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The role of nitric oxide in the local antiallodynic and antihyperalgesic effects and expression of δ-opioid and cannabinoid-2 receptors during neuropathic pain in mice

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Running title: Opioid and cannabinoid receptor regulation by nitric oxide

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Abbreviations: AM630, 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone; CB2R, cannabinoid 2 receptor; CCI, chronic constriction of the sciatic nerve; cGMP, guanosine 3′,5′-cyclic monophosphate; DPDPE ([d-Pen(2),d-Pen(5)]-Enkephalin); DOPr, δ-opioid receptor; JWH-015 ((2-methyl-1-propyl-1H-indol-3-yl)-1-naphthalenylmethanone); KO, knockout; L-NIL, L-N(6)-(1-iminoethyl)-lysine; NANT, N-[(4S)-4-amino-5-[(2-aminoethyl)amino]pentyl]-N′-nitroguanidine tris(trifluoroacetate) salt; NOS1, neuronal nitric oxide synthase; NOS2, inducible nitric oxide synthase; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; PKG, cGMP-dependent protein kinase; Rp-8-pCPT-cGMPs, (Rp)-8-(para-chlorophenylthio)guanosine-3',5'-cyclic monophosphorothioate; WT, wild type.

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ABSTRACT

Both δ-opioid receptor (DOPr) and cannabinoid 2 receptor (CB2R) agonists attenuate neuropathic pain but the precise mechanism implicated in these effects is not completely elucidated. We investigated if nitric oxide synthesized by neuronal (NOS1) or inducible (NOS2) nitric oxide synthases could modulate DOPr and/or CB2R antiallodynic and antihyperalgesic effects through the peripheral nitric oxide-cGMP-protein kinase G (PKG) pathway activation and affect their expression during neuropathic pain. In wild type (WT) mice at 21 days after chronic constriction of sciatic nerve (CCI), we evaluated the effects of [d-Pen(2),d-Pen(5)]-Enkephalin (DPDPE), (2-methyl-1-propyl-1H-indol-3-yl)-1-naphthalenylmethanone (JWH-015) and a NOS1 (N-[(4S)-4-amino-5-[(2-aminoethyl)amino]pentyl]-N′-nitroguanidine tris(trifluoroacetate) salt; NANT), NOS2 (L-N(6)-(1-iminoethyl)-lysine; L-NIL), L-guanylate cyclase (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; ODQ) or a PKG ((Rp)-8-(para-chlorophenylthio)guanosine-3',5'-cyclic monophosphorothioate; Rp-8-pCPT-cGMPs) inhibitor administered alone or combined. Expression of DOPr and CB2R mRNA in the spinal cord and dorsal root ganglia of naive and nerve injured WT, NOS1-KO and NOS2-KO mice, was also assessed. The subplantar administration of NANT, L-NIL, ODQ or Rp-8-pCPT-cGMPs dose-dependently inhibited neuropathic pain and enhanced the local effects of DPDPE or JWH-015. Moreover, although the basal levels of DOPr and CB2R mRNA were similar between WT and NOS-KO animals, nerve injury only decreased (DOPr) or increased (CB2R) their expression in the dorsal root ganglia of WT and NOS2-KO mice, but not in NOS1-KO. Results suggest that inactivation of nitric oxide-cGMP-PKG peripheral pathway triggered by NOS1 and NOS2 enhanced the peripheral actions of DOPr and CB2R agonists and that nitric oxide synthesized by NOS1 is implicated in the peripheral regulation of DOPr and CB2R gene transcription during neuropathic pain.

Introduction

Neuropathic pain is a clinical manifestation characterized by the presence of allodynia and hyperalgesia and it is difficult to treat with the most potent analgesic compounds. Recent works have demonstrated that δ-opioid receptor (DOPr) agonists elicit antiallodynic and antihyperalgesic effects in several models of neuropathic pain (Mika et al., 2001; Kabli and Cahill, 2007), although the possible changes in the expression of DOPr following nerve injury is controversial. Thus, from no changes (Besse et al., 1992), to an increase (Kabli and Cahill, 2007) or a decrease (Stone et al., 2004; Obara et al., 2009) in their expression, in the dorsal root ganglia and spinal cord from sciatic nerve injured animals, have been reported. In addition to DOPr, other studies also showed that the cannabinoid-2 receptor (CB2R) activation is effective in attenuating neuropathic pain (Bridges et al., 2001; Fox et al., 2001) and that their expression increases after nerve injury (Zhang et al., 2003; Costa et al., 2004). Even so, the precise mechanisms implicated in the peripheral actions of DOPr and CB2R agonists as well as in the expression of their receptors during neuropathic pain are not completely elucidated.

Several studies have shown that nitric oxide synthesized by neuronal (NOS1) or inducible (NOS2) nitric oxide synthases via central guanosine 3′, 5′-cyclic monophosphate (cGMP)-protein kinase G (PKG) pathway activation mediates numerous neuropathic pain symptoms (Meller et al., 1992). Accordingly, the expression of NOS1 and NOS2 is up-regulated in the spinal cord and dorsal root ganglia after nerve injury (Levy et al., 1999; De Alba et al., 2006). Moreover, the systemic administration of selective NOS or guanylate cyclase inhibitors might reverse the hypersensitivity to pain induced by the spinal or sciatic nerve injury (De Alba et al., 2006; LaBuda et al., 2006; Guan et al., 2007), but the involvement of the peripheral nitric oxide-cGMP-PKG pathway in the maintenance of thermal and mechanical hypersensitivity induced by the chronic constriction of the sciatic nerve, is not completely established.

It is well known that the nitric oxide-cGMP-PKG pathway activation modulates the peripheral antinociceptive effects induced by certain drugs during inflammatory pain, including opioids (Ferreira et al., 1991; Pol, 2007; Hervera et al., 2009; Leánez et al., 2009) and cannabinoids (Lopes et al., 2009). Nitric oxide also regulates the transcription of μ- and κ-opioid receptor

genes under basal and inflammatory conditions (Park et al., 2002; Pol et al., 2005) but the exact role of nitric oxide in the peripheral actions and expression of DOPr and CB2R during neuropathic pain is not known.

Thus, in order to study if the nitric oxide-cGMP-PKG peripheral pathway activation triggered by NOS1 and NOS2 could modulate the local effects of DOPr and CB2R agonists in nerve injured wild type (WT) mice, at 21 days after the chronic constriction of the sciatic nerve (CCI), we evaluated: 1) the mechanical antiallodynic, thermal antihyperalgesic and thermal antiallodynic effects of the subplantar administration of a specific DOPr ([d-Pen(2),d-Pen(5)]-Enkephalin; DPDPE) or CB2R ((2-methyl-1-propyl-1H-indol-3-yl)-1-naphthalenylmethanone; JWH-015) agonist 2) the reversibility of these effects by their co-administration with a specific DOPr (naltrindole) or a CB2R (6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone; AM630) antagonist; 3) the antiallodynic and antihyperalgesic effects produced by selective NOS1 (N-[(4S)-4-amino-5-[(2-aminoethyl)amino]pentyl]-N′-nitroguanidine tris(trifluoroacetate) salt; NANT), NOS2 (L-N(6)-(1-iminoethyl)-lysine; L-NIL), soluble guanylate cyclase (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; ODQ) or PKG ((Rp)-8-(para-chlorophenylthio)guanosine-3',5'-cyclic monophosphorothioate; Rp-8-pCPT-cGMPs) inhibitors subplantarly administered, alone or combined, with DPDPE or JWH-015.

To evaluate the role played by nitric oxide synthesized by NOS1 and NOS2 in the expression of DOPr and CB2R during neuropathic pain, the expression of DOPr and CB2R mRNA in the spinal cord and dorsal root ganglia of sciatic nerve injured WT, NOS1-KO and NOS2-KO mice at 21 days after surgery, was also evaluated.

Material and Methods

Animals: Male NOS1-knockout mice (C57BL/6J background) and NOS2-knockout mice (C57BL/6J background) were purchased from Jackson Laboratories (Bar Harbor, ME, USA) while WT mice with the same genetic background (C57BL/6J) were acquired from Harlan Laboratories (France). All mice weighing 21 to 25 g were housed under 12-h/12-h light/ dark conditions in a room with controlled temperature (22º 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 conducted between 9:00 AM and 5:00 PM. The study protocol was approved by the local Committee of Animal Use and Care of the Autonomous University of Barcelona in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health.

Induction of neuropathic pain: Neuropathic pain was induced by the chronic constriction of the sciatic nerve. 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 (1988). 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 was prevented from applying a too strong ligation, taking care to preserve epineural circulation. Sham-operated mice that underwent exposure of the right sciatic nerve without ligature and non-operated (naive) mice were used as controls.

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 21 days after CCI.

Nociceptive behavioral tests

Mechanical allodynia was quantified by measuring the hind paw withdrawal response to von Frey filament stimulation. In brief, animals were placed in a Plexiglas® box (20 cm high, 9 cm

diameter) with a wire grid bottom through which the von Frey filaments (North Coast Medical, Inc., San Jose, CA, USA) bending force range from 0.008 to 3.5 g, were applied by using a modified version of the up–down paradigm, as previously reported by Chaplan et al. (1994). 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 were considered nociceptive-like responses. 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. The baseline values were between 1.3 and 1.5 g.

Thermal hyperalgesia was assessed as previously reported by Hargreaves et al. (1988). Paw withdrawal latency in response to radiant heat was measured using the plantar test apparatus (Ugo Basile, Italy). Briefly, mice were placed in Plexiglas boxes (20 cm high x 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 12s was used to prevent tissue damage in absence of response. The mean paw withdrawal latencies from the ipsilateral and contralateral hind paws were determined from the average of 3 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. The baseline values were between 8.4 and 9.0 s.

Thermal allodynia to cold stimulus was assessed by using the hot/cold-plate analgesia meter (Ugo Basile, Italy), previously described by Bennett and Xie (1988). The number of elevations of each hind paw was recorded in the mice exposed to the cold plate (4 ± 0.5 ºC) for 5 minutes. The baseline values were between 0 and 1 paw lifts.

Gene expression studies

Tissue isolation and total RNA extraction. Animals were sacrificed at 0 and 21 days after CCI-induction by cervical dislocation. Tissues from the ipsilateral lumbar spinal cord and dorsal root ganglia of WT, NOS1-KO and NOS2-KO mice were removed immediately after sacrifice, frozen in liquid nitrogen and stored at -80ºC until assay. Samples from two or three animals were pooled into one experimental sample for each spinal cord or dorsal root ganglia tissues, respectively. All tissues were homogenized in ice-cold with a homogenizer (Ultra-Turf, T8; Ika Werke, Staufen, Germany) and the total RNA was extracted with TRIzol reagent (Invitrogen, Renfrewshire, England). The amount of the purified RNA (A_{260}/A_{280} ratio ≥ 1.9) was determined by spectrophotometry.

Reverse transcription. In all experiments, 1 µg of total RNA was reverse transcribed into cDNA using SuperScript II RNAse H^- reverse transcriptase (Invitrogen, Renfrewshire, UK) in a final volume of 10 µl. Negative controls were performed in which all of the components were included except reverse transcriptase.

TaqMan probe real-time polymerase chain reaction (PCR). The expression of DOPr and CB2R was determined by real-time PCR using pre-developed mice TaqMan® gene expression assays (Applied Biosystems, CA, USA) for these genes; Mm00443063_m1 (DOPr) and Mm00438286_m1 (CB2R). A probe against GAPDH (Mm 99999915_g1) was used as endogenous control and reactions without RNA were included as negative controls to ensure the specificity. PCR reactions were set up in 96-well plates containing the corresponding cDNA, 0.9 µmol/L of each forward and reverse primers, 0.25 µmol/L of TaqMan® MGB probe and a final concentration of 1x universal master mix (Applied Biosystems, CA, USA), which provides the PCR buffer, $MgCl_2$, dNTPs, and the thermal stable AmpliTaq Gold DNA polymerase. The assay was conducted using the Applied Biosystems ABI PRISM 7000 Sequence Detection System. All samples were assayed in duplicate. Relative expression of the

target genes was calculated by means of the comparative threshold cycle method (Livak and Schmittgen, 2001).

Experimental protocol. In a first set of experiments we assessed the expression of neuropathic pain by using the chronic constriction injury model of Bennett and Xie (1988). WT mice were habituated for 1 h to the environment of the different experimental tests during 4 days. 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 as previously described and animals were tested in each paradigm at 21 days after surgery by using the same sequence as for baseline responses. In the initial experiments we used sham-operated and non-operated (naive) mice as controls. However, since the results obtained in sham-operated and naive mice were very similar we used the latter as a true control in all subsequent experiments.

In a second set of experiments, we investigated the mechanical antiallodynic, thermal antihyperalgesic and thermal antiallodynic effects of the subplantar administration of different doses of an specific DOPr agonist, DPDPE (38.7-232.3 nmol; Clark et al., 1986), an specific CB2R agonist, JWH-015 (15.3-91.6 nmol; Huffman, 2000) or their corresponding vehicle in the ipsilateral and contralateral paws of sciatic nerve injured WT mice at 21 days after surgery. The effects of both agonists in the contralateral and ipsilateral paws of naive mice have been also evaluated. Animals were tested in each paradigm pre and post drug administration using the same sequence as mentioned before.

In another set of experiments, the specificity of the mechanical antiallodynic, thermal antihyperalgesic and thermal antiallodynic effects produced by DPDPE and JWH-015 in sciatic nerve injured WT mice was assessed by evaluating the reversibility of the effects produced by a dose of each agonist that produced the maximal inhibition of allodynia or hyperalgesia (154.8 nmol for DPDPE and 91.6 nmol for JWH-015) with the peripheral co-administration with an specific DOPr (naltrindole, 110.9 nmol; Portoghese et al., 1990) or CB2R (AM630, 59.5 nmol; Ross et al., 1999) antagonist. The effects of these antagonists administered alone were also

tested in sciatic nerve injured WT mice.

The possible involvement of the peripheral nitric oxide-cGMP-PKG pathway activated by NOS1 and NOS2 in the local antiallodynic and antihyperalgesic effects of DOPr and CB2R agonists has been evaluated in an extra group of WT mice. For this purpose, the local effects produced by different doses of NANT (50.9-254.5 nmol) a selective NOS1 inhibitor (Hah et al., 2003), L-NIL (134.1-894.1 nmol) a selective NOS2 inhibitor (Moore et al., 1994), ODQ (13.4-53.4 nmol) a selective soluble guanylyl cyclase inhibitor (Garthwaite et al., 1995), Rp-8-pCPT-cGMPs (4.1-16.5 nmol) a PKG inhibitor (Butt et al., 1994) or vehicle, in the ipsilateral and contralateral paws of sciatic nerve injured WT mice, were initially evaluated. After that, the effects of the subplantar co-administration of NANT (50.9 nmol), L-NIL (223.5 nmol), ODQ (13.4 nmol), Rp-8-pCPT-cGMPs (4.1 nmol) or vehicle with DPDPE (38.7 nmol) or JWH-015 (15.3 nmol) on the mechanical allodynia, thermal hyperalgesia and thermal allodynia induced by sciatic nerve injury in WT mice at 21 days after CCI induction, have been also evaluated. The doses of all tested drugs were selected according to previous experiments as the ones which produce the lowest antiallodynic and antihyperalgesic effects. The effects produced by these inhibitors, alone or combined, in the contralateral and ipsilateral paws of naive mice were also evaluated

In all 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 post drug administration are compared and calculated according to the following equation:

Maximal possible effect (%) = [(drug– baseline) / (cut-off – baseline)] x 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] x 100.

For each drug and test, the ED_{50} value defined as the dose that produces a 50% effect based on the Emax estimated from the double reciprocal plot, was also calculated.

Finally, the relative DOPr and CB2R mRNA expression in the ipsilateral site of the spinal cord and dorsal root ganglia from naive and sciatic nerve ligated WT, NOS2-KO and NOS1-KO

mice, at 21 days after CCI induction, were also evaluated by using real time PCR.

Drugs. JWH-015, AM630 and L-NIL were acquired from Tocris (Ellisville, MI). DPDPE, naltrindole hydrochloride, NANT, ODQ and Rp-8-pCPT-cGMPs were purchased from Sigma-Aldrich (St. Louis, MO). DPDPE, naltrindole hydrochloride, NANT, L-NIL and Rp-8-pCPT-cGMPs were dissolved in saline solution (0.9% NaCl), ODQ in DMSO (10% solution in saline) and AM630 in DMSO (50% solution in saline). All drug combinations were diluted in the highest required concentration of DMSO. All drugs administered alone or combined were injected in a final volume of 30 µl. In all experiments, drugs were administered into the plantar side of the right paw, 20 min before behavioral testing. For each group treated with a drug the respective control group received the same volume of vehicle.

Statistical analysis. Data are expressed as mean ± standard error of the mean (SEM). For each test and paw, the comparison of the nociceptive values obtained in naive, sham-operated and sciatic nerve injured WT mice was assessed by using a one-way ANOVA followed by the Student Newman Keuls test.

For each test and dose, the comparison of the effects produced by DPDPE, JWH-015, NANT, L-NIL, ODQ or Rp-8-pCPT-cGMPs vs. the effects produced by their respective vehicle in the ipsilateral paw of nerve injured mice, was evaluated by using a Student's t test. The ED_{50} values (dose that produced a 50% of the maximal effect) plus 95% confidence limits were determined by linear regression analysis of dose-response relations based on at least 5-6 mice per dose.

For each test, the reversion of the antiallodynic and antihyperalgesic effects of DOPr or CB2R agonists by their specific antagonists and the effects produced by each antagonist administered alone in the ipsilateral paw of sciatic nerve injured WT mice were analyzed by using a one way ANOVA followed by the Student Newman Keuls test.

The comparison between the effects produced by the combination of one specific agonist (DPDPE or JWH-015) plus an specific inhibitor (NANT, L-NIL, ODQ or Rp-8-pCPT-cGMPs) with the effects produced by each of these agonists administered alone in the mechanical and

thermal allodynia as well as thermal hyperalgesia induced by CCI, in the ipsilateral paw of nerve injured WT mice, were performed by using a one way ANOVA followed by the Student Newman Keuls test.

The changes in the expression of DOPr and CB2R in the spinal cord and dorsal root ganglia of naive or nerve injured WT, NOS1-KO and NOS2-KO mice at 21 after CCI, were analyzed by using a two-way ANOVA (genotype and surgery as between factors of variation), followed by the corresponding one way ANOVA or Student's t test when required. A value of $p < 0.05$ was considered as a significant.

Results

Expression of neuropathic pain in WT mice. Our results showed that the total sciatic nerve ligation produced mechanical allodynia, thermal hyperalgesia and thermal allodynia (Table 1). Thus, sciatic nerve injury led to a significant decrease of the threshold for evoking paw withdrawal to a mechanical stimulus, a decrease of paw withdrawal latency to thermal stimulus and an increase in the number of paw elevations to cold thermal stimulus in the ipsilateral paw of animals as compared to naive (non-operated) or sham-operated mice ($p < 0.001$; one way ANOVA followed by the Student Newman Keuls test). Sham operation did not produce any modification of nociceptive responses in the three behavioral tests. In all tests, non significant changes were observed in the contralateral paw when compared sciatic nerve injured, sham-operated or naive mice.

Effects of the subplantar administration of specific DOPr and CB2R agonists alone or co-administered with selective receptor antagonists in the mechanical allodynia, thermal hyperalgesia and thermal allodynia induced by sciatic nerve injury in WT mice.

The subplantar administration of DPDPE or JWH-015 into the ipsilateral paw dose-dependently inhibited the mechanical allodynia (Fig. 1A), thermal hyperalgesia (Fig 1B) and thermal allodynia (Fig. 1C) induced by sciatic nerve injury. Thus, the mechanical antiallodynic and thermal antihyperalgesic effects produced by different doses of DPDPE (77.4-232.3 nmol) or JWH-015 (30.5-91.6 nmol) in the ipsilateral paws of sciatic nerve injured WT mice were significantly higher than those obtained in their corresponding vehicle treated groups ($p < 0.05$; Student's t test). However, while the thermal antiallodynic effects of JWH-015 were significantly higher than that obtained in vehicle treated mice ($p < 0.05$; Student's t test; JWH-015 vs. vehicle), the thermal antiallodynic effects of DPDPE in the ipsilateral paw were only modestly improved as compared to the effects produced by vehicle in the same paw. Moreover, analyzing the ED_{50} values of DPDPE and JWH-015 our data showed that the potency of the CB2R agonist on the inhibition of mechanical and thermal sensitivity induced by sciatic nerve injury was between 3.6-5.9 times higher than that of the DOPr agonist (Table 2), indicating that

JHW-015 is markedly more potent than DPDPE on the inhibition of neuropathic pain.

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

In all tests, the antiallodynic and antihyperalgesic effects produced by DPDPE (Table 3A) or JWH015 (Table 3B) in the ipsilateral paw of sciatic nerve injured WT mice were completely reversed by the subplantar co-administration with a selective DOPr (naltrindole) or CB2R (AM630) antagonist, respectively ($p < 0.05$; one way ANOVA followed by the Student Newman Keuls test). The subplantar administration of naltrindole, AM630 or vehicle alone in sciatic nerve injured WT mice did not show any significant effect on the three different nociceptive responses evaluated in this study. In addition, the subplantar administration of CTAP, a selective μ-opioid receptor antagonist, was unable to revert the local antiallodynic and antihyperalgesic effects produced by 154.8 nmol of DPDPE, confirming the specific involvement of DOPr in the effects produced by high doses of DPDPE in these experimental conditions (data not shown).

Involvement of the peripheral nitric oxide–cGMP–PKG pathway triggered by NOS1 and NOS2 in the mechanical allodynia, thermal hyperalgesia and thermal allodynia induced by the sciatic nerve injury in WT mice.

Our results showed that the subplantar administration of different doses of NANT, L-NIL, ODQ or Rp-8-pCPT-cGMPs dose-dependently inhibited the mechanical allodynia (Fig. 2A), thermal hyperalgesia (Fig. 2B) and thermal allodynia (Fig. 2C) induced by sciatic nerve injury in WT mice. Thus, in all behavioral tests the antiallodynic and antihyperalgesic effects of all inhibitors in the ipsilateral paw of sciatic nerve injured WT mice were significantly higher than those obtained in their corresponding vehicle treated groups ($p < 0.05$; Student's t test).

Moreover, the subplantar administration of NANT, L-NIL, ODQ, Rp-8-pCPT-cGMPs or vehicle did not have any significant antinociceptive effect neither on the contralateral paw of sciatic nerve injured mice nor in the ipsilateral or contralateral paw of naive animals (data not

shown).

Furthermore analyzing the ED_{50} values of NOS1, NOS2, ODQ and PKG inhibitors our data showed that the potency of the NOS1 inhibitor (NANT) on the inhibition of mechanical and thermal sensitivity induced by sciatic nerve injury was between 3.7-4.5 times higher than the NOS2 (L-NIL) inhibitor (Table 4). Our results also showed that the potency of the PKG inhibitor (Rp-8-pCPT-cGMPs) on the inhibition of mechanical and thermal sensitivity induced by sciatic nerve injury was between 2.4-3.6 times higher than that of the L-guanylate cyclase inhibitor (ODQ). Moreover, the potency of the peripheral cGMP-PKG pathway blockers on the inhibition of mechanical and thermal allodynia as well as thermal hyperalgesia induced by sciatic nerve injury was higher than the peripheral NOS inhibitors.

Role of the peripheral nitric oxide-cGMP-PKG pathway activated by NOS1 and NOS2 on the local antiallodynic and antihyperalgesic effects produced by DPDPE or JWH015 in sciatic nerve injured WT mice.

The role of the peripheral nitric oxide-cGMP-PKG pathway activated by NOS1 and NOS2 in the local antiallodynic and antihyperalgesic effects induced by DPDPE or JWH015 during neuropathic pain was assessed by evaluating the effects of the co-administration of DPDPE (38.7 nmol) or JWH015 (15.3 nmol) with NANT (50.9 nmol), L-NIL (223.5 nmol), ODQ (13.4 nmol), Rp-8-pCPT-cGMPs (4.1 nmol) or vehicle in sciatic nerve injured WT mice at 21 days after CCI induction.

Our results showed that the co-administration of DPDPE plus NANT, L-NIL, ODQ or Rp-8-pCPT-cGMPs significantly increases the local mechanical antiallodynic (Fig. 3A), thermal antihyperalgesic (Fig. 3B) and thermal antiallodynic (Fig. 3C) effects produced by DPDPE alone in the ipsilateral paw of sciatic nerve injured mice ($p < 0.001$, one way ANOVA followed by Student-Newman-Keuls test). In a similar way, the co-administration of JWH-015 plus NANT, L-NIL, ODQ or Rp-8-pCPT-cGMPs significantly increases the local mechanical antiallodynic (Fig. 4A), thermal antihyperalgesic (Fig. 4B) and thermal antiallodynic (Fig. 4C) effects produced by JWH-015 alone in the ipsilateral paw of sciatic nerve injured mice ($p <$

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0.001, one way ANOVA followed by Student-Newman-Keuls test).

The local co-administration of DPDPE or JWH-015 plus vehicle, NANT, L-NIL, ODQ or Rp-8-pCPT-cGMPs did not have any significant effect neither on the contralateral paw of sciatic nerve injured WT mice nor in the ipsilateral or contralateral paw of naive animals (data not shown).

Expression of DOPr and CB2R mRNA in the spinal cord and dorsal root ganglia of sciatic nerve injured WT, NOS2-KO and NOS1-KO mice.

The expression of DOPr mRNA in the spinal cord of WT and NOS knockout mice is shown in Fig. 5A. The two way ANOVA showed a significant effect of the surgery ($p < 0.006$) as well as a significant interaction between genotype and surgery ($p < 0.045$). Thus, sciatic nerve injury did not affect the expression of DOPr in the spinal cord of WT or NOS2-KO animals, but significantly increased their expression in NOS1-KO mice ($p < 0.037$, Student's t test; as compared to their respective naive mice). Our results did not show any significant difference between genotypes when compared the expression of DOPr mRNA among theme in naive or sciatic nerve injured mice.

In the dorsal root ganglia, the two way ANOVA also showed a significant effect of the surgery ($p < 0.008$) as well as a significant interaction between genotype and surgery ($p < 0.050$). Thus, sciatic nerve injury significantly reduced the expression of DOPr in the dorsal root ganglia of WT ($p < 0.001$, Student's t test) and NOS2-KO mice ($p < 0.047$, Student's t test), but not in NOS1 knockout animals, as compared to their respective naive mice (Fig. 5B). Non significant differences were found between genotypes when compared the expression of DOPr mRNA among theme in naive or sciatic nerve injured mice.

The CB2R mRNA expression in the spinal cord of WT and NOS knockout mice is shown in Fig. 5C. The two way ANOVA showed a significant effect of the surgery ($p < 0.001$) and a marginal significant interaction between genotype and surgery ($p < 0.072$). Sciatic nerve injury significantly increased the expression of CB2R in the spinal cord of WT ($p < 0.050$, Student's t test) and NOS2-KO ($p < 0.009$, Student's t test), but not in NOS1-KO mice, as compared to

their respective naive mice. Our results also showed that in naive mice, the CB2R mRNA expression in NOS2-KO mice was significantly lower than in WT or NOS1-KO animals ($p < 0.002$, one way ANOVA followed by Student-Newman-Keuls test).

In the dorsal root ganglia, the two way ANOVA only showed a significant effect of the surgery ($p < 0.012$). Thus, while sciatic nerve injury significantly increased the expression of CB2R in the dorsal root ganglia of WT ($p < 0.029$, Student's t test; vs. naive WT) and NOS2-KO mice ($p < 0.040$, Student's t test; vs. naive NOS2-KO), non significant changes were observed in NOS1-KO (Fig. 5D). Our results did not show any significant difference between genotypes when compared the expression of CB2R mRNA among them in naive or sciatic nerve injured mice.

Furthermore, the expression of DOPr in the spinal cord and dorsal root ganglia was significantly higher than that of CB2R in all genotypes ($p < 0.01$, Student's t test).

Discussion

The local administration of specific DOPr and CB2R agonists, nitric oxide synthases or cGMP-PKG pathway inhibitors, dose-dependently inhibited the mechanical and thermal allodynia as well as the thermal hyperalgesia induced by sciatic nerve injury. Interestingly, the local antiallodynic and antihyperalgesic effects of DPDPE and JWH-015 were significantly enhanced by their co-administration with nitric oxide synthases or cGMP-PKG pathway blockers. This study also showed that nitric oxide synthesized by NOS1 is implicated in the peripheral down and up-regulation of DOPr and CB2R gene transcription during neuropathic pain.

In a model of CCI-induced neuropathic pain our results confirmed the mechanical antiallodynic effects of DOPr and CB2R agonists locally administered (Elmes et al., 2004; Kabli and Cahill, 2007; Obara et al., 2009) and further demonstrated the thermal antihyperalgesic and antiallodynic effects of both agonists in these experimental conditions. Moreover, while JWH-015 had a similar potency in the inhibition of mechanical and thermal allodynia as well as thermal hyperalgesia, the capability of DPDPE to reduce mechanical allodynia and thermal hyperalgesia was higher than that of reducing thermal allodynia. Comparing the ED_{50} values of DPDPE and JWH-015 our data revealed that the DOPr agonist is effective at doses 3.6 – 4.1 times higher than the CB2R agonist in reversing the mechanical allodynia and thermal hyperalgesia induced by sciatic nerve injury. Curiously, a higher relative efficacy of JWH-015 than DPDPE was observed on the inhibition of thermal allodynia, where the potency of JWH-015 is 5.9 times higher than that of DPDPE. The specificity of the peripheral antiallodynic and antihyperalgesic effects of DOPr and CB2R agonists after sciatic nerve injury was demonstrated by the completed reversion of their effects with selective antagonists who did not have any effect in the absence of agonists. Moreover, the highest doses of DPDPE or JWH-015 did not produce any significant effect in the contralateral paw of sciatic nerve injured mice indicating a peripheral site of action.

It is well accepted that nitric oxide mediates some neuropathic pain symptoms (LaBuda et al., 2006). Thus, several works using pharmacological and genetic approaches have demonstrated that nitric oxide mediates the maintenance of neuropathic pain through the activation of spinal

nitric oxide-cGMP-PKG pathway triggered by NOS1 and NOS2, where the soluble guanylyl cyclase and PKG enzymes are essentially required for the pronociceptive action of nitric oxide in the spinal cord (Guan et al., 2007; Schmidtko et al., 2008). Besides it, the peripheral involvement of this nitric oxide signal pathway in the maintenance of thermal and mechanical hypersensitivity induced by the chronic constriction of the sciatic nerve has not been fully clarified. Our results showed that the subplantar administration of NOS1 and NOS2 inhibitors as well as soluble guanylate cyclase or PKG blockers generates potent dose-dependent antiallodynic and antihyperalgesic effects after peripheral nerve injury. Where the potency of the peripheral downstream cGMP-PKG pathway blockers attenuating neuropathic pain was much higher than that of NOS1 or NOS2 inhibitors. These data suggest that nitric oxide produced by NOS1 and NOS2 mediates the maintenance of neuropathic pain induced by CCI through the peripheral nitric oxide-cGMP-PKG pathway activation.

In other pain models, such as acute and inflammatory, a clear relationship between the antinociceptive effects of opioids and the nitric oxide-cGMP-PKG pathway activation has been extensively demonstrated. In accordance, a significant reduction in the antinociceptive effects of opioids was observed when neuronal and/or inducible NOS are inhibited, either pharmacological or using knockout mice for these enzymes (Li and Clark, 2001; Pol, 2007; Leánez et al., 2009). Moreover, the co-administration of a DOPr agonist with a nitric oxide donor significantly enhances the antinociceptive potency of DPDPE in a mouse model of inflammatory pain (Hervera et al., 2009). In the present study, the involvement of the peripheral nitric oxide-cGMP-PKG pathway as a possible mechanism of action of DOPr and CB2R agonists during neuropathic pain was also investigated. Interestingly and in contrast to inflammatory pain, the local pharmacological blockage of the nitric oxide-cGMP-PKG pathway potentiated the peripheral antiallodynic and antihyperalgesic effects of DOPr and CB2R agonists during neuropathic pain. That is, the inhibitory effects induced by DPDPE or JWH-015 plus NANT, L-NIL, ODQ or Rp-8-pCPT-cGMPs were higher than those produced by each drug administered alone in all paradigms evaluated. These results suggest that the activated nitric oxide-cGMP-PKG peripheral pathway was implicated as a mechanism limiting the local

antiallodynic and antihyperalgesic efficiency of DOPr and CB2R agonists under neuropathic pain conditions. Therefore, the local co-administration of opioids or cannabinoids with a NOS1, NOS2, guanylate cyclase or a PKG inhibitor might represent a useful therapeutic strategy for the treatment of neuropathic pain.

The possible alteration of DOPr and CB2R gene expression by neuropathic pain has also been evaluated in this study. Our data indicated a decreased abundance of DOPr mRNA in the dorsal root ganglia of WT mice in day 21 after CCI. According to these results, Obara et al. (2009) also demonstrated that the dorsal root ganglia DOPr mRNA levels decreased in days 3 and 14 after total sciatic nerve ligation in WT mice, although their expression did not change in day 16 after the partial sciatic nerve ligation (Pol et al., 2006). These findings suggest that the DOPr expression changes induced by neuropathic pain in the dorsal root ganglia could be more related to the nerve injury model (partial vs. CCI) than the post-injury time. Moreover, although sciatic nerve injury did not alter the transcription of DOPr gene in the spinal cord, an enhanced transcription of CB2R in the spinal cord and dorsal root ganglia of WT mice at 21 days following CCI, has been demonstrated. In accordance to our results, an increased immunoreactivity and CB2R mRNA expression in the spinal cord and the nerve sections proximal to the spinal nerve ligation site, have been also demonstrated by other authors (Zhang et al., 2003; Wotherspoon et al., 2005). In summary and taking account that DOPr are mainly located in neurons and CB2R in glial cells, the nerve injury-induced degeneration of C fibers (Ossipov et al., 2000) and glial activation (Mika et al., 2009) could be the principal responsible for the decreased and increased synthesis of peripheral DOPr and CB2R, that leads the lower peripheral potency of DPDPE compared to JWH-015 during neuropathic pain.

Finally, the role of nitric oxide synthesized by NOS1 or NOS2 enzymes in DOPr and CB2R gene expression changes observed at 21 days after CCI-induced neuropathic pain has been evaluated by using knockout mice. Thus, and similarly that occur in WT mice, nerve injury also decreased DOPr and increased CB2R expression in the dorsal root ganglia of NOS2-KO mice and did not alter (DOPr) or enhanced (CB2R) their expression in the spinal cord of NOS2-KO mice, but not in NOS1-KO. These findings indicated that nitric oxide synthesized by NOS1

plays a dual role in the modulation of DOPr and CB2R gene transcription after sciatic nerve injury, since it is implicated in the decreased or not altered DOPr mRNA expression as well as in the increased transcription of CB2R that take place in the peripheral and central nervous system of animals with neuropathic pain.

In summary, our data demonstrate that the inactivation of nitric oxide-cGMP-PKG peripheral pathway triggered by NOS1 and NOS2 enhanced the peripheral actions of DOPr and CB2R agonists and that nitric oxide synthesized by NOS1 is implicated in the peripheral regulation of DOPr and CB2R gene transcription during neuropathic pain.

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Footnotes:

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b) Part of these results has been presented as a communication to the 4th International Conference on cGMP, held in Regensburg, Germany, June, 2009 and the 38th Annual Meeting of Society for Neuroscience, held in Chicago, USA, October, 2009.

Legends for figures

Fig. 1. Effects of the subplantar administration of different doses (logarithmic axis) of an specific DOPr agonist (DPDPE), an specific CB2R agonist (JWH-015) and their corresponding vehicle (dotted lines) in the mechanical allodynia (A), thermal hyperalgesia (B) and thermal allodynia (C) induced by CCI in the ipsilateral paw of WT mice at 21 days after surgery. Both agonists were administered 20 min before starting behavioral testing. Data are expressed as mean values of maximal possible effect (%) for mechanical allodynia and thermal hyperalgesia and inhibition (%) for thermal allodynia ± SEM (5-6 animals for each dose and drug tested). In all tests, for each drug and dose, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ denotes significant differences when compared each agonist vs. their corresponding vehicle treated group (Student's t test).

Fig. 2. Effects of the subplantar administration of different doses (logarithmic axis) of an specific NOS1 (NANT), NOS2 (L-NIL), guanylate cyclase (ODQ), PKG (Rp-8-pCPT-cGMPs; Rp-8) inhibitor and their corresponding vehicle (dotted lines) in the mechanical allodynia (A), thermal hyperalgesia (B) and thermal allodynia (C) induced by CCI in the ipsilateral paw of WT mice at 21 days after surgery. All inhibitors were administered 20 min before starting behavioral testing. Data are expressed as mean values of maximal possible effect (%) for mechanical allodynia and thermal hyperalgesia and inhibition (%) for thermal allodynia ± SEM (5-6 animals for each dose and drug tested). In all tests, for each drug and dose, * $p < 0.05$; ** $p < 0.01$, and *** $p < 0.001$ denotes significant differences when compared each inhibitor vs. their corresponding vehicle treated group (Student's t test).

Fig. 3. Effects of the subplantar co-administration of DPDPE (38.7 nmol) plus vehicle, NANT (50.9 nmol), L-NIL (223.5 nmol), ODQ (13.4 nmol) or Rp-8-pCPT-cGMPs (4.1 nmol; Rp-8) in the ipsilateral paw of sciatic nerve injured WT mice at 21 days after CCI. All drugs were co-administered 20 min before starting behavioral testing. Data are expressed as mean values of the maximal possible effect (%) for mechanical allodynia (A) and thermal hyperalgesia (B) and as

inhibition (%) for thermal allodynia (C) ± SEM (5-6 animals per group). For each behavioral test, * $p < 0.05$ denotes significant differences vs. group treated with DPDPE + vehicle (one way ANOVA followed by Student Newman Keuls test).

Fig. 4. Effects of the subplantar co-administration of JWH-015 (15.3 nmol) plus vehicle, NANT (50.9 nmol), L-NIL (223.5 nmol), ODQ (13.4 nmol) or Rp-8-pCPT-cGMPs (4.1 nmol; Rp-8) in the ipsilateral paw of WT mice at 21 days after CCI. All drugs were co-administered 20 min before starting behavioral testing. Data are expressed as mean values of the maximal possible effect (%) for mechanical allodynia (A) and thermal hyperalgesia (B) and as inhibition (%) for thermal allodynia (C) ± SEM (5-6 animals per group). For each behavioral test, * $p < 0.05$ denotes significant differences vs. group treated with JWH-015 + vehicle (one way ANOVA followed by Student Newman Keuls test).

Fig. 5. Relative DOPr mRNA expression in the spinal cord (A) and dorsal root ganglia (B) of naive (white columns) and total sciatic nerve ligated (striped columns) WT, NOS2-KO and NOS1-KO mice. The figure also shows the relative CB2R mRNA expression in the spinal cord (C) and dorsal root ganglia (D) of naive (white columns) and total sciatic nerve ligated (striped columns) WT, NOS2-KO and NOS1-KO mice. Data are expressed as mean values ± SEM (5-6 samples per group). For each genotype, * $p < 0.05$; ** $p < 0.001$, and *** $p < 0.0001$ denotes significant differences between naive and sciatic nerve injured mice (Student's t test). For each experimental group, different letters (a, b) indicate significant differences between genotypes (* $p < 0.05$; one way ANOVA followed by the Student Newman Keuls test).

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Table 1. Mechanical allodynia (basal response, %), thermal hyperalgesia (withdrawal latency, sec) and thermal allodynia (paw lifts, number) in the contralateral and ipsilateral paw of naive, sham-operated and sciatic nerve injured WT mice at 21 days after surgery.

Tests	**Paw**	**Naive**	**Sham-operated**	**Sciatic nerve injured**
Mechanical allodynia (basal response, %)	Contralateral	100.0 ± 7.6	92.4 ± 8.0	85.5 ± 5.5
	Ipsilateral	100.0 ± 7.3	95.8 ± 7.9	39.9 ± 1.4 *
Thermal hyperalgesia (withdrawal latency, sec)	Contralateral	8.4 ± 0.1	8.3 ± 0.2	7.7 ± 0.2
	Ipsilateral	8.7 ± 0.1	8.6 ± 0.1	3.7 ± 0.1 *
Thermal allodynia (paw lifts, number)	Contralateral	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.1
	Ipsilateral	0.0 ± 0.0	0.3 ± 0.2	5.5 ± 0.7 *

Results are shown as mean values ± SEM; n= 8-10 animals per experimental group. For each test and paw, * $p < 0.05$ denotes significant differences vs. naive or sham-operated mice (one way ANOVA, followed by the Student Newman Keuls test).

Table 2. Comparison of the potencies (ED_{50}) of the subplantar administration of DPDPE or JHW015 to suppress the mechanical allodynia (von Frey test), thermal hyperalgesia (plantar test) and thermal allodynia (cold plate test) induced by nerve injury in WT mice at 21 days after CCI induction.

Tests	DPDPE	JHW-015	Ratio (DPDPE /JHW-015)
Mechanical allodynia	76.2 (48.5-119.2)	21.2 (11.0-40.3)	3.6
Thermal hyperalgesia	85.6 (54.2-135.0)	20.8 (10.1-42.5)	4.1
Thermal allodynia	115.0 (90.0-147.0)	19.6 (18.9-19.9)	5.9

Data are expressed as ED_{50} values (nmol) with 95% confidence limits determined on the quantal data of 5-6 animals per dose. For each test, the ratio of the ED_{50} values between agonists is also indicated.

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Table 3. Reversal of the effects of DPDPE (154.8 nmol, **A**) and JWH-015 (91.6 nmol, **B**) on the mechanical allodynia, thermal hyperalgesia and thermal allodynia induced by nerve injury in the ipsilateral paw of WT mice, at 21 days after CCI, by the subplantar administration of specific DOPr (naltrindole; 110.9 nmol) or CB2R (AM630; 59.5 nmol) antagonists. The effects of the subplantar administration of vehicle, naltrindole or AM630 alone have also shown.

A	Mechanical allodynia (MPE, %)	Thermal hyperalgesia (MPE, %)	Thermal allodynia (Inhibition, %)
vehicle	3.5 ± 3.5	3.0 ± 1.0	5.5 ± 5.5
DPDPE + vehicle	53.5 ± 3.7 *	60.4 ± 3.7 *	42.5 ± 12.6*
DPDPE + naltrindole	3.1 ± 1.8	4.2 ± 2.5	7.1 ± 7.1
naltrindole + vehicle	5.3 ± 4.0	3.61 ± 1.7	8.3 ± 8.3

B	Mechanical allodynia (MPE, %)	Thermal hyperalgesia (MPE, %)	Thermal allodynia (Inhibition, %)
vehicle	4.6 ± 1.9	4.9 ± 1.7	4.9 ± 3.2
JWH-015 + vehicle	61.5 ± 7.4 *	62.9 ± 2.1*	70.4 ± 10.8 *
JWH-015 + AM630	2.7 ± 2.7	13.5 ± 2.5	17.4 ± 10.2
AM630 + vehicle	6.0 ± 3.5	5.4 ± 3.3	4.2 ± 4.2

Results are shown as mean values ± SEM; n= 5-7 animals per group. For each test, * represents significant differences compared to other groups ($p < 0.05$; one way ANOVA, followed by the Student Newman Keuls test). MPE = maximal possible effect.

Table 4. Comparison of the potencies (ED_{50}) of the subplantar administration of L-NIL, NANT, ODQ or Rp-8-pCPT-cGMPs (RP-8) to suppress the mechanical allodynia (von Frey test), thermal hyperalgesia (plantar test) and thermal allodynia (cold plate test) induced by nerve injury in WT mice at 21 days after CCI induction.

Tests	L-NIL	NANT	ODQ	RP-8	Ratio (L-NIL/ NANT)	Ratio (ODQ/ RP-8)
Mechanical allodynia	465.6 (423.7-511.6)	118.0 (115.1-120.9)	22.5 (12.5-40.2)	7.7 (6.3-9.3)	3.9	2.9
Thermal hyperalgesia	462.2 (394.7-541.3)	101.4 (97.4-105.5)	21.7 (13.0-36.4)	9.0 (8.1-10.1)	4.5	2.4
Thermal allodynia	334.8 (283.4-395.6)	90.6 (61.3-133.9)	26.6 (19.8-35.6)	7.3 (6.1-8.8)	3.7	3.6

Data are expressed as ED_{50} values (nmol) with 95% confidence limits determined on the quantal data of 5-6 animals per dose.

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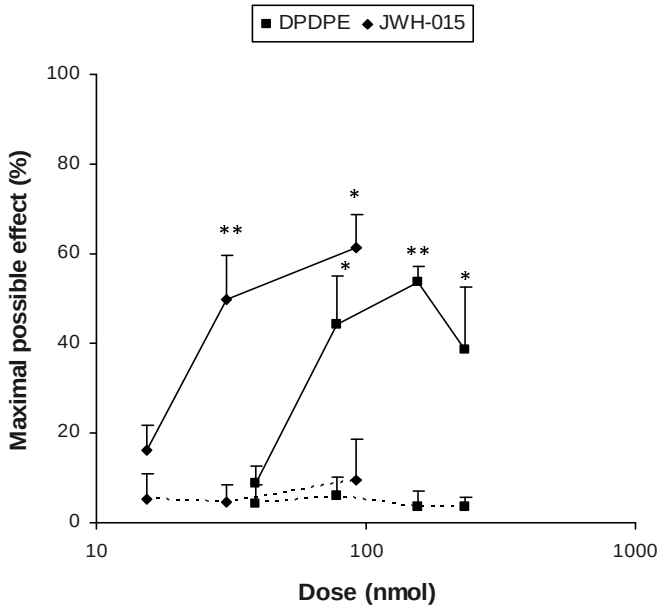
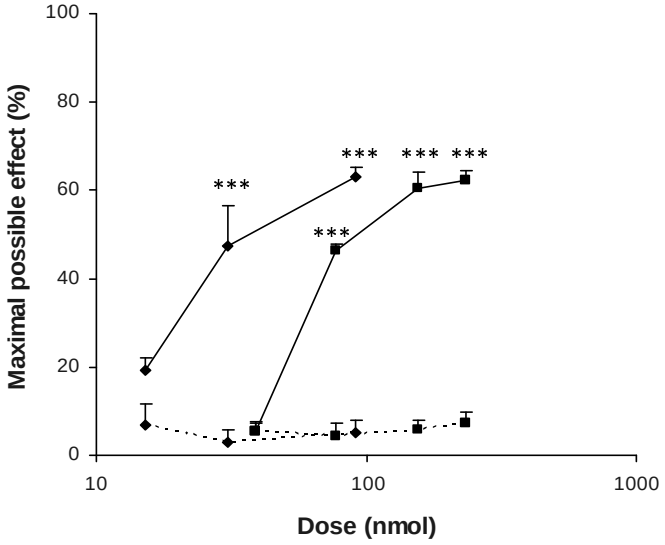
FIGURE 1

A



B



C

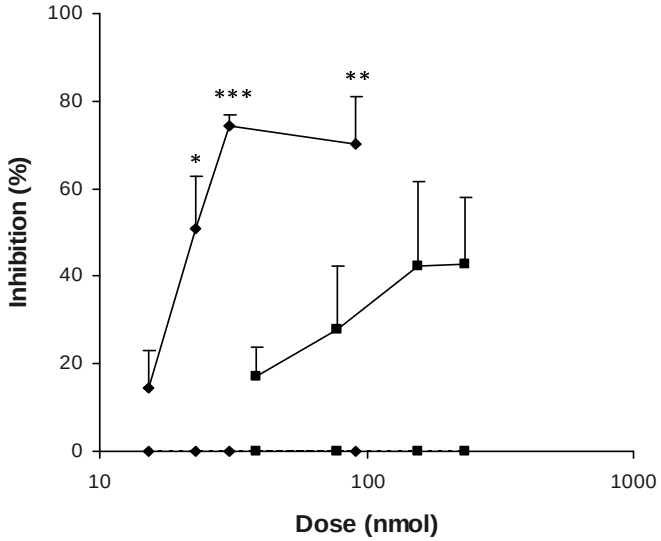


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FIGURE 2

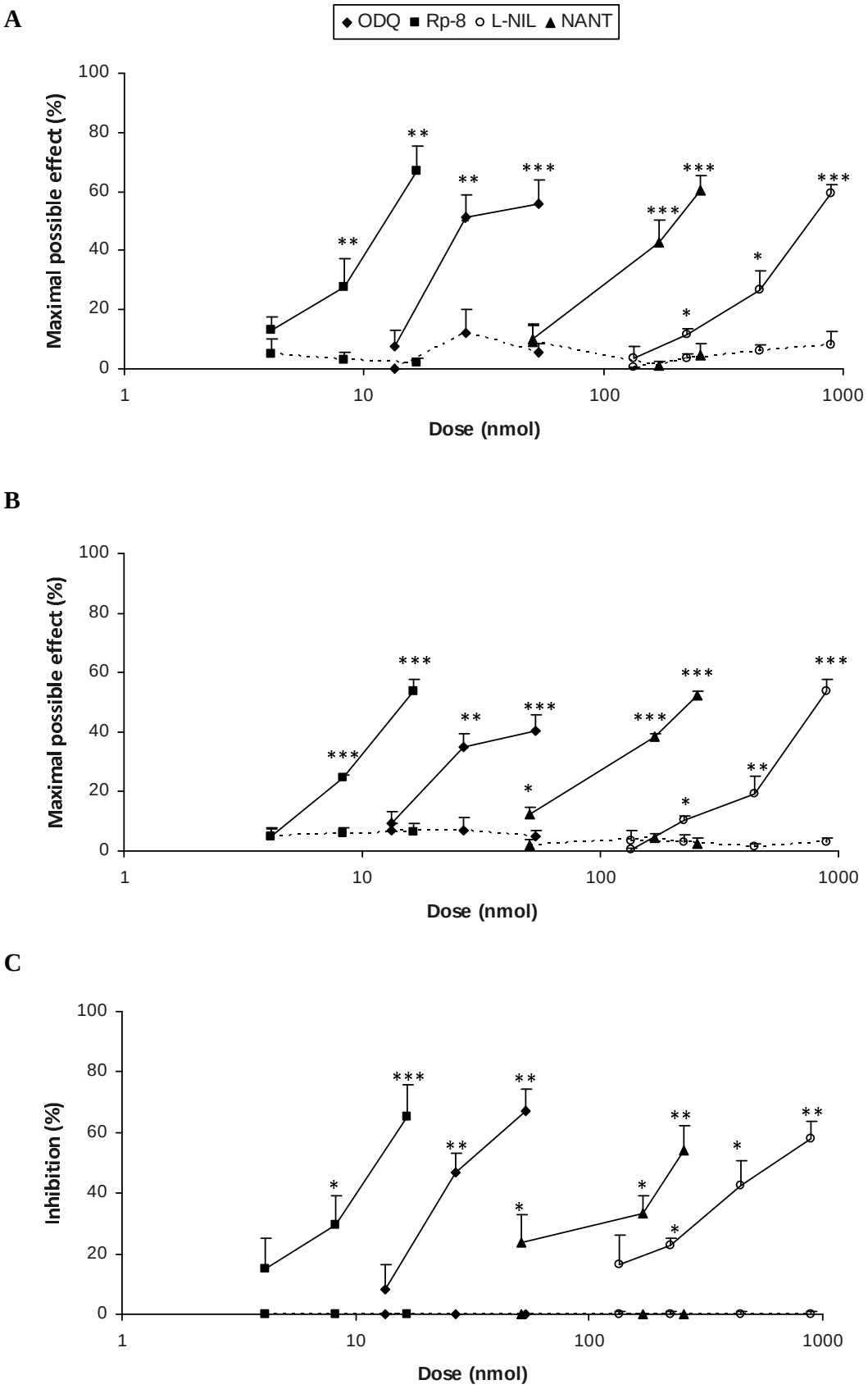

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FIGURE 3

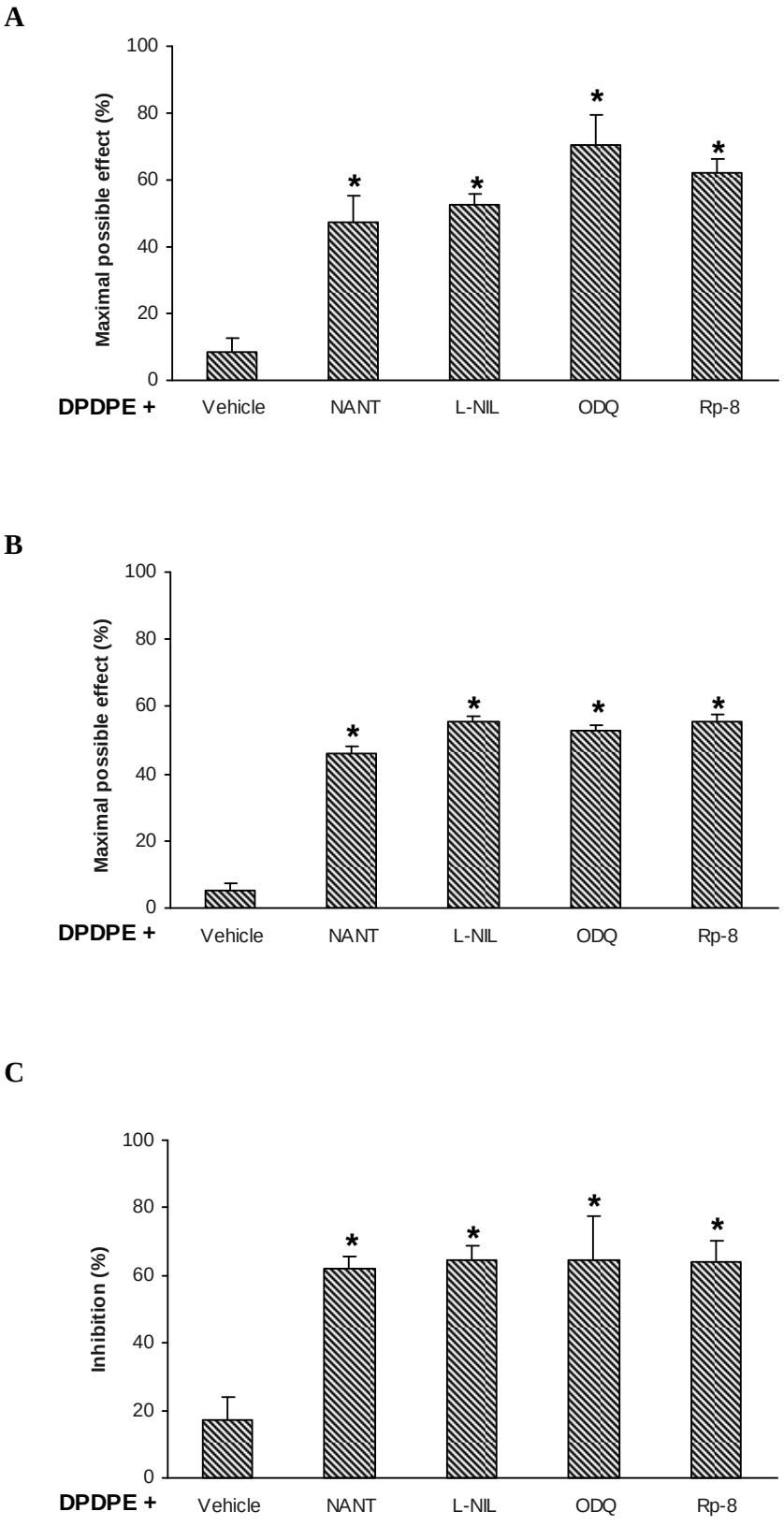

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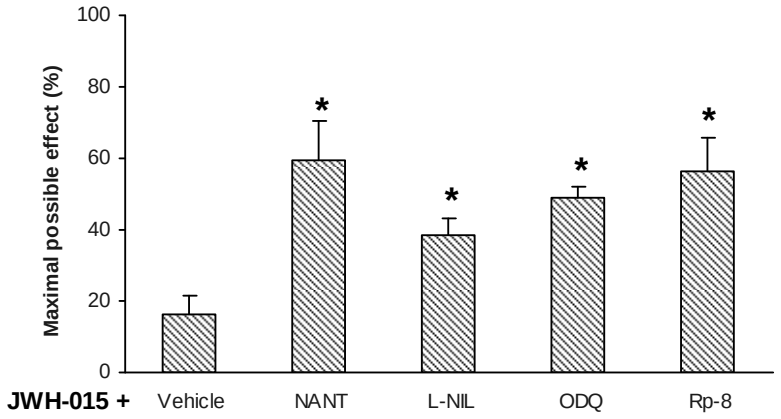
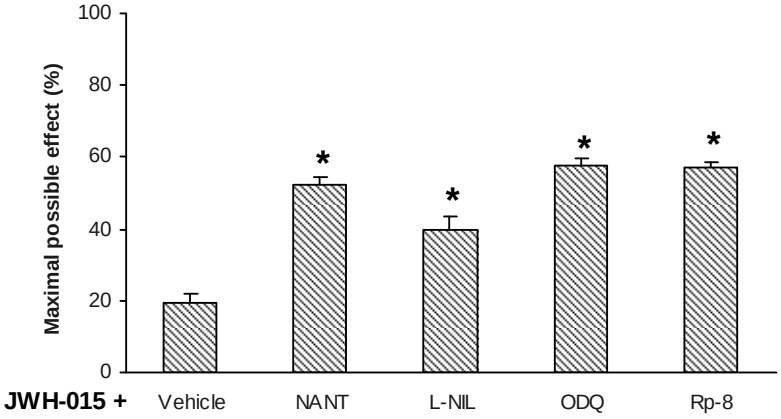
FIGURE 4

A



B



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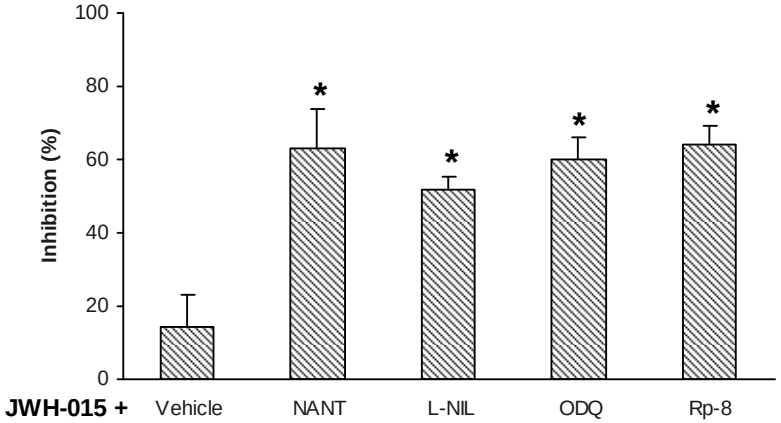

FIGURE 5