). For each behavioral test, \* denotes significant differences versus control group treated with vehicle ( $P < 0.05$ , one-way ANOVA followed by Student Newman–Keuls test) and + denotes significant differences versus control group treated with JWH-015 ( $P < 0.05$ , one-way ANOVA followed by the Student Newman–Keuls test).

nerve-injured mice were completely reversed by its subplantar coadministration with selective MOR (CTAP, 120  $\mu$ g) or peripheral opioid receptor (NX-ME, 20  $\mu$ g) antagonists ( $P < 0.05$ ; one-way ANOVA, followed by Student Newman–Keuls test, table 3). In a similar way, the mechanical and thermal antiallodynic as well as the antihyperalgesic effects produced by 100  $\mu$ g of DPDPE in the ipsilateral paw of sciatic nerve-injured mice were completely reversed by its subplantar coadministration with a selective DOR (naltrindole, 50  $\mu$ g) or a peripheral opioid receptor (NX-ME, 20  $\mu$ g) antagonist ( $P < 0.049$ ; one-way ANOVA, followed by Student Newman–Keuls test). In addition, the mechanical antiallodynic, thermal antihyperalgesic, and thermal antiallodynic effects produced by 30  $\mu$ g of JWH-015 in the ipsilateral paw of sciatic nerve-injured mice were also completely reversed by its subplantar coadministration with a selective CB2R antagonist (AM630, 30  $\mu$ g;  $P < 0.040$ ; one-way ANOVA, followed by Student Newman–Keuls test).

The subplantar administration of AM251 (a selective CB1R antagonist; 150  $\mu$ g) was unable to revert the local antiallodynic and antihyperalgesic effects produced by JWH-015.

The mechanical and thermal antiallodynic as well as the antihyperalgesic effects produced by the subcutaneous administration of 5 mg/kg of morphine in the ipsilateral paw of sciatic nerve-injured mice were completely reversed by its subcutaneous coadministration with CTAP (4 mg/kg) but not with NX-ME (1 mg/kg;  $P < 0.005$ ; one-way ANOVA, followed by Student Newman–Keuls test, table 4). In contrast, the inhibitory effects produced by 5 mg/kg of DPDPE on the ipsilateral paw of sciatic nerve-injured mice were completely reversed by its coadministration with naltrindole (2 mg/kg) or NX-ME (1 mg/kg;  $P < 0.009$ ; one-way ANOVA, followed by Student Newman–Keuls test). Finally, the mechanical antiallodynic, thermal antihyperalgesic, and thermal antiallodynic effects produced by 3 mg/kg of JWH-015 on the ipsilateral paw of sciatic nerve-injured mice were completely reversed



**Fig. 5.** Effects of tin protoporphyrin IX (SnPP) treatment on the antiallodynic and antihyperalgesic responses to morphine, [d-Pen(2),d-Pen(5)]-enkephalin (DPDPE) or (2-methyl-1-propyl-1H-indol-3-yl)-1-naphthalenylmethanone (JWH-015). Local (A, B, C) and systemic (D, E, F) mechanical antiallodynic (A, D), thermal antihyperalgesic, (B, E) and thermal antiallodynic (C, F) effects of the subplantar or systemic administration of morphine (100  $\mu$ g or 5 mg/kg), DPDPE (25  $\mu$ g or 0.5 mg/kg), or JWH-015 (5  $\mu$ g or 0.15 mg/kg) combined with SnPP (290  $\mu$ g or 10 mg/kg) at 10 days after surgery are shown. The effects of the subplantar or subcutaneous administration of morphine, DPDPE, JWH-015, SnPP, or vehicle alone are also represented. Data are expressed as mean values of the maximal possible effect (%) for mechanical allodynia and thermal hyperalgesia and as inhibition (%) for thermal allodynia  $\pm$  SEM (6 animals per group). For each behavioral test, \* denotes significant differences *versus* group treated with vehicle ( $P < 0.05$ , one-way ANOVA followed by Student Newman–Keuls test), + denotes significant differences *versus* group treated with SnPP plus vehicle ( $P < 0.05$ , one-way ANOVA followed by the Student Newman–Keuls test), and # denotes significant differences *versus* group treated with morphine plus vehicle ( $P < 0.05$ , one-way ANOVA followed by the Student Newman–Keuls test).

by its subcutaneous coadministration with AM630 (1 mg/kg) but not with AM251 (5 mg/kg;  $P < 0.007$ ; one-way ANOVA, followed by Student Newman–Keuls test).

The subplantar or subcutaneous administration of the different antagonists alone in sciatic nerve-injured mice (tables 3 and 4) as well as in the contralateral and ipsilateral paw of sham-operated mice or in the contralateral paw of sciatic nerve-injured mice (data not shown) did not have any significant effect on the different nociceptive responses evaluated in this study. In addition, the subplantar or subcutaneous administration of all tested agonists alone or combined with their respective antagonists did not produce any significant effect in the contralateral and ipsilateral paw of sham-operated mice nor in the contralateral paw of sciatic nerve-injured mice (data not shown).

#### Effect of CORM-2 and CoPP on MOR, DOR, and CB2R Protein Expression in the Dorsal Root Ganglia from Sciatic Nerve-Injured Mice

The protein levels of MOR, DOR, and CB2R in the dorsal root ganglia from sciatic nerve-injured mice treated with vehicle, CORM-2, or CoPP as well as from sham-operated mice treated with vehicle are shown in figure 6. Our results show that the expression of MOR (fig. 6A) was significantly increased by CORM-2 or CoPP treatments ( $P < 0.001$ ; one-way ANOVA *vs.* sham-operated and sciatic nerve-injured vehicle treated mice). The unchanged protein levels of DOR in the dorsal root ganglia from sciatic nerve-injured mice were not altered by CORM-2 or CoPP treatments (fig. 6B), while the enhanced peripheral expression of CB2R induced by nerve injury was significantly reduced by both treatments

**Table 3.** Effects of the Subplantar Administration of Morphine (100 µg), DPDPE (100 µg), or JWH-015 (30 µg) Alone or Combined with CTAP (120 µg) or NX-ME (20 µg), Naltrindole (50 µg), or NX-ME (20 µg), and AM630 (30 µg) or AM251 (150 µg), Respectively, on the Mechanical Allodynia, Thermal Hyperalgesia, and Thermal Allodynia Induced by Injury in the Ipsilateral Paw of Sciatic Nerve-injured Animals

Treatments		Mechanical Allodynia Von Frey Filaments Strength, g	Thermal Hyperalgesia Withdrawal Latency, s	Thermal Allodynia Paw Lifts, No.
Vehicle	Vehicle	1.0 ± 0.1	3.6 ± 0.1	5.7 ± 0.6
Morphine	Vehicle	2.7 ± 0.4*	7.0 ± 0.1*	2.2 ± 0.5*
	CTAP	1.2 ± 0.1	3.7 ± 0.1	5.2 ± 0.5
	NX-ME	1.0 ± 0.1	3.4 ± 0.1	4.7 ± 0.5
Vehicle	CTAP	1.3 ± 0.1	3.1 ± 0.1	4.2 ± 0.2
	NX-ME	1.1 ± 0.2	3.4 ± 0.1	5.0 ± 0.8
Vehicle	Vehicle	1.2 ± 0.1	3.2 ± 0.3	5.0 ± 0.9
DPDPE	Vehicle	2.4 ± 0.1*	8.5 ± 0.4*	2.7 ± 0.5*
	Naltrindole	1.2 ± 0.1	3.5 ± 0.3	4.2 ± 0.5
	NX-ME	1.2 ± 0.1	3.4 ± 0.1	4.7 ± 0.7
Vehicle	Naltrindole	1.4 ± 0.2	4.1 ± 0.1	5.5 ± 0.9
	NX-ME	1.1 ± 0.2	3.4 ± 0.1	5.0 ± 0.8
Vehicle	Vehicle	1.2 ± 0.1	3.1 ± 0.3	5.2 ± 0.8
JWH-015	Vehicle	2.6 ± 0.2*	8.7 ± 0.2*	1.7 ± 0.6*
	AM630	1.0 ± 0.1	4.6 ± 0.2	5.2 ± 0.3
	AM251	2.4 ± 0.1*	7.7 ± 0.2*	2.4 ± 0.2*
Vehicle	AM630	1.4 ± 0.2	3.7 ± 0.2	5.7 ± 0.7
	AM251	1.4 ± 0.1	3.4 ± 0.1	5.0 ± 0.8

For each test and drug tested.

\*  $P < 0.05$  denotes significant differences vs. their respective vehicle plus vehicle treated group (one-way ANOVA, followed by the Student Newman-Keuls test). Results are shown as mean values ± SEM;  $n = 6$  animals per experimental group.

CTAP = H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH<sub>2</sub>; DPDPE = [d-Pen(2),d-Pen(5)]-enkephalin; JWH-015 = (2-methyl-1-propyl-1H-indol-3-yl)-1-naphthalenylmethanone; NX-ME = naloxone methiodide.

(fig. 6C;  $P < 0.001$ ; one-way ANOVA vs. sham-operated vehicle treated mice).

#### **Effect of CORM-2 and CoPP on HO-1, HO-2, CD11b/c, NOS1, and NOS2 Protein Expression in the Dorsal Root Ganglia and/or Spinal Cord from Sciatic Nerve-Injured Mice**

Our results show that the dorsal root ganglia expression of HO-1 (fig. 7A) was significantly increased by CORM-2 or CoPP treatments ( $P < 0.001$ ; one-way ANOVA vs. sham-operated and nerve-injured vehicle treated mice). In contrast, the dorsal root ganglia overexpression of HO-2 induced by sciatic nerve injury (fig. 7B) was unaltered by CORM-2 or CoPP treatments ( $P < 0.001$ ; one-way ANOVA compared to sham-operated vehicle treated mice).

We also investigated whether the increased spinal cord expression of CD11b/c induced by nerve injury could be altered by CORM-2 and CoPP treatments (fig. 7C;  $P < 0.001$ ; one-way ANOVA vs. sham-operated vehicle treated mice). Our results show that both CORM-2 and CoPP treatments inhibited the increased expression of CD11b/c in sciatic nerve-injured mice ( $P < 0.001$ ; one-way ANOVA vs. sciatic nerve-injured mice treated with vehicle).

The protein levels of NOS1 (fig. 7D) and NOS2 (fig. 7E) in the spinal cord from sciatic nerve-injured mice treated with vehicle, CORM-2, or CoPP are also shown. The

expression of NOS1 and NOS2 from sham-operated wild-type mice treated with vehicle has been also represented. Sciatic nerve injury significantly increased the protein levels of NOS1 and NOS2 ( $P < 0.001$ ; one-way ANOVA vs. sham-operated vehicle treated mice), which expression was significantly reduced by the intraperitoneal administration of CORM-2 and CoPP ( $P < 0.001$ ; one-way ANOVA vs. sciatic nerve-injured mice treated with vehicle).

#### **Discussion**

In this study, we demonstrated that the intraperitoneal administration of CORM-2, CORM-3, or CoPP attenuates the mechanical allodynia, thermal hyperalgesia, and thermal allodynia induced by sciatic nerve injury in mice. Our results also indicate that treatments with CO-RMs or CoPP increased the local antiallodynic and antihyperalgesic effects produced by the subplantar, but not systemic, administration of morphine, while decreased those produced by DPDPE and JWH-015. In consequence, the specific HO-1 inhibitor SnPP only decreased the antiallodynic and antihyperalgesic effects produced by the subplantar administration of morphine. Moreover, CORM-2 and CoPP treatments increased the expression of MOR and HO-1, did not change DOR and HO-2 expression, and decreased the overexpression of CB2R, CD11b/c, NOS1, and NOS2 induced by nerve injury.



**Table 4.** Effects of the Subcutaneous Administration of Morphine (5 mg/kg), DPDPE (5 mg/kg), or JWH-015 (3 mg/kg) Alone or Combined with CTAP (4 mg/kg) or NX-ME (1 mg/kg), Naltrindole (2 mg/kg), or NX-ME (1 mg/kg), and AM630 (1 mg/kg) or AM251 (5 mg/kg), Respectively, on the Mechanical Allodynia, Thermal Hyperalgesia, and Thermal Allodynia Induced by Injury in the Ipsilateral Paw of Sciatic Nerve-injured Animals

Treatments		Mechanical Allodynia Von Frey Filaments Strength, g	Thermal Hyperalgesia Withdrawal Latency, s	Thermal Allodynia Paw Lifts, No.
Vehicle	Vehicle	1.6 ± 0.1	3.6 ± 0.1	4.0 ± 0.3
Morphine	Vehicle	2.6 ± 0.1*	8.6 ± 0.6*	1.5 ± 0.2*
	CTAP	1.6 ± 0.1	4.2 ± 0.2	4.3 ± 0.2
	NX-ME	2.5 ± 0.2*	8.4 ± 0.3*	1.6 ± 0.2*
	CTAP	1.5 ± 0.1	4.0 ± 0.2	4.4 ± 0.4
Vehicle	NX-ME	1.6 ± 0.1	4.2 ± 0.3	4.4 ± 0.4
	Vehicle	1.5 ± 0.1	3.5 ± 0.2	4.7 ± 0.6
	DPDPE	2.6 ± 0.2*	8.3 ± 0.2*	2.2 ± 0.2*
	Naltrindole	1.6 ± 0.1	4.5 ± 0.1	4.3 ± 0.3
Vehicle	NX-ME	1.8 ± 0.1	3.8 ± 0.2	4.7 ± 0.6
	Naltrindole	1.5 ± 0.1	4.3 ± 0.2	4.2 ± 0.4
	NX-ME	1.6 ± 0.1	3.9 ± 0.2	4.6 ± 0.4
	Vehicle	1.5 ± 0.1	3.8 ± 0.1	4.6 ± 0.2
JWH-015	Vehicle	2.6 ± 0.1*	8.4 ± 0.1*	1.2 ± 0.2*
	AM630	1.6 ± 0.1	4.5 ± 0.1	4.0 ± 0.3
	AM251	2.4 ± 0.1*	8.2 ± 0.1*	1.8 ± 0.4*
	Vehicle	1.6 ± 0.1	4.4 ± 0.2	4.2 ± 0.2
Vehicle	AM630	1.6 ± 0.1	4.4 ± 0.2	4.2 ± 0.2
	AM251	1.5 ± 0.1	4.3 ± 0.2	4.2 ± 0.4

For each test and drug tested.

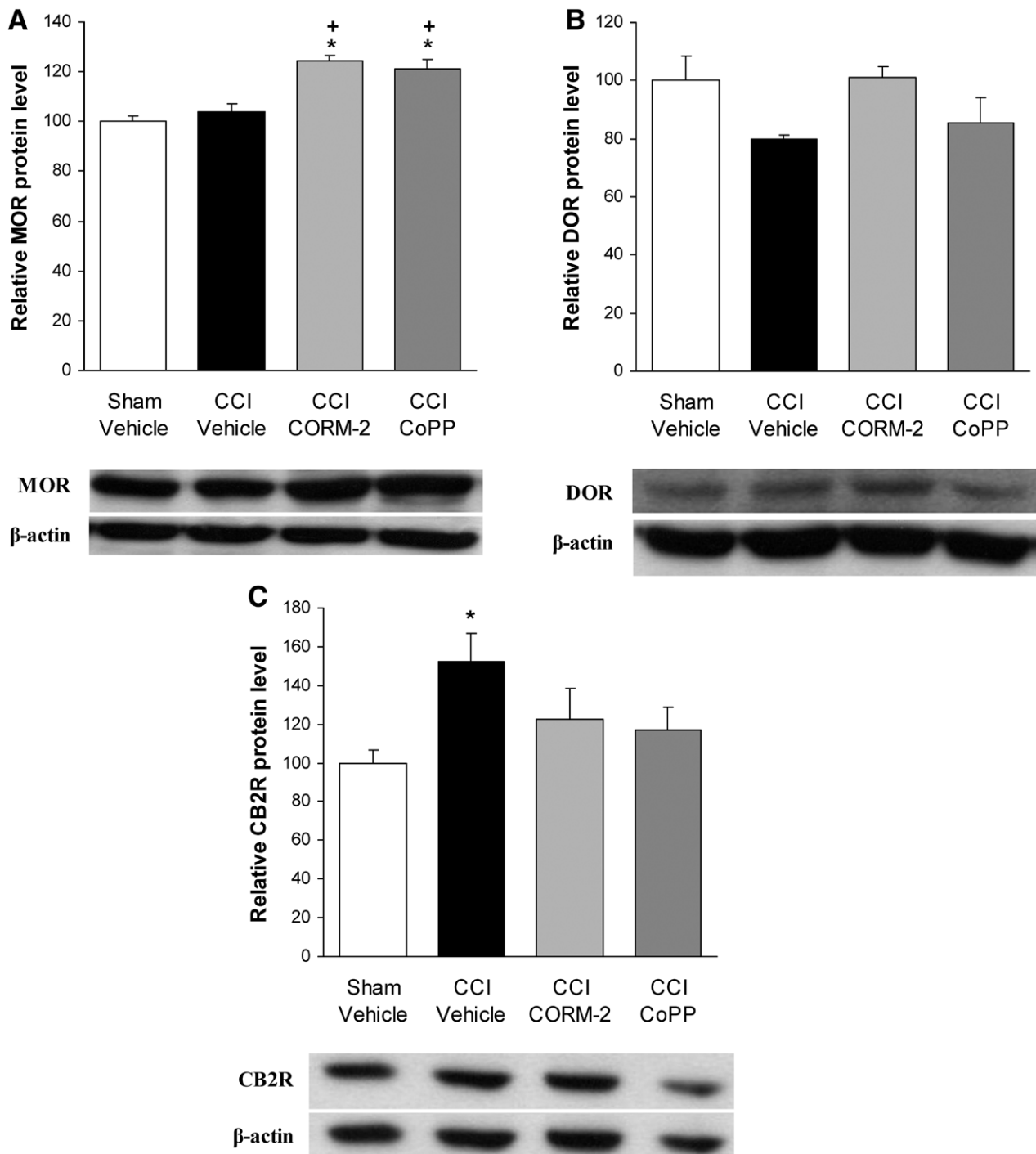
\*  $P < 0.05$  indicates significant differences vs. their respective vehicle plus vehicle treated group (one-way ANOVA, followed by the Student Newman-Keuls test). Results are shown as mean values ± SEM;  $n = 6$  animals per experimental group.

CTAP = H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH<sub>2</sub>; DPDPE = [d-Pen(2),d-Pen(5)]-enkephalin; JWH-015 = (2-methyl-1-propyl-1H-indol-3-yl)-1-naphthalenylmethanone; NX-ME = naloxone methiodide.

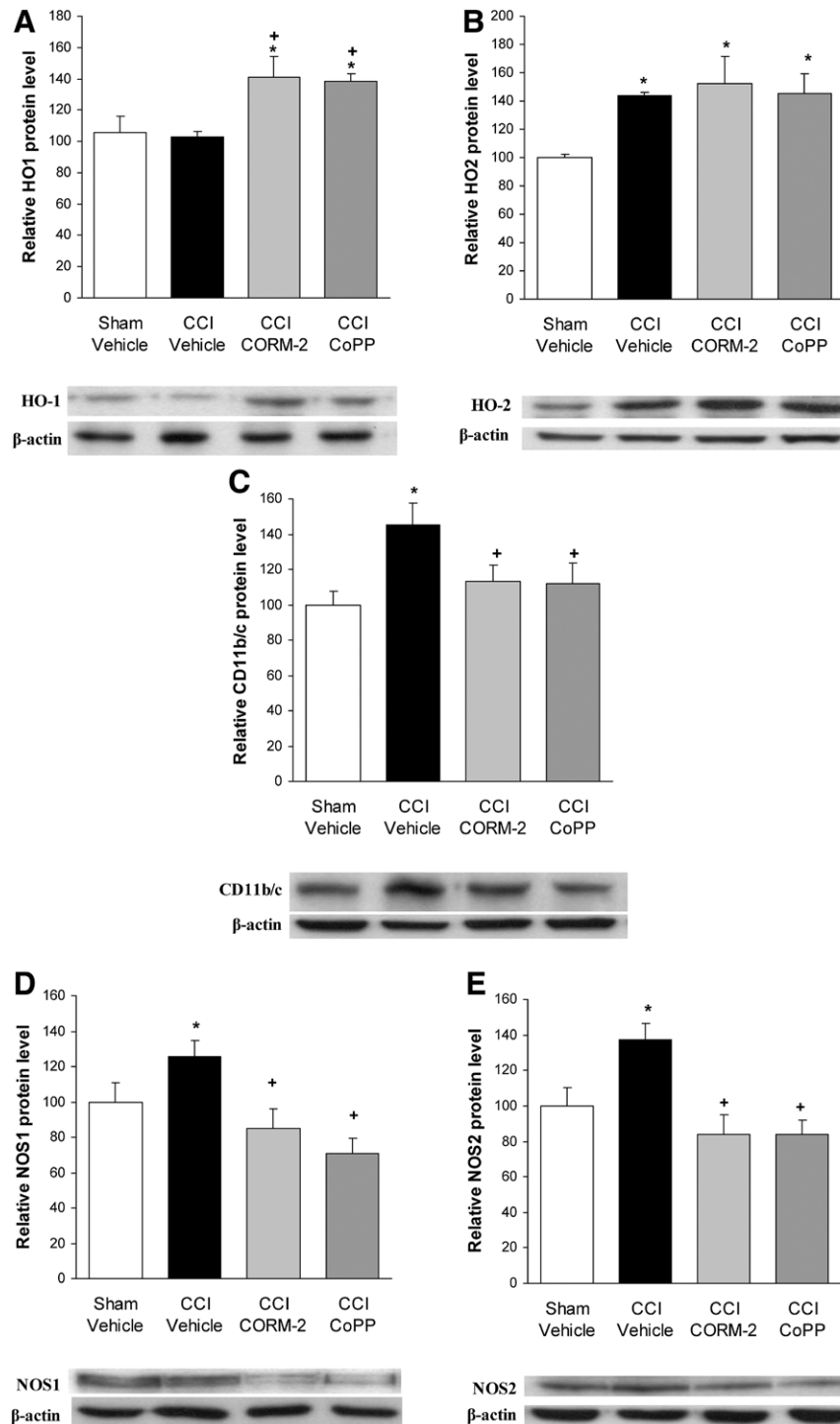
The antinociceptive and antiinflammatory effects produced by CO-RMs or CoPP during inflammatory diseases have been previously reported.<sup>15,21</sup> In accordance with these findings, our results further demonstrate that the administration of CORM-2, CORM-3, or CoPP inhibited the mechanical and thermal hypersensitivity induced by the chronic constriction of sciatic nerve in mice. The lack of antinociceptive effects produced by inactive CO-RMs (iCORM-2 and iCORM-3), unable to release carbon monoxide,<sup>29</sup> supports the hypothesis that the antinociceptive effects produced by CORM-2 and CORM-3 after sciatic nerve injury could probably be due to the release of carbon monoxide. This study also reveals, for first time, that the intraperitoneal administration of CO-RMs or CoPP significantly enhanced the antiallodynic and antihyperalgesic effects produced by the peripheral, but not systemic, administration of morphine after sciatic nerve injury. Moreover, while the peripheral antiallodynic and antihyperalgesic effects produced by morphine were significantly decreased by the subplantar administration of SnPP (HO-1 inhibitor), the inhibitory effects produced by morphine systemically administered remain unaffected after their coadministration with SnPP. These findings indicate that whereas HO-1 participates in the antinociceptive effects produced by the peripheral administration of morphine after sciatic nerve injury, the systemic administration of this drug did not use this pathway to induce their

antiallodynic and antihyperalgesic effects during neuropathic pain. The mechanism activated by MOR agonists to produce antinociception after their subcutaneous administration during neuropathic pain is under investigation in our laboratory.

Our results also reveal that while the administration of CORM-2, CORM-3, or CoPP blocks the inhibitory effects produced by the subplantar and systemic administration of DOR or CB2R agonists, the administration of SnPP did not alter their antiallodynic and antihyperalgesic effects following nerve injury. In accordance with these results, a clear relationship between the local antinociceptive effects of MOR agonists, but not DOR or CB2R, and the nitric oxide–cyclic guanosine monophosphate–PKG–adenosine triphosphate–sensitive potassium signaling peripheral pathway activation was previously demonstrated under neuropathic pain conditions.<sup>5,6</sup> Thus, while the local pharmacologic inhibition of the nitric oxide–cyclic guanosine monophosphate–PKG signaling pathway attenuated the peripheral antinociceptive effects of morphine, its blockage potentiated the peripheral antiallodynic and antihyperalgesic effects of DOR and CB2R agonists after neuropathic pain. These results agree with the ideas proposed by other authors that the activation of DOR reduces the principal symptoms of neuropathic pain by reducing the voltage-gated sodium channels through the activation of protein kinase C,<sup>34,35</sup> while the activation of CB2R



**Fig. 6.** Effect of tricarbonyldichloro ruthenium(II) dimer (CORM-2) and cobalt protoporphyrin IX (CoPP) on  $\mu$ -opioid receptors (MOR),  $\delta$ -opioid receptors (DOR), and cannabinoid 2 receptors (CB2R) protein expression from sciatic nerve-injured mice. The protein expression of MOR (A), DOR (B), and CB2R (C) in the ipsilateral site of the dorsal root ganglia from sciatic nerve-injured (CCI) mice treated with vehicle, CORM-2, or CoPP at 10 days after surgery are represented. The expression of these receptors in the dorsal root ganglia from sham-operated mice treated with vehicle has been also represented as controls (sham-vehicle). For each protein, \* indicates significant differences when compared *versus* sham-operated vehicle treated mice ( $P < 0.05$ , one-way ANOVA followed by Student Newman–Keuls test) and + indicates significant differences when compared *versus* sciatic nerve-injured vehicle treated mice ( $P < 0.05$ , one-way ANOVA followed by Student Newman–Keuls test). Representative examples of Western blots for MOR, DOR, and CB2R proteins, in which  $\beta$ -actin was used as a loading control, are also shown. Data are expressed as mean values  $\pm$  SEM;  $n = 5$  samples per group.



**Fig. 7.** Effect of tricarbonyldichloro ruthenium(II) dimer (CORM-2) and cobalt protoporphyrin IX (CoPP) on the HO-1, HO-2, CD11b/c, neuronal nitric oxide synthase (NOS1), and inducible nitric oxide synthase (NOS2) protein expression from sciatic nerve-injured mice. The protein expression of HO-1 (inducible heme oxygenase; A) and HO-2 (constitutive heme oxygenase; B) in the ipsilateral site of the dorsal root ganglia and those of CD11b/c (C), NOS1 (D), and NOS2 (E) in the ipsilateral lumbar spinal cord from sciatic nerve-injured (CCI) mice treated with vehicle, CORM-2 or CoPP at 10 days after surgery are represented. The expression of these proteins in the dorsal root ganglia or spinal cord from sham-operated mice treated with vehicle has been also represented as controls (sham-vehicle). For each protein, \* indicates significant differences when compared *versus* sham-operated vehicle treated mice ( $P < 0.05$ , one-way ANOVA followed by Student Newman-Keuls test) and + indicates significant differences when compared *versus* sciatic nerve-injured vehicle treated mice ( $P < 0.05$ , one-way ANOVA followed by Student Newman-Keuls test). Representative examples of Western blots for HO-1, HO-2, CD11b/c, NOS1, and NOS2 proteins, in which  $\beta$ -actin was used as a loading control, are also shown. Data are expressed as mean values  $\pm$  SEM;  $n = 5$  samples per group.

reduces neuropathic pain by inhibiting the activated microglia induced by nerve injury.<sup>36</sup> Moreover, the fact that CO-RMs or CoPP treatments did not alter the antiallodynic and antihyperalgesic effects produced by the subcutaneous administration of morphine might support the evidence that nitric oxide counteracts the analgesic actions produced by the subcutaneous and spinal administration of morphine during acute and prolonged pain.<sup>37–39</sup> In summary, these data indicate that different pathways are activated by morphine to attenuate neuropathic pain according to their administration site.

The specificity of the antiallodynic and antihyperalgesic effects produced by the local or systemic administration of morphine and DPDPE after sciatic nerve injury was demonstrated by the complete reversal of their effects with their coadministration with selective antagonists (CTAP and naltrindole). It is interesting to note that the mitigation of neuropathic pain symptoms induced by the subplantar administration of morphine or DPDPE is produced by interaction with peripheral opioid receptors as demonstrated with the reversal of their effects by the coadministration with the nonselective peripherally acting opioid receptor antagonist (NX-ME). In contrast to DPDPE, the antiallodynic and antihyperalgesic effects produced by the systemic administration of morphine were not antagonized by NX-ME, indicating the involvement of central MOR in their effects. The specificity of the antiallodynic and antihyperalgesic effects of the subplantar and systemic administration of JWH-015 after sciatic nerve injury was also demonstrated by the complete reversal of their effects with their coadministration with a selective CB2R (AM630), but not a CB1R (AM251), antagonist. The subplantar or systemic administration of all tested antagonists did not have any effect when they were administered alone.

In accordance with other studies, our results indicate that while the peripheral expression of MOR and DOR did not change after sciatic nerve injury, the expression of CB2R is increased.<sup>5,7,40–42</sup> This study also demonstrates that treatments with CORM-2 or CoPP enhance the peripheral expression of MOR, although both treatments did not modify DOR expression and decreased the overexpression of CB2R induced by sciatic nerve injury. Thus, the enhanced peripheral expression of MOR after sciatic nerve injury induced by CORM-2 or CoPP treatments might be responsible for the increased local antiallodynic and antihyperalgesic effects produced by morphine after these treatments, although a reduction of the overall inflammation produced by both treatments<sup>20,21,43</sup> could be also implicated in the enhanced local antinociceptive effects produced by morphine after CORM-2 or CoPP treatments.

In order to identify the possible mechanisms implicated in the peripheral regulation of MOR expression by CORM-2 and CoPP, we evaluated the effects of these treatments on the expression of HO, NOS isoforms, and a microglial marker (CD11b/c) in the dorsal root ganglia and/or spinal cord from sciatic nerve-injured mice. In accordance with other inflammatory models,<sup>17,43</sup> our results confirmed that the expression

of HO-1 was significantly increased in the dorsal root ganglia of sciatic nerve-injured mice treated with CORM-2 or CoPP. However, the increased expression of HO-2 in the dorsal root ganglia from sciatic nerve-injured mice remained unaltered after CORM-2 or CoPP treatments. These data indicate that the enhanced local antiallodynic and antihyperalgesic effects of morphine produced by both treatments are produced by the activation of HO-1 expression, but not through the inhibition of HO-2 overexpression induced by nerve injury.

It is well known that microglial cells play an important role in the development of chronic pain. In accordance, spinal microglia is strongly activated after nerve injury,<sup>44</sup> and the administration of microglial activation inhibitors significantly reduced the behavioral symptoms of neuropathic pain in animals.<sup>27</sup> Moreover, the activated microglia promotes the consolidation and progression of neuropathic pain state by the upregulation of several inflammatory mediators including nitric oxide. Activated microglia after nerve injury can also modify the opioid-specific signaling that diminished the antinociceptive potency of morphine after nerve injury. Accordingly, treatment with glial inhibitors enhanced the effectiveness of morphine in animals with neuropathic pain.<sup>45</sup>

Interestingly, we found that CORM-2 and CoPP treatments were able to reverse the nerve injury-induced microglial activation, as well as the NOS1 and NOS2 overexpression. In accordance with these data, the suppression of microglial activation and/or the nitric oxide synthesis by CORM-2 or CoPP treatments may also be responsible for the improvement of morphine efficacy produced by these treatments under neuropathic pain conditions.<sup>6,27</sup> Therefore, the increased peripheral antiallodynic and antihyperalgesic effects of morphine induced by CORM-2 and CoPP treatments might be, at least in part, explained by the enhancement of peripheral MOR expression and the inhibition of inflammatory responses that are linked to microglia activation in the spinal cord. This is in agreement with other *in vitro* studies showing that CORM-3 is effective in reducing the production of cytokines and nitric oxide in microglia activated with endotoxin or thrombin as well as under hypoxic conditions.<sup>46</sup>

In summary, this study suggests for first time that treatments with CO-RMs or CoPP enhance the local antinociceptive effects of morphine through enhancing the peripheral MOR expression and inhibiting the spinal microglial activation and NOS1/NOS2 overexpression.

## References

1. Obara I, Przewlocki R, Przewlocka B: Local peripheral effects of mu-opioid receptor agonists in neuropathic pain in rats. *Neurosci Lett* 2004; 360:85–9
2. Obara I, Makuch W, Spetea M, Schütz J, Schmidhammer H, Przewlocki R, Przewlocka B: Local peripheral antinociceptive effects of 14-O-methyloxymorphone derivatives in inflammatory and neuropathic pain in the rat. *Eur J Pharmacol* 2007; 558:60–7

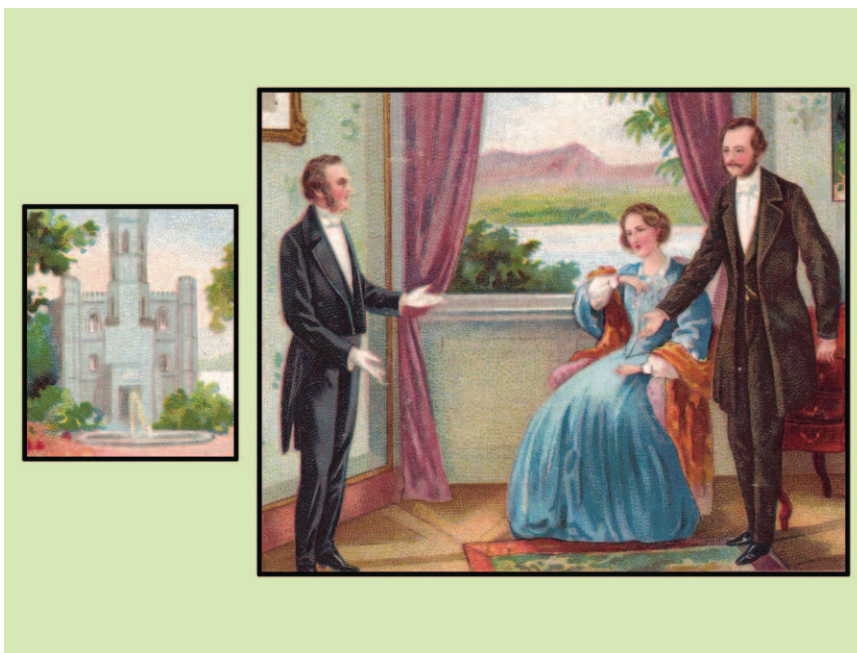


3. Guan Y, Johanek LM, Hartke TV, Shim B, Tao YX, Ringkamp M, Meyer RA, Raja SN: Peripherally acting mu-opioid receptor agonist attenuates neuropathic pain in rats after L5 spinal nerve injury. *Pain* 2008; 138:318–29
4. Obara I, Parkitna JR, Korostynski M, Makuch W, Kaminska D, Przewlocka B, Przewlocki R: Local peripheral opioid effects and expression of opioid genes in the spinal cord and dorsal root ganglia in neuropathic and inflammatory pain. *Pain* 2009; 141:283–91
5. Hervera A, Negrete R, Leáñez S, Martín-Campos J, Pol O: The role of nitric oxide in the local antiallodynic and antihyperalgesic effects and expression of delta-opioid and cannabinoid-2 receptors during neuropathic pain in mice. *J Pharmacol Exp Ther* 2010; 334:887–96
6. Hervera A, Negrete R, Leáñez S, Martín-Campos JM, Pol O: Peripheral effects of morphine and expression of  $\mu$ -opioid receptors in the dorsal root ganglia during neuropathic pain: Nitric oxide signaling. *Mol Pain* 2011; 7:25
7. Truong W, Cheng C, Xu QG, Li XQ, Zochodne DW: Mu opioid receptors and analgesia at the site of a peripheral nerve injury. *Ann Neurol* 2003; 53:366–75
8. Kabli N, Cahill CM: Anti-allodynic effects of peripheral delta opioid receptors in neuropathic pain. *Pain* 2007; 127:84–93
9. Steiner AA, Branco LG, Cunha FQ, Ferreira SH: Role of the haeme oxygenase/carbon monoxide pathway in mechanical nociceptor hypersensitivity. *Br J Pharmacol* 2001; 132:1673–82
10. Nascimento CG, Branco LG: Role of the peripheral heme oxygenase-carbon monoxide pathway on the nociceptive response of rats to the formalin test: Evidence for a cGMP signaling pathway. *Eur J Pharmacol* 2007; 556:55–61
11. Li X, Clark JD: The role of heme oxygenase in neuropathic and incisional pain. *Anesth Analg* 2000; 90:677–82
12. Li X, Clark JD: Heme oxygenase type 2 participates in the development of chronic inflammatory and neuropathic pain. *J Pain* 2003; 4:101–7
13. Gordh T, Sharma HS, Azizi M, Alm P, Westman J: Spinal nerve lesion induces upregulation of constitutive isoform of heme oxygenase in the spinal cord. An immunohistochemical investigation in the rat. *Amino Acids* 2000; 19:373–81
14. Fan W, Huang F, Wu Z, Zhu X, Li D, He H: Carbon monoxide: A gas that modulates nociception. *J Neurosci Res* 2011; 89:802–7
15. Rosa AO, Egea J, Lorrio S, Rojo AI, Cuadrado A, López MG: Nrf2-mediated haeme oxygenase-1 up-regulation induced by cobalt protoporphyrin has antinociceptive effects against inflammatory pain in the formalin test in mice. *Pain* 2008; 137:332–9
16. Egea J, Rosa AO, Lorrio S, del Barrio L, Cuadrado A, López MG: Haeme oxygenase-1 overexpression via nAChRs and the transcription factor Nrf2 has antinociceptive effects in the formalin test. *Pain* 2009; 146:75–83
17. Maicas N, Ferrándiz ML, Devesa I, Motterlini R, Koenders MI, van den Berg WB, Alcaraz MJ: The CO-releasing molecule CORM-3 protects against articular degradation in the K/BxN serum transfer arthritis model. *Eur J Pharmacol* 2010; 634:184–91
18. Sawle P, Foresti R, Mann BE, Johnson TR, Green CJ, Motterlini R: Carbon monoxide-releasing molecules (CO-RMs) attenuate the inflammatory response elicited by lipopolysaccharide in RAW264.7 murine macrophages. *Br J Pharmacol* 2005; 145:800–10
19. De Backer O, Elinck E, Blanckaert B, Leybaert L, Motterlini R, Lefebvre RA: Water-soluble CO-releasing molecules reduce the development of postoperative ileus via modulation of MAPK/HO-1 signalling and reduction of oxidative stress. *Gut* 2009; 58:347–56
20. Ferrándiz ML, Maicas N, Garcia-Arnandis I, Terencio MC, Motterlini R, Devesa I, Joosten LA, van den Berg WB, Alcaraz MJ: Treatment with a CO-releasing molecule (CORM-3) reduces joint inflammation and erosion in murine collagen-induced arthritis. *Ann Rheum Dis* 2008; 67:1211–7
21. Guillén MI, Megías J, Clérigues V, Gomar F, Alcaraz MJ: The CO-releasing molecule CORM-2 is a novel regulator of the inflammatory process in osteoarthritic chondrocytes. *Rheumatology (Oxford)* 2008; 47:1323–8
22. Li X, Clark JD: Spinal cord nitric oxide synthase and heme oxygenase limit morphine induced analgesia. *Brain Res Mol Brain Res* 2001; 95:96–102
23. Liang D, Li X, Lighthall G, Clark JD: Heme oxygenase type 2 modulates behavioral and molecular changes during chronic exposure to morphine. *Neuroscience* 2003; 121:999–1005
24. Bennett GJ, Xie YK: A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* 1988; 33:87–107
25. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL: Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods* 1994; 53:55–63
26. Hargreaves K, Dubner R, Brown F, Flores C, Joris J: A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* 1988; 32:77–88
27. Mika J, Osikowicz M, Rojewska E, Korostynski M, Wawrzczak-Bargiela A, Przewlocki R, Przewlocka B: Differential activation of spinal microglial and astroglial cells in a mouse model of peripheral neuropathic pain. *Eur J Pharmacol* 2009; 623:65–72
28. Motterlini R, Clark JE, Foresti R, Sarathchandra P, Mann BE, Green CJ: Carbon monoxide-releasing molecules: Characterization of biochemical and vascular activities. *Circ Res* 2002; 90:E17–24
29. Clark JE, Naughton P, Shurey S, Green CJ, Johnson TR, Mann BE, Foresti R, Motterlini R: Cardioprotective actions by a water-soluble carbon monoxide-releasing molecule. *Circ Res* 2003; 93:e2–8
30. Devesa I, Ferrándiz ML, Terencio MC, Joosten LA, van den Berg WB, Alcaraz MJ: Influence of heme oxygenase 1 modulation on the progression of murine collagen-induced arthritis. *Arthritis Rheum* 2005; 52:3230–8
31. Hervera A, Negrete R, Leáñez S, Pol O: Potential therapeutic role of carbon monoxide synthesized by heme oxygenase-1 in the attenuation of neuropathic pain via microglial inactivation. *Eur J Pain Suppl* 2011; 5:54
32. Hervera A, Negrete R, Leáñez S, Pol O: Carbon monoxide synthesized by heme oxygenase-1 enhanced the effects and expression of  $\mu$ -opioid receptors during neuropathic pain in mice. *Basic Clin Pharmacol Toxicol* 2011; 109:21–2
33. Hervera A, Leáñez S, Pol O: The inhibition of the nitric oxide-cGMP-PKG-JNK signaling pathway avoids the development of tolerance to the local antiallodynic effects produced by morphine during neuropathic pain. *Eur J Pharmacol* 2012; 685:42–51
34. Chattopadhyay M, Mata M, Fink DJ: Continuous delta-opioid receptor activation reduces neuronal voltage-gated sodium channel (NaV1.7) levels through activation of protein kinase C in painful diabetic neuropathy. *J Neurosci* 2008; 28:6652–8
35. Gaveriaux-Ruff C, Nozaki C, Nadal X, Hever XC, Weibel R, Matifas A, Reiss D, Filliol D, Nassar MA, Wood JN, Maldonado R, Kieffer BL: Genetic ablation of delta opioid receptors in nociceptive sensory neurons increases chronic pain and abolishes opioid analgesia. *Pain* 2011; 152:1238–48
36. Romero-Sandoval A, Natile-McMenemy N, DeLeo JA: Spinal microglial and perivascular cell cannabinoid receptor type 2 activation reduces behavioral hypersensitivity without tolerance after peripheral nerve injury. *ANESTHESIOLOGY* 2008; 108:722–34
37. Machelska H, Labuz D, Przewlocki R, Przewlocka B: Inhibition of nitric oxide synthase enhances antinociception mediated by mu, delta and kappa opioid receptors in acute and prolonged pain in the rat spinal cord. *J Pharmacol Exp Ther* 1997; 282:977–84

38. Ozek M, Uresin Y, Güngör M: Comparison of the effects of specific and nonspecific inhibition of nitric oxide synthase on morphine analgesia, tolerance and dependence in mice. *Life Sci* 2003; 72:1943–51
39. Toda N, Kishioka S, Hatano Y, Toda H: Modulation of opioid actions by nitric oxide signaling. *ANESTHESIOLOGY* 2009; 110:166–81
40. Zhang J, Hoffert C, Vu HK, Groblewski T, Ahmad S, O'Donnell D: Induction of CB2 receptor expression in the rat spinal cord of neuropathic but not inflammatory chronic pain models. *Eur J Neurosci* 2003; 17:2750–4
41. Costa B, Colleoni M, Conti S, Trovato AE, Bianchi M, Sotgiu ML, Giagnoni G: Repeated treatment with the synthetic cannabinoid WIN 55,212-2 reduces both hyperalgesia and production of pronociceptive mediators in a rat model of neuropathic pain. *Br J Pharmacol* 2004; 141:4–8
42. Pol O, Murtra P, Caracul L, Valverde O, Puig MM, Maldonado R: Expression of opioid receptors and c-fos in CB1 knockout mice exposed to neuropathic pain. *Neuropharmacology* 2006; 50:123–32
43. Megías J, Guillén MI, Clérigues V, Rojo AI, Cuadrado A, Castejón MA, Gomar F, Alcaraz MJ: Heme oxygenase-1 induction modulates microsomal prostaglandin E synthase-1 expression and prostaglandin E(2) production in osteoarthritic chondrocytes. *Biochem Pharmacol* 2009; 77:1806–13
44. Watkins LR, Milligan ED, Maier SF: Glial activation: A driving force for pathological pain. *Trends Neurosci* 2001; 24:450–5
45. Mika J, Osikowicz M, Makuch W, Przewlocka B: Minocycline and pentoxifylline attenuate allodynia and hyperalgesia and potentiate the effects of morphine in rat and mouse models of neuropathic pain. *Eur J Pharmacol* 2007; 560:142–9
46. Bani-Hani MG, Greenstein D, Mann BE, Green CJ, Motterlini R: Modulation of thrombin-induced neuroinflammation in BV-2 microglia by carbon monoxide-releasing molecule 3. *J Pharmacol Exp Ther* 2006; 318:1315–22

## ANESTHESIOLOGY REFLECTIONS FROM THE WOOD LIBRARY-MUSEUM

### No Swan Song for Baron Justus von Liebig



Giessen Professor Justus Liebig was elevated to *Freiherr* (Baron) in 1845, the year that Bavaria's future "Swan King", Prince Ludwig II, was born. The prince's parents, King Maximilian II and Queen Marie are depicted (*right*) formally receiving Liebig 7 years later, in 1852, after the Baron had accepted their royal invitation to a professorship at the University of Munich. A pioneering professor of organic and agricultural chemistry, Justus von Liebig would also curate Germany's oldest continuously maintained botanical gardens and consult on other royal gardens. He passed away in 1873, about 11 years before the Swan King (now known as "Mad King" Ludwig II) could have used Liebig's expert advice—for it was in 1884 that King Ludwig II began moving into Neuschwanstein ("new swan stone"), the fantastic fortress after which many Disney theme park castles have been patterned. (Copyright © the American Society of Anesthesiologists, Inc.)

George S. Bause, M.D., M.P.H., Honorary Curator, ASA's Wood Library-Museum of Anesthesiology, Park Ridge, Illinois, and Clinical Associate Professor, Case Western Reserve University, Cleveland, Ohio. UJYC@aol.com.