Role of gaseous neurotransmitters in the effects and expression of opioid and cannabinoid receptors during neuropathic pain

PhD Thesis

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Treball realitzat a l'Institut d'Investigació Biomèdica Sant Pau i l'Institut de Neurociències de la Universitat Autònoma de Barcelona, sota la direcció de la Dra. Olga Pol Rigau

Doctorand | Directora de tesi

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1. Abstract

Neuropathic pain is caused by a lesion or disease of the somatosensory nervous system, and is characterized by the presence of allodynia and hyperalgesia. Nowadays, its treatment is based upon opioids, but high doses are necessary to alleviate symptoms and they have several undesirable side effects. Therefore, it is important to investigate new targets and mechanisms to improve the current opioid treatments in order to reduce dosage and avoid side effects. In this study, we investigated the role played by the two main gaseous neurotransmitters, nitric oxide (NO) and carbon monoxide (CO) in the development of neuropathic pain, as well as their effects in the mu (MOR) and delta (DOR) opioid, and cannabinoid 2 (CB2) receptors mediated therapies. Indeed, by using the chronic constriction of the sciatic nerve, as a mouse model of neuropathic pain, we demonstrated that: I) the peripheral NO-sGC-PKG signaling pathway, triggered by NOS1 and NOS2, plays a key role in the development and expression of the main symptoms of neuropathic pain, II) the peripheral, but not systemic, antinociceptive effects of MOR agonists during neuropathic pain are produced through the activation of the HO1/NOS1/NOS2-sGC-PKG-K^+ATP signaling pathway, and the NO, synthesized by NOS1 and NOS2, is implicated in the peripheral down regulation of MOR, III) the peripheral antinociceptive effects of DOR and CB2 receptor agonists during neuropathic pain can be increased by the inactivation of the NOS1/NOS2-sGC-PKG signaling pathway. Moreover the NO, synthesized by NOS1, is implicated in the peripheral down- and up-regulation of DOR and CB2 receptor during neuropathic pain, IV) the inhibition of the NO-sGC-PKG-JNK signaling pathway avoids the development of tolerance to the local antiallodynic effects produced by morphine during neuropathic pain, V) CO, synthesized by HO1, inhibits neuropathic pain by the attenuation of NOS1/NOS2 overexpression and microglial activation induced by nerve injury, and VI) the treatment with CO, exogenously deliberated or endogenously synthesized by HO1, enhances the peripheral antinociceptive effects of MOR agonists by up-regulating the peripheral expression of MOR and inhibiting the microglial activation. In summary, both NO and CO systems have an essential role in the expression of neuropathic pain and both modulate the effects and expression of MOR, DOR and CB2 receptors after sciatic nerve injury, but while MOR elicits its peripheral antinociceptive effects through the activation of the HO1/NOS-sGC-PKG-K^+ATP signaling pathway, DOR or CB2 receptor do not use this pathway to produce their effects. Finally, although this study shows different strategies to increase the local antinociceptive effects produced by opioids and cannabinoids and avoid the development of tolerance during neuropathic pain, the investigation of new mechanisms of action of these drugs is essential to improve their therapeutic actions in neuropathic pain.

2. List of abbreviations

Δ9-THC	Δ9-tetrahydrocannabinol
2-AG	2-arachidonoylglycerol
5'-GMP	5'-guanosine monophosphate
AC	Adenylate cyclase
AEA	Ananadamide
AM251	N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide
AM630	6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-ethoxyphenyl)methanone
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
CB1	Cannabinoid receptor 1
CB2	Cannabinoid receptor 2
CCI	Chronic constriction injury
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CO	Carbon monoxide
CoPP	Cobalt-protoporphyrin IX
CORM	Carbon monoxide releasing molecules
CORM-2	Tricarbonyldichlororuthenium (II) dimer
CORM-3	Tricarbonylchloro(glycinato)ruthenium (II)
CREB	cAMP response element-binding protein
DAMGO	[D-Ala^2, NMe-Phe^4, Gly-ol^5]-enkephalin
DOR	Delta opioid receptor
DPDPE	[D-Pen^2,D-Pen^5]-enkephalin
DRG	Dorsal root ganglia
GABA	γ-Aminobutyric acid
GDP	Guanosine diphosphate
GPCR	G-protein coupled receptor
GTP	Guanosine triphosphate
HO	Heme oxygenase
HO1	Inducible heme oxygenase

HO2	Constitutive heme oxygenase
HO3	Oxygen sensing heme oxygenase
JNK	c-Jun N-terminal kinase
JWH-015	(2-Methyl-1-propyl-1*H*-indol-3-yl)-1-naphthalenylmethanone
K^+ATP	ATP-sensitive potassium channel
KO	Knock-out
KOR	Kappa opioid receptor
MAPK	Mitogen associated protein kinase
MOR	Mu opioid receptor
NADA	N-Arachidonoyl dopamine
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NOS	Nitric oxide synthase
NOS1/nNOS	Neuronal nitric oxide synthase
NOS2/iNOS	Inducible nitric oxide synthase
NOS3/eNOS	Endothelial nitric oxide synthase
ORL1	Opioid-like receptor 1
PAG	Periaqueductal grey matter
PDE	Phosphodiesterase
PI3K	Phosphatidylinositol 3-kinase
PKG	cGMP-dependent protein kinase or Protein Kinase G
RVM	Rostral ventromedial medulla
sGC	Soluble guanylate cyclase
SR-144,528	N-[(1S)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan2-yl]-5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-1H-pyrazole-3-carboxamide
TRPV	Transient Receptor Potential Vanilloid
TRPV1	Transient receptor potential cation channel subfamily V member 1
WIN55,212-2	(R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-napthalenylmethanone

3. Introduction

3.1. Pain

Pain is defined as "*An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage* ", by the International Association for the Study of Pain (www.iasp-pain.org).

Pain is an essential ability of the organism that allows detecting potential injury, triggers protective responses and alerts the organism from injured tissue with an aim to prevent further injury.

However, sometimes, there is an abnormal sensitivity in the somatosensory system that can result in chronic pain. Pain is referred as chronic pain when the symptoms last longer than the time required for healing the tissue damage, or when it is associated with some pathological states that do not heal.

Several epidemiological studies from different countries have reported widely varying prevalence rates for chronic pain, ranging from 12-80% of the population (Abu-Saad, 2010).

3.1.1. Nociceptive processing

3.1.1.1. Peripheral nociceptors

Pain processing begins with specialized sensory neurons called nociceptors that are able to distinguish and preferentially respond to noxious stimuli. Peripheral nociceptors are free nerve endings in the skin, muscle, articulation, fascia, and viscera that respond to noxious stimulation. Unlike other types of receptors, peripheral nociceptors are multimodal, which means that can respond to multiple stimulus modalities, such as thermal, mechanical or chemical (Van and Gybels, 1981).

3.1.1.2. Primary afferents

Primary afferent fibers divided into physiologically distinct layers called laminae transmit sensory information from the peripheral nociceptors to the dorsal horn of the spinal cord.

Different fiber types form synapses in different layers and either glutamate or substance P as the neurotransmitters. Fibers that innervate regions of the head arise from cell bodies in the trigeminal ganglia, whereas in the rest of the body the cell bodies are located in the dorsal root ganglia (DRG). These fibers are categorised based on their myelination, the modality of stimulation that evokes a response and the characteristics of the response (Figure 1): Aδ fibers form synapses in laminae I and V, C fibers connect with neurons in lamina II, Aβ fibers connect with lamina I, III, & V. While Aδ fibers are related to the sensation of a immediate well located shallow pain,

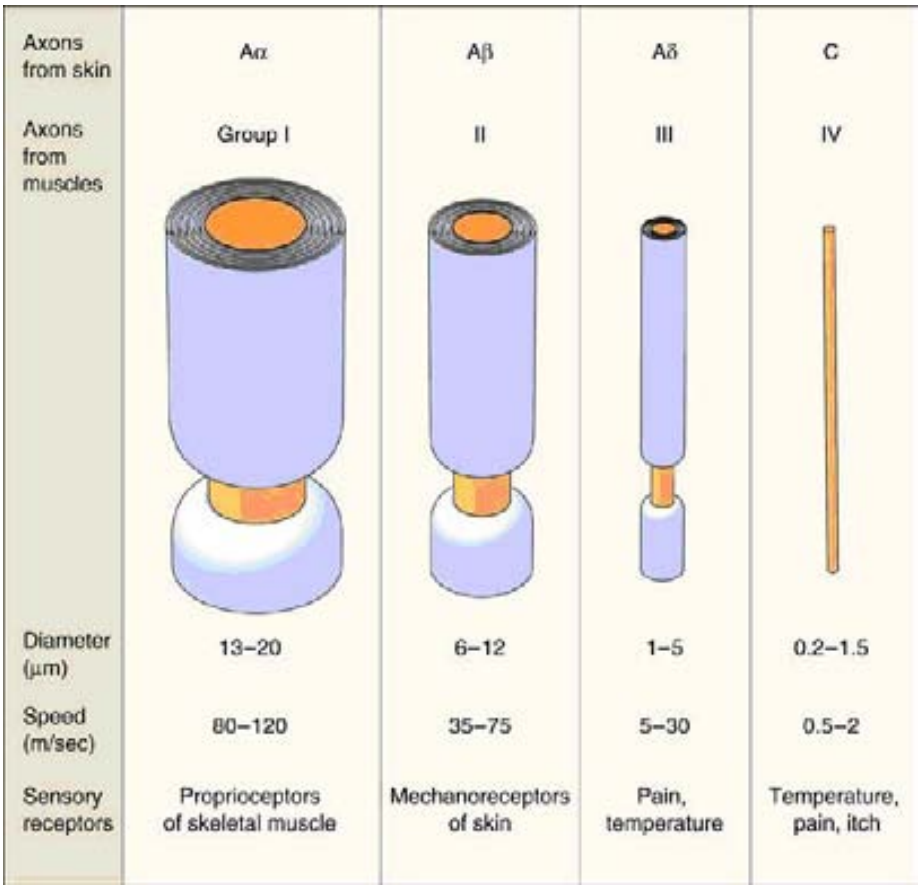

Figure 1. Types of afferent fibers by size and conduction velocity *(from http://alexandria.healthlibrary.ca/)*

responding to weaker stimulus, C fibers respond to stronger intensities of stimulus leading a secondary and slower, but deeper and spread out over an unspecific area of pain (Kandel, et al., et al., 1991).

3.1.1.3. Processing pathway

The afferent pain signals reach the dorsal root ganglia, cross the synapse to a higher neuron in the dorsal horn of the spinal cord and connect with interneuron cells. This is not a simple, passive, neuron-neuron transmission. The signal processing in the dorsal root ganglia and the dorsal horn of the spinal cord is complex and only partly understood. There are many factors, including descending stimulatory and inhibitory signals from the brain, as well as modulation of neurotransmitter substances responsible of the central modulation of the processing. The synapse between primary afferents and dorsal horn interneurons is one of the most targeted sites for analgesic drugs.

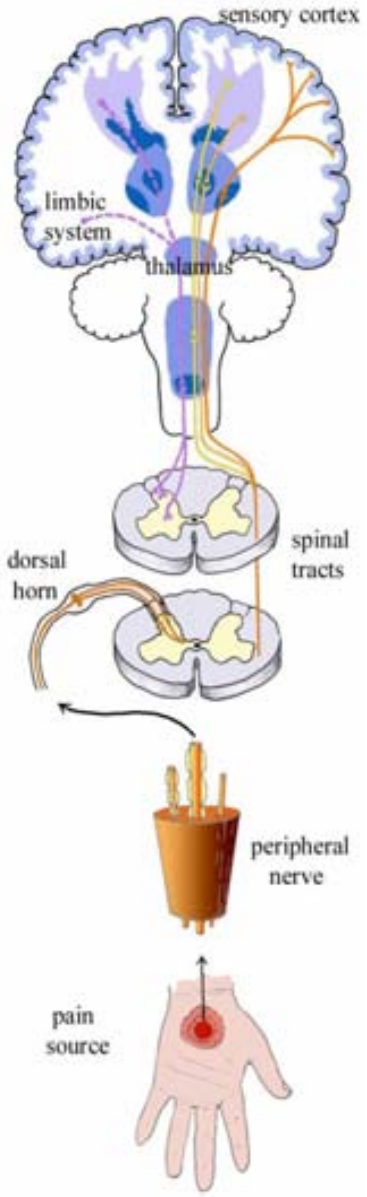

Figure 2. Anatomic Diagram of Pain Pathways *(from http:// www.perioperativepain.com/)*

These interneurons act as a secondary afferents that either make synapse in thalamic nucleus which subsequently make synaptic contact with tertiary neurons, or synapse with neurons of some nucleus from the brainstem, such as periaqueductal gray or the raphe magnus, areas involved in descending modulation. Tertiary neurons from the thalamus send afferent fibers to the somatosensory cortex which is involved in the sensory quality of pain and to limbic structures implicated in the emotional components of pain (Figure 2). There are also some descending pathways that can modulate pain sensory. Indeed, the brain, through the hypothalamus, can request the release of endogenous molecules with analgesic effects that reduce or inhibit the pain sensation (Willis and Westlund, 1997).

3.1.2. Neuropathic pain

Neuropathic pain is defined by the International Association for the Study of Pain (www.iasp-pain.org) as *"Pain caused by a lesion or disease of the somatosensory nervous system"* and it can have its origin in the central or the peripheral nervous system. Central neuropathic pain is mainly a consequence from spinal cord injury, stroke or multiple sclerosis. Peripheral neuropathic pain results from lesions to the peripheral nervous system, mainly caused by mechanical trauma, metabolic diseases, neurotoxic chemicals, infections or tumoural invasions.

Neuropathic pain is characterized by the presence of exaggerated response to painful stimuli (hyperalgesia), pain response to normally innocuous stimuli (allodynia), and ectopic and spontaneous pain. The mechanisms which can contribute to neuropathic pain syndromes are multiple and complex, and, in big part still unknown and controversial. Specifically, during peripheral neuropathic pain syndromes a lesion in a peripheral nerve triggers changes initially in the peripheral sensory system and successively in the central nervous system.

After peripheral nerve damage, injured and non-injured neighboring sensory neurons can generate ectopic discharges in the absence of any stimulus. Important factors causing the development of these discharges are the up-regulation of voltage gated sodium channels (Wood, et al., 2004), and down-regulation of potassium channels (Everill and Kocsis, 1999). Moreover, the development of neuropathic pain involves not only neuronal pathways, but also Schwann cells, satellite cells in the dorsal root ganglia, components of the peripheral immune system, spinal microglia and astrocytes, that cause an inflammatory process following nerve injury (Scholz and Woolf, 2007).

Another critical change responsible for the appearance of some of the main pain symptoms, such as allodynia or hyperalgesia, is the phenotypic switch of the sensory neurons, so that neurons belonging to a specific neuronal subtype start expressing molecules which are normally distinctively expressed by other neuronal subtypes. For example, the neuromodulator substance P, which is normally expressed only in C-fibres, begins to be expressed in A-fibers neurons after peripheral nerve injury (Noguchi, et al., 1995). These changes induce non-pain sensory neurons to generate

pain-like sensations when stimulated with non-noxious stimulus (Song, et al., 2012). As a consequence of this peripheral hyperactivity, subsequent changes take place in the spinal cord. Indeed, dorsal horn neurons increase their responsiveness to synaptic inputs, these inputs from the primary sensory neurons are stronger, and consequently the central responsiveness increases, also there is an apoptotic loss of inhibitory neurons in the superficial dorsal horn of the spinal cord. Furthermore, nerve injury also activates the spinal cord glia which enhance pain by the release of proinflammatory cytokines, nitric oxide, glutamate, ATP and BDNF, among others (Woolf, 2004).

3.2. Nitric oxide

Nitric oxide (NO) is one of the smallest and simplest biologically active molecules in nature and one of the most ubiquitous substances in mammalian species. As one of the most widespread signaling molecule in mammal, NO is a major player in the modulation of nearly every cellular and tissue process in the organism. Indeed, this little molecule can act as a neurotransmitter, autacoid, constitutive or inducible mediator, cytoprotective or cytotoxic molecule among others (Ignarro, 2005).

Although alternative mechanisms exist for the generation of NO (acidification or reduction of nitrite), the vast majority of mammalian NO is derived from nitric oxide synthase (NOS) enzymes. This family of enzymes converts L-arginine to citrulline and NO in a NADPH/O_2 dependent manner. Three NOS isoforms exist to provide a wide range of concentration and temporal NO profiles. Two of these isoforms are constitutive (NOS1 or neuronal nNOS and NOS3 or endothelial eNOS), while the third is inducible (NOS2 or iNOS) (Stuehr, 1997). The signal transduction activated by NO involves different important physiological processes, including smooth muscle relaxation and neurotransmission. NO interacts with the hemoproyein, soluble guanylate cyclase (sGC), a heterodimeric enzyme that converts guanosine triphosphate to cyclic guanosine monophosphate (cGMP), being a critical component of this signaling pathway. It is through the specific interaction with the sGC heme group that other neurotransmitters like carbon monoxide activate this enzyme (Denninger and Marletta, 1999).

This increase in the cGMP levels activates the cGMP-dependent protein kinase or Protein Kinase G (PKG) (Figure 3). PKG is a serine/threonine-specific protein kinase that phosphorylates a large number of biologically important targets, frequently resulting in changes into activity or function, subcellular localization or regulatory features. The proteins that are modified by PKG are involved in the regulation of several processes such as calcium homeostasis, calcium sensitivity, platelet activation and adhesion, smooth muscle contraction, cardiac function, gene expression, feedback of the NO-signaling pathway, inflammatory cascades, pain signaling and other processes. Numerous cyclic nucleotide phosphodiesterases (PDE) can degrade cGMP by hydrolyzing cGMP into 5'-GMP. PDE 5, -6 and -9 are cGMP-specific while PDE1, -2, -3, -10 and -11 can hydrolyse both cAMP and cGMP. Therefore, these enzymes are essential regulators of the downstream signaling of cGMP (Francis, et al., 2010).

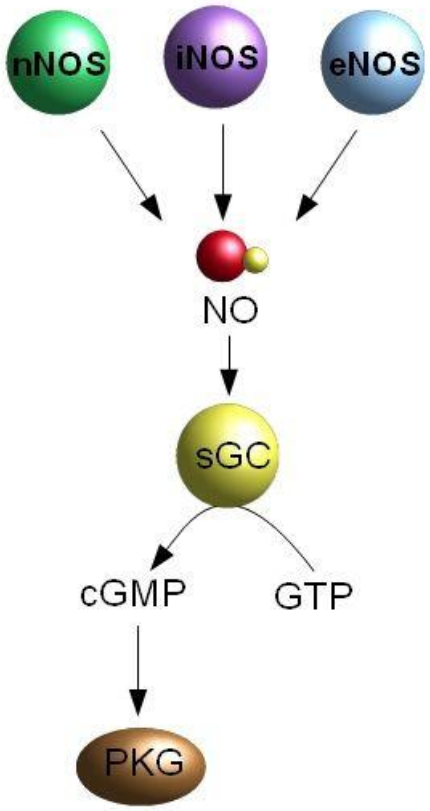

Figure 3. Schematic NO signaling pathway

3.2.1. Nitric oxide in pain

Pharmacologic, electrophysiologic, and immunohistochemical studies revealed a significant role of NO in nociception processing. Recent studies have indicated that NO may modulate spinal and sensory neuron excitability through multiple mechanisms that may underlie its distinctive roles in different pain states. Differential regulation of the different isoenzymes of NOS, contributes mainly to the complexity underlying the role of NO in nociception (Luo and Cizkova, 2000).

NO modulates pain generation and transmission throughout the central and peripheral nervous systems (including brain and spinal cord as well as perivascular tissue and peripheral nerve terminals) and locally released pain mediators responsible for the

initiation of inflammation and formation of vascular edema, although the precise mechanisms remain still unclear. NO signaling is also involved in the analgesic activity of many drugs such as: nonsteroidal anti-inflammatory drugs, opioids, cannabinoids or local anesthetics (Janicki and Jeske-Janicka, 1998).

Indeed, several studies revealed that NO synthesized by NOS1 or NOS2 mediates numerous neuropathic pain symptoms through the activation of the sGC-cGMP-PKG pathway (Meller, et al., 1992). Thus, the expression of NOS1 and NOS2 increases in the spinal cord and dorsal root ganglia after nerve injury (Levy, et al., 1999; De Alba, et al., 2006). Moreover, the systemic or spinal administration of selective NOS or sGC-PKG pathway inhibitors could reverse the hypersensitivity to painful stimulus induced by the spinal or sciatic nerve injury (De Alba, et al., 2006; LaBuda, et al., 2006; Guan, et al., 2007; Tanabe, et al., 2009).

3.3. Carbon monoxide

Carbon monoxide (CO), is a colorless, odorless, and tasteless gas, slightly lighter than air and commonly known by its toxicity when encountered in higher concentrations, however it is also produced in normal animal metabolism, acting as one of the small gaseous molecules that naturally modulate cellular and tissue functions (Li, et al., 2009).

CO is known by its highly toxicity. This toxicity is caused by the ability of the CO to combine with the hemoglobin to produce carboxyhemoglobin, which is ineffective for delivering oxygen to the tissues. Concentrations as low as 667 ppm are enough to inactivate up to 50% of the body's hemoglobin, levels enough for causing seizure, coma, and even death (Tikuisis, et al., 1992).

However, CO is produced endogenously by the organisms as a signaling molecule. CO arises in

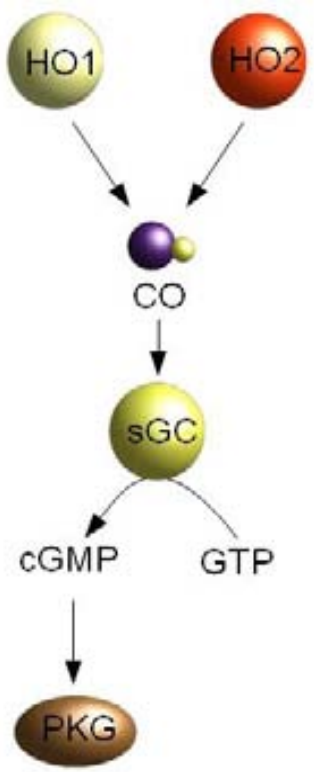

Figure 4. Schematic CO signaling pathway

biological systems during the oxidative catabolism of heme by the heme oxygenase (HO) enzymes. HO exists as constitutive (HO2, HO3) and inducible (HO1) isoforms, responding to regulation by multiple stress-stimuli. HO1 confers protection in vitro and in vivo against oxidative cellular stress. The main effects of CO as a neurotransmitter are performed by the same signaling pathway than NO. Accordingly, CO effects depend on the activation of the guanylate cyclase activity by direct binding of CO to the heme moiety of the enzyme, stimulating the production of intracellular cGMP, which, as a result, activate PKG and its downstream targets (Figure 4) (Ryter, et al., 2002).

3.3.1. Carbon monoxide in pain

CO synthesized by HO1 and HO2, is a gaseous neurotransmitter also implicated in the modulation of nociceptive pathways. However, while HO2 seems to exert a pronociceptive effect during neuropathic pain (Fan, et al., 2011), HO1 plays an important role in the modulation of acute inflammatory pain (Steiner, et al., 2001; Rosa, et al., 2008). Consequently, the expression of HO2 increases after nerve injury, and the mechanical and thermal hypersensitivity to pain induced by nerve injury has been shown to be markedly decreased in HO2 knock-out mice (Li and Clark, 2000; Li and Clark, 2003). In contrast, the overexpression of HO1 is associated with potent anti-inflammatory and antinociceptive effects during inflammatory pain (Rosa, et al., 2008 ; Fan, et al., 2011). Moreover, CO-releasing molecules (CORMs) are a new class of chemical agents able to reproduce several biological effects of HO1-derived CO (Motterlini, et al., 2002; Clark, et al., 2003; Sawle, et al., 2005; Motterlini and Otterbein, 2010) and several authors have shown that the administration of CORMs or cobalt protoporphyrin IX (CoPP), an HO1 overexpression inducer, exerts potent anti-inflammatory effects in vivo (Guillen, et al., 2008; Fan, et al., 2011).

3.4. Opioids and cannabinoids

Opioids and cannabinoids play a central role in nociception and pain signaling. Endogenous opioids and endocannabinoids provide an anti-nociceptive tone and

regulate the experience of pain (Marvizon, et al., 2010). Moreover, exogenous opioids have become the most widespread used analgesics, whereas cannabinoid substances seem to arise as s new therapeutical approach in pain management. Opioids have been used in the treatment of pain for thousands of years with some dating the first use of opioid poppy extracts for analgesia back to 3000 BC (Hutchinson, et al., 2007). Regardless of such widespread use, current opioid therapeutics can result in unfavorable side effects and are not effective in all types of pain. Thus, novel approaches are needed aiming to optimize the opioid therapy for pain treatment (Christrup, et al., 2009). Interestingly, since 2003 a cannabis-based medicinal extract product was approved to be developed as a drug for multiple sclerosis, which alleviates neuropathic pain, spasticity, overactive bladder, and other symptoms (Wade, et al., 2004).

3.4.1. Opioid receptors

Opioid receptors belong to the superfamily of G protein coupled receptors (GPCRs). Like all GPCRs the opioid receptors contain seven hydrophobic transmembrane domains interconnected by short loops and display an extracellular N-terminal domain and an intracellular C-terminal tail. The opioid receptor family consists of four receptors: μ (MOR), δ (DOR), κ (KOR) and opioid-like receptor 1 (ORL1). These receptors are highly homologous with their transmembrane domains and intracellular loops (86-100%) (Waldhoer, et al., 2004).

All four receptors are widely and differentially distributed throughout the central and peripheral nervous systems as well as many endocrine and immune cells. Therefore, drugs that modulate their activity induce a variety of physiological and behavioural effects.

Up to 75% of the opioid receptors are found pre-synaptically on the C-fibers terminal of the dorsal horn (lamina I and II) of the spinal cord and are predominantly MOR or DOR. The contribution of MOR, DOR and KOR to the analgesic effects of opioids in the spinal cord are estimated at 70, 24 and 6% respectively at a predominantly (> 70%) at the presynaptic location (Besse, et al., 1990). Therefore, is not surprising that opioid receptors, particularly MOR, mediate analgesia in the spinal cord. This is also reflected

by the fact that the most potent opioids are MOR ligands. The main mechanisms of spinal opioid analgesia, whether it be endogenously or exogenously mediated, are produced by the activation of presynaptic opioid receptors.

Opioid receptors are also located in the serotoninergic and noradrenergic cores of the brain stem and midbrain including the raphe nuclei, RVM, PAG and the locus coeruleus among others (Przewlocki and Przewlocka, 2001).

3.4.1.1. Opioid receptor ligands

3.4.1.1.1. Endogenous opioid peptides

The opioid receptors have their own signalling messengers in the organism, they are activated by endogenous opioid peptides, of which there are close to 30, all providing from the same 3 precursors proteins, that after post-translational proteolytic processing at specific sites, will generate peptides with opiate-like activity from these large proteins, giving them their different affinity for the different subtypes of opioid receptors. The endogenous opioid peptides are classified according to their affinity to specific receptor: endorphins (MOR), enkephalins (DOR), dynorphins (KOR) and nociceptins (ORL1) (Weber, et al., 1983).

3.4.1.1.2. Exogenous opioids

Exogenous opioids consist of a large group of chemical compounds that share their ability to bind opioid receptors, but diverge in many physical and pharmacological properties, such as opioid receptor specificity, affinity, chemical structure (peptidic or non-peptidic), metabolic stability, hydrophobicity, compound efficacy as agonists, antagonists or inverse agonists as well as possible interactions with non-opioid receptors.

There different classes of opioids depending on their origin:

- Natural opiates: alkaloids contained in the resin of the opium poppy, such as morphine or codeine.
- Morphine derivates: chemically altered morphine prodrugs (esters), such as heroin or nicomorphine.
- Semi-synthetic opioids: chemically altered natural opiates, such as oxycodone or buprenorphine.

- Fully synthetic opioids: such as fentanyl or methadone.

There are also other drugs like tramadol or tapentadol that are not opioids, but do have properties as opioid receptors agonists.

3.4.1.2. Mechanisms of action

The main mechanisms by which opioids produce analgesia are through the inhibitory action on peripheral and central neurons, however it is known that there are significant differences in mechanisms of action whether opioids are administered chronically or acutely.

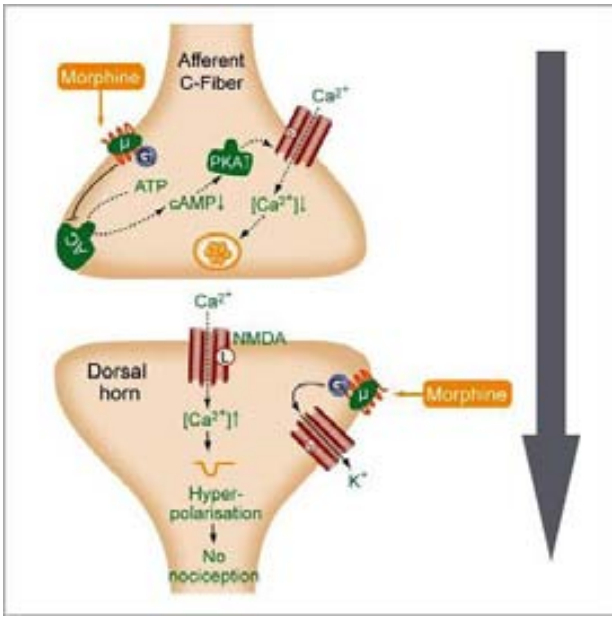

Figure 5. Synaptic scheme of opioid signaling *(from http://http://www.changepain-emodules.com/)*

3.4.1.2.1. Acute administration

Opioid receptors are coupled to inhibitory G-proteins, Gi/o. Upon receptor activation, GDP is transformed to GTP leading to the uncoupling of the α from the βγ subunit of the G protein, this subunit interacts with adenyl cyclase (AC), inhibiting its activity and decreasing the cAMP levels as a result. Moreover, voltage-dependent calcium channels are inhibited whereas inwardly rectifying potassium channels are activated leading to an inhibition of the neurotransmitter release (Figures 5 and 6) (Ikeda, et al., 2002). In primary afferent C fibres the activation of opioid receptors leads to a hyperpolarisation, decreasing the neuron firing which inhibits glutamate and substance P release in the dorsal horn of the spinal cord resulting in a decreased neuronal excitability (Freye and Latasch, 2003). Indeed, intrathecal administration of morphine is able to reduce the levels of substance P and CGRP released after noxious stimulation (Go and Yaksh, 1987). Moreover, the post-synaptic opioid receptors inhibit neuronal firing and hyperpolarise the dendrites of projection neurons, interneurons and disinhibit inhibitory interneurons; as a result there is an inhibition of the C-fibre induced activity. Briefly, spinal activation of opioid receptors produce analgesia by pre-synaptic modulation of

the activity of primary afferent fibres and by post-synaptic inhibition of dorsal horn neurons (Kohno, et al., 1999; Marker, et al., 2005; Zhou, et al., 2008). Opioids also activate the PKC and MAPK cascades which also affect cytoplasmic events and the transcriptional activity of cells (Williams, et al., 2001).

Supraspinal mechanisms for analgesia are still poorly understood but seem to be basically related to a disinhibition of inhibitory interneurons, such as GABA-neurons. Once activated, supraspinal receptors modulate the activity of the descending pathways to the spinal cord, consequently contributing to analgesia. Morphine administration in some regions of the brain or the brainstem, produces analgesia through activation of the inhibitory descending control pathways (Fields, 2000). Opioids also have actions at other levels such as the thalamus, the amygdala or the sensory cortex which are relevant to the conscious and emotional effects of these drugs (Dickenson and Suzuki, 2005).

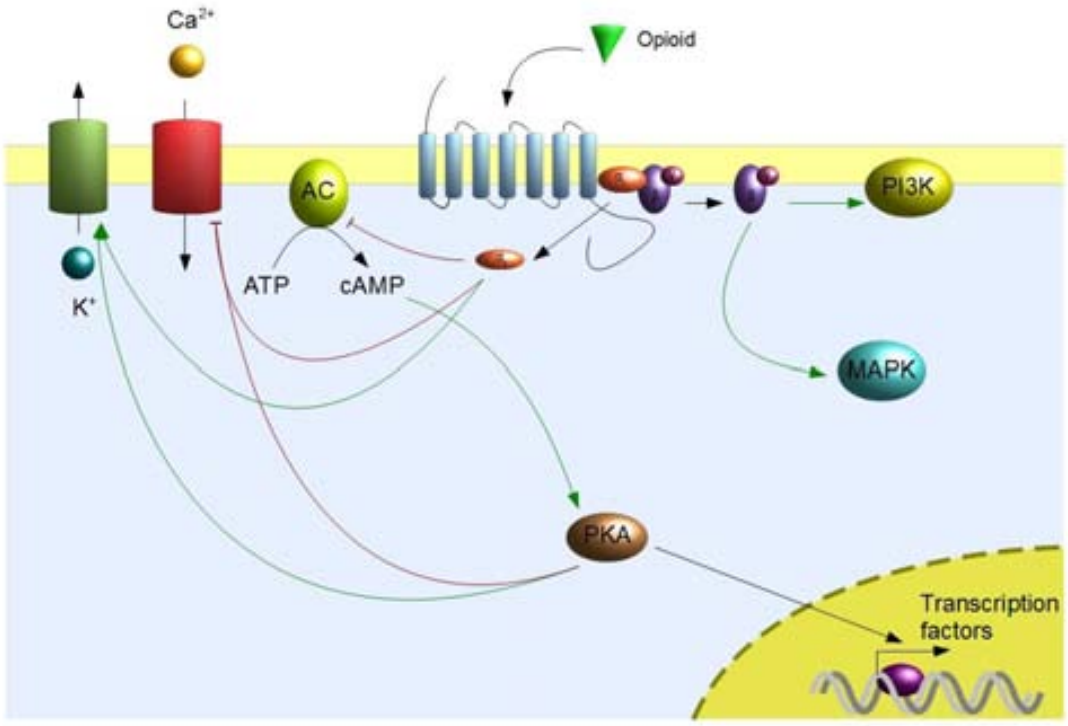

Figure 6. Schematic summary of opioid receptor downstream cell signaling

In the last years, new approaches to pain control and novel strategies to develop drugs for the treatment of pain have been developed. Indeed, novel opioid ligands acting exclusively in the periphery, therefore avoiding central side effects, are being developed and tested in clinical trials. A common approach is the use of hydrophilic

compounds in order to reduce their ability in crossing the blood–brain barrier. Interestingly, several studies point out that great part of the analgesic effects produced by systemically administered opioids should be mediated by peripheral opioid receptors (Stein and Lang, 2009). Moreover, human studies have shown that peripherally acting opioid receptor agonists can have the same analgesic efficacy as conventional opioids in patients with neuropathic or visceral pain, but the main mechanisms by which peripheral opioids exert their effects are still unknown (Wallace, et al., 2006; Mangel, et al., 2008; van Dorp, et al., 2008).

3.4.1.2.2. Chronic administration

Although opioids still stay as the analgesics of choice in the clinical practice for the treatment of chronic pain, an essential drawback to their chronic administration is the development of tolerance, being one of the most unwanted effects of opioid therapy. Tolerance is defined by a significant decrease in analgesia after repeated administration of an opioid drug, requiring a higher dosage in order to maintain the analgesic effect (Foley, 1995). As the dosage is increased, so there are the side effects, such as respiratory depression or constipation. Therefore, to understand the mechanism of opioid tolerance is essential to be able to avoid or at least attenuate its development.

It is interesting to point out, that the development of opioid tolerance in humans could vary depending on the receptor activated, the agonist, the route of administration or the disease for which opioids are prescribed.

It seems to be well accepted that, repeated opioid receptor activation leads to the receptor phosphorylation by GPCR kinases followed by the binding of β-arrestins, which uncouples the opioid receptor from the G-protein, desensitizing the receptor, and targets the receptors to clathrin-coated vesicles, to their posterior internalization (Chakrabarti, et al., 1997; Appleyard, et al., 1999). Upon the internalisation of the receptor, it can go to the membrane leading to resensitisation, or be targeted for degradation, leading to receptor down-regulation (Littleton, 2001). These difference in the signalling may be caused depending on the agonist used, for example DAMGO, which induces receptor internalization and elicits less tolerance compared to morphine

which does not induce receptor internalisation (Chakrabarti, et al., 1997; Chu, et al., 2010).

Other studies point out that β-arrestin have functions also as a scaffolding molecule essential for the membrane recruitment of many other signalling molecules, such as MAPK, c-jun N-terminal kinase (JNK) (Ma and Pei, 2007) suggesting that β-arrestin could has a role in tolerance independent from receptor internalisation.

Moreover, other studies showed that rats undergoing prolonged treatment with morphine peripherically administered do not develop tolerance in the presence of painful paw inflammation. In the DRG neurons of these animals the internalization of MOR was significantly increased, and G-protein coupling to MOR as well as inhibition of cAMP accumulation were preserved. However, opioid receptor internalization and signalling were reduced and tolerance was restored when endogenous opioid peptides in inflamed tissue were removed with antibodies (Stein and Lang, 2009). However, more studies need to be done under neuropathic pain conditions.

3.4.2. Cannabinoid receptors

Cannabinoid receptors were named because of their affinity for the agonist Δ9-tetrahydrocannabinol (Δ9-THC), a ligand found in organic extracts from *Cannabis sativa*. The cannabinoid receptors are a class of GPCRs. As is typical from GPCRs, cannabinoid receptors contain seven hydrophobic transmembrane domains interconnected by short loops as well as an extracellular N-terminal domain and an intracellular C-terminal tail (Howlett, 2002). At present, there are two known types of cannabinoid receptors, termed as cannabinoid receptor type 1 (CB1) and cannabinoid receptor type 2 (CB2), however an increasing number of therapeutic actions of cannabinoids are being reported that do not appear to be mediated by either of these cannabinoid receptors. Indeed, studies in CB1, CB2, or CB1/CB2 receptors double knockout mice revealed that non-CB1/CB2 receptor-mediated responses to cannabinoids, both in the central and peripheral nervous systems (Howlett, et al., 2002).

3.4.2.1. Cannabinoid receptor type 1

Although CB1 receptors are known to be found primarily in the brain, recent studies have demonstrated its widespread expression among almost every organ and tissue in the organism (Terry, et al., 2010).

In terms of neurotransmission, the CB1 receptor is often localised in axon terminals, and its activation leads to inhibition of transmitter release. The consequence is inhibition of neurotransmission via a presynaptic mechanism on glutamatergic, GABAergic, glycinergic, cholinergic, noradrenergic and serotonergic neurons in many regions of the central nervous system. Moreover, in the peripheral nervous system, CB1 receptor-mediated inhibition of adrenergic, cholinergic and sensory neuroeffector transmission has been frequently observed (Szabo and Schlicker, 2005).

3.4.2.2. Cannabinoid receptor type 2

Originally, it was thought that CB2 receptor expression patterns were exclusively from the peripheral tissues of immune system, localized on immune cells such as monocytes, macrophages, B-cells, and T-cells. But, recent investigations revealed that CB2 receptor is also expressed in the brain and spinal cord, though not as densely as the CB1 receptor, and unlike the CB1 receptor, CB2 receptors are primarily found on glia (Onaivi, 2006). CB2 receptors are also found throughout the gastrointestinal system, where they modulate intestinal inflammatory response (Wright, et al., 2008). In the peripheral nervous system is where they are more widely expressed, although they are not expressed in nociceptive sensory neurons, but on glial and immune cells mediating cytokine release (Pertwee, 2006).

3.4.2.3. Cannabinoid receptor ligands

3.4.2.3.1. Endocannabinoids

Endocannabinoids are lipophilic molecules produced from within the body that activate cannabinoid receptors. Endocannabinoids serve as intercellular 'lipid messengers', signaling molecules that are released from one cell and activating the cannabinoid receptors present on other nearby cells. Anandamide (AEA), the first

endogenous ligand to be reported at the end of 1992, acts as a partial CB1 agonist but only as a weak CB2 receptor agonist (Devane, et al., 1992). Other endocannabinoids, all derived from arachidonic acid, were later identified. First came the finding of 2-arachidonoylglycerol (2-AG), which activates both CB1 and CB2 receptors, and more recently, 2-arachidonyl-glyceryl ether (noladin), a selective CB1 agonist, O-arachidonoyl-ethanolamine (virhodamine), a partial CB2 receptor agonist and a CB1 antagonist, and N-arachidonoyl-dopamine (NADA), a selective CB1 agonist and a potent agonist of vanilloid receptors (TRPVs), were discovered. AEA and 2-AG are synthesized and released only on demand, explicitly when and where necessary, following physiological or pathological stimuli, in a Ca^{2+}-dependent phospholipid remodeling manner. No conclusive data on the biosynthetic mechanisms underlying the formation of noladin, virodhamine and NADA have been reported so far. Besides, the physiological role of the endocannabinoids remain still unclear. However, the endocannabinoids AEA and 2-AG, since their finding, have been implicated in a wide range of physiological and pathological processes (Bisogno, et al., 2005).

3.4.2.3.2. Exogenous cannabinoids

There are different classes of exogenous cannabinoid depending on their origin:

- Phytocannabinoids: lipophillic moleculles concentrated in a viscous resin produced in *Cannabis* and *Echinacea* plants. There are more than 85 different cannabinoids from the *Cannabis* plants, and at least 25 different cannabinoids have been isolated from *Echinacea*s. The best known herbal cannabinoids are Δ9-THC from *Cannabis sp.* and the lipophilic alkamides (alkylamides) from *Echinacea sp.* (Woelkart, et al., 2008).
- Synthetic cannabinoids: laboratory synthetic molecules based on the structure of herbal cannabinoids or endogenous cannabinoids. The laboratory synthesis allows to create specific molecules that bind to one receptor much more specifically over the other, and change its interaction with the receptor, creating specific antagonists or inverse agonists. For example, JWH-015 is a synthetic potent selective CB2 receptor agonist, with around 50x more selectivity for CB2 over CB1 receptors. Other relevant synthetic cannabinoids

are: WIN 55,212-2 (a full agonist of that has much higher affinity than THC), SR-144,528 (acts as a potent and highly selective CB2 receptor inverse agonist), AM251 (an antagonist of CB1 receptor), AM630 (an antagonist of CB2 receptor), among others.

3.4.2.4. Mechanisms of action

3.4.2.4.1. Cannabinoid receptor type 1

CB1 receptors are coupled through Gi/o proteins and inhibit AC with α subunit and activate MAPK with the βγ subunits (Figure 7). In addition, CB1 receptors inhibit presynaptic N- and P/Q-type calcium channels and activate inwardly rectifying potassium channels. As they are presynaptic receptor, calcium entry is necessary for vesicle release, therefore its activation will lead to a decrease in the neurotransmitter release (Figure 8) (Howlett, et al., 2002) .

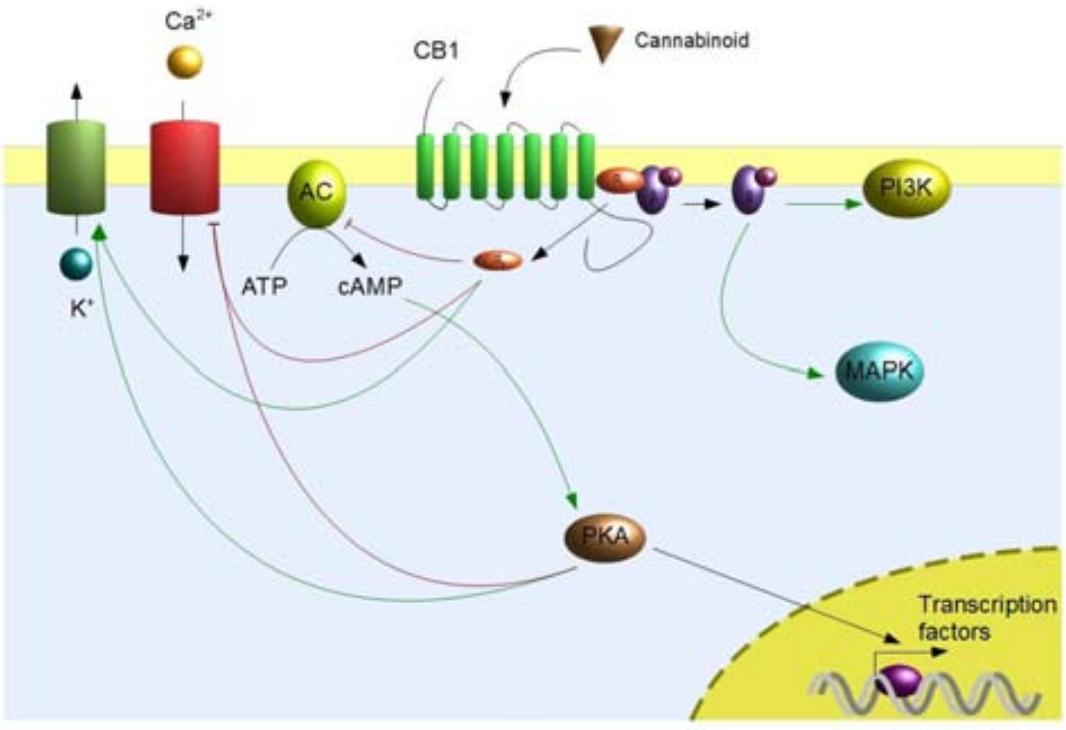

Figure 7. Schematic summary of CB1 receptor downstream cell signaling

3.4.2.4.2. Cannabinoid receptor type 2

Like the CB1 receptors, CB2 receptors inhibit the activity of AC through their Gi/o α subunits, and activate MAPK and PI3K pathways through their βγ subunits, which results in changes in cell migration as well as in several transcriptional signaling changes.. The demonstrated suppression immune response by a decrease in the cytokine release is mainly mediated through inhibition of AC in immune and glial cells (Figure 9) (Howlett, et al., 2002).

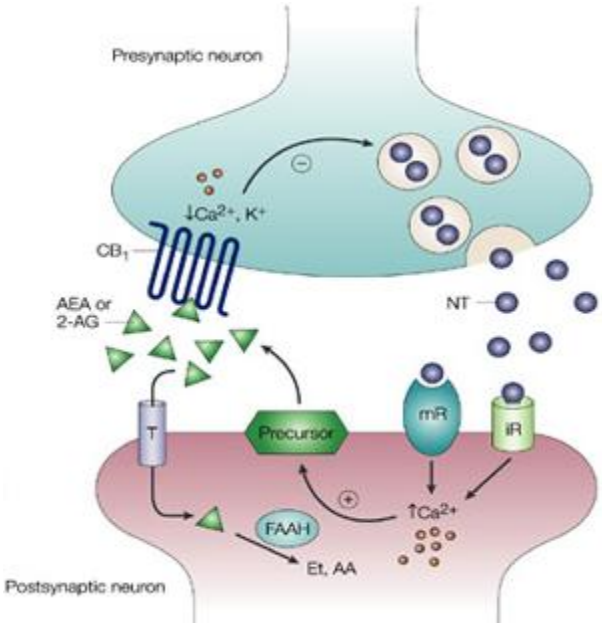

Figure 8. Synaptic scheme of CB1 signaling

(Modified from (Guzman, 2003)

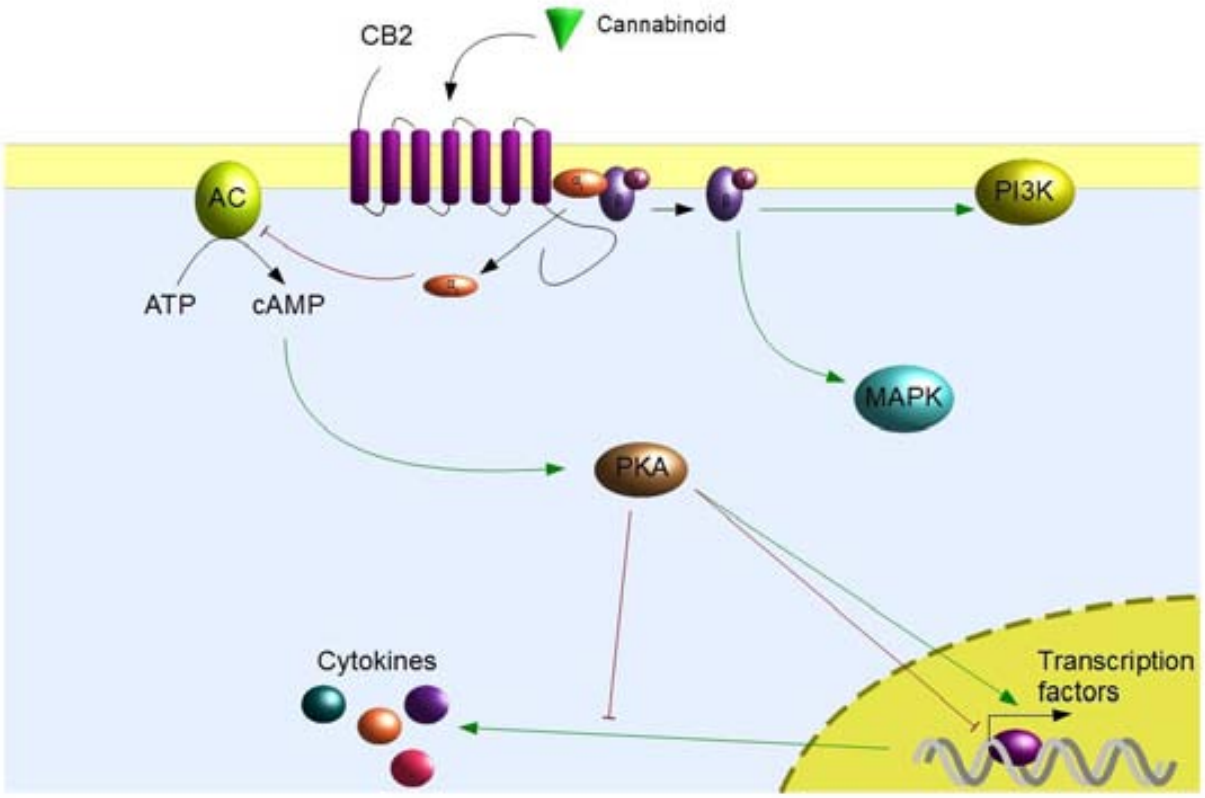

Figure 9. Schematic summary of CB2 receptor downstream cell signaling

4. Objectives

In a neuropathic pain model induced by the chronic constriction of sciatic nerve in mice, the main aims of this thesis are to evaluate:

- The role played by the NO-sGC-PKG signaling pathway, triggered by NOS1 and NOS2, on the expression of neuropathic pain as well as in the antinociceptive effects and expression of MOR, DOR and CB2 receptors after sciatic nerve injury

- The involvement of the NO-sGC-PKG-JNK signaling pathway, triggered by NOS2, on the development of morphine tolerance during neuropathic pain

- The role played by CO, exogenously administered or HO1-derived, in the modulation of neuropathic pain as well as in the antinociceptive effects and expression of MOR, DOR and CB2 receptors in these experimental conditions

5. Manuscripts

5.1. *The spinal cord expression of neuronal and inducible nitric oxide synthases and their contribution in the maintenance of neuropathic pain in mice.*

Hervera A, Negrete R, Leánez S, Martín-Campos JM, Pol O.

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The Spinal Cord Expression of Neuronal and Inducible Nitric Oxide Synthases and Their Contribution in the Maintenance of Neuropathic Pain in Mice

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Abstract

Background: Nitric oxide generated by neuronal (NOS1), inducible (NOS2) or endothelial (NOS3) nitric oxide synthases contributes to pain processing, but the exact role of NOS1 and NOS2 in the maintenance of chronic peripheral neuropathic pain as well as the possible compensatory changes in their expression in the spinal cord of wild type (WT) and NOS knockout (KO) mice at 21 days after total sciatic nerve ligation remains unknown.

Methodology/Principal Findings: The mechanical and thermal allodynia as well as thermal hyperalgesia induced by sciatic nerve injury was evaluated in WT, NOS1-KO and NOS2-KO mice from 1 to 21 days after surgery. The mRNA and protein levels of NOS1, NOS2 and NOS3 in the spinal cord of WT and KO mice, at 21 days after surgery, were also assessed. Sciatic nerve injury led to a neuropathic syndrome in WT mice, in contrast to the abolished mechanical allodynia and thermal hyperalgesia as well as the decreased or suppressed thermal allodynia observed in NOS1-KO and NOS2-KO animals, respectively. Sciatic nerve injury also increases the spinal cord expression of NOS1 and NOS2 isoforms, but not of NOS3, in WT and NOS1-KO mice respectively. Moreover, the presence of NOS2 is required to increase the spinal cord expression of NOS1 whereas an increased NOS1 expression might avoid the up-regulation of NOS2 in the spinal cord of nerve injured WT mice.

Conclusions/Significance: These data suggest that the increased spinal cord expression of NOS1, regulated by NOS2, might be responsible for the maintenance of chronic peripheral neuropathic pain in mice and propose these enzymes as interesting therapeutic targets for their treatment.

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Introduction

Neuropathic pain is a clinical manifestation characterized by the presence of exaggerated response to painful stimuli (hyperalgesia), pain response to normally innocuous stimuli (allodynia), and spontaneous pain. It is well accepted that nitric oxide synthesized by three nitric oxide synthases (neuronal, NOS1; inducible, NOS2 and endothelial, NOS3) regulates several cellular processes, such as pathological pain [1–2]. Therefore, several works using pharmacological and genetic approaches have demonstrated that selective NOS1 and NOS2 inhibitors might reverse the mechanical hypersensitivity to pain induced by spinal and peripheral neuropathy [3–5]. However and besides that these studies suggest a potential role of nitric oxide in the modulation of nerve injury-induced mechanical hypersensitivity, the exact contribution of NOS1 and NOS2 in the modulation of the thermal hyperalgesia and thermal allodynia induced by sciatic nerve injury remains unknown. Thus, the first aim of our study is to compare the pain-related behavior induced by the chronic constriction of the sciatic nerve in NOS1-KO, NOS2-KO and WT mice from days 1 to 21 after nerve injury.

Several works have demonstrated that nerve injury after sciatic nerve ligation evoked an increased NOS1 and NOS2, but not of NOS3, protein expression in the ipsilateral site of the dorsal root ganglia and the sciatic nerve [4,6–11]. In contrast, the possible changes induced by peripheral neuropathic pain in the spinal cord expression of these genes remain controversial. That is, from no changes to an increased NOS protein expression in the spinal cord of sciatic nerve-injured animals at days 7^{th} to 26^{th} after surgery have been reported [4,7–8,11]. Therefore, our subsequent aim is to evaluate the mRNA and protein expression of NOS1 and NOS2 isoenzymes in the spinal cord of WT mice at 21 days after sciatic nerve ligation and correlate it with their corresponding behavior responses.

It is well known that the expression of other NOS isoforms is up-regulated in a compensatory manner in the spinal cord of NOS1 and NOS2 knockout mice under basal and inflammatory pain conditions [12–14]. However, the possible changes in the

expression of NOS1, NOS2 and NOS3 isoforms that might occurs in the spinal cord of NOS1 and NOS2 knockout mice after total sciatic nerve ligation remains unknown. Thus, the mRNA and protein levels of these three isoenzymes (NOS1, NOS2 and NOS3) in the spinal cord of NOS2-KO and NOS1-KO mice at day 21 after sciatic nerve injury were also evaluated.

Results

Effect of NOS1 and NOS2 deletion on the development of neuropathic pain in mice

Mechanical allodynia. Sciatic nerve ligature led to a significant decrease of the threshold for evoking paw withdrawal to a mechanical stimulus only in WT mice and this response was abolished in mice lacking NOS1 or NOS2 (Fig. 1A). Baseline values were similar in all genotypes. Sham operation did not produce any modification of the nociceptive responses in WT, NOS1-KO or in NOS2-KO mice for the whole duration of the experiment.

For NOS1-KO mice, the two-way ANOVA revealed a significant effect of the genotype ($p<0.002$) at days 4, 10, 14 and 21 after surgery, a significant effect of surgery ($p<0.008$) on days 1, 4, 7, 14 and 21 and a significant interaction between genotype and surgery from day 1 to day 21 ($p<0.030$) after surgery in the ipsilateral paw of NOS1-KO mice as compared to WT group.

In WT mice, nerve injury led to a significant decrease of the threshold for evoking hind paw withdrawal to a mechanical stimulus on the injured side. This mechanical allodynia appeared on the first measurement after sciatic nerve ligation (day 1) and persisted for the whole duration of the experiment. Indeed, a significant effect of sciatic nerve ligation was revealed on day 1 ($p<0.001$), day 4 ($p<0.001$), day 7 ($p<0.039$), day 10 ($p<0.017$), day 14 ($p<0.007$) and day 21 ($p<0.001$) after surgery (one-way ANOVA vs. WT sham-operated animals). In contrast, a significant decrease of mechanical allodynia was observed in NOS1-KO mice on day 1 ($p<0.008$), day 4 ($p<0.001$), day 7 ($p<0.001$), day 10 ($p<0.001$), day 14 ($p<0.001$) and day 21 ($p<0.001$) when compared to the WT group (one-way ANOVA).

The possible development of mechanical allodynia in NOS2-KO mice after sciatic nerve injury has been also evaluated. The two-way ANOVA revealed a significant effect of the genotype ($p<0.001$) at days 4, 7, 10, 14 and 21 after surgery, a significant effect of the surgery ($p<0.023$) on days 1, 10 and 21 and a significant interaction between genotype and surgery from day 1 to day 21 ($p<0.008$) after surgery in the ipsilateral paw of NOS2-KO mice as compared to WT group. Indeed, a significant decrease of mechanical allodynia was also observed in NOS2-KO mice on day 1 ($p<0.008$), day 4 ($p<0.001$), day 7 ($p<0.001$), day 10 ($p<0.001$), day 14 ($p<0.001$) and day 21 ($p<0.001$) when compared to WT group (one-way ANOVA).

Withdrawal latencies of the contralateral paw were not modified in any experimental group (data not shown).

Thermal hyperalgesia. Sciatic nerve ligature decreased paw withdrawal latency to thermal stimulus only in WT mice and this response was abolished in mice lacking NOS1 or NOS2 genes (Fig. 1B). Baseline values were similar in all genotypes. Sham operation did not produce any modification of nociceptive responses in WT, NOS1-KO or in NOS2-KO mice for the whole duration of the experiment.

Regarding NOS1-KO mice, the two-way ANOVA revealed a significant effect of the genotype ($p<0.001$), surgery ($p<0.001$) and their interaction ($p<0.005$) from day 1 to day 21 after surgery in the ipsilateral paw of NOS1-KO mice as compared to the WT

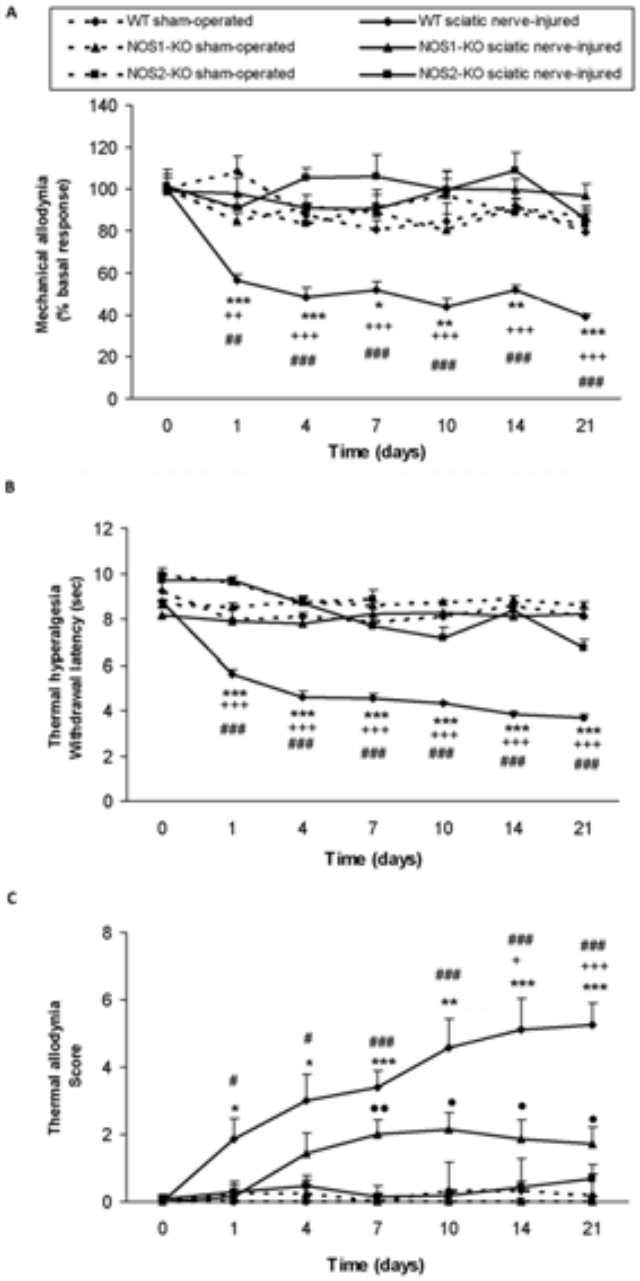

Figure 1. Effect of NOS1 and NOS2 deletion on sciatic nerve injury induced mechanical allodynia, thermal hyperalgesia and thermal allodynia in mice. Development of mechanical allodynia (**A**), thermal hyperalgesia (**B**) and thermal allodynia (**C**) in the ipsilateral paw of WT, NOS1-KO and NOS2-KO mice at 0, 1, 4, 7, 10, 14 and 21 days after sciatic nerve ligation. For each test and time tested, * indicates significant differences when compared sciatic nerve-injured WT mice vs. sham-operated WT mice (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, one-way ANOVA followed by Scheffe test), + when compared sciatic nerve-injured NOS1-KO mice vs. sciatic nerve-injured WT mice (+ $p<0.05$, ++ $p<0.01$, +++ $p<0.001$, one-way ANOVA followed by Scheffe test) and # when compared sciatic nerve-injured NOS2-KO mice vs. sciatic nerve-injured WT mice (# $p<0.05$, ## $p<0.01$, ### $p<0.001$, one-way ANOVA followed by Scheffe test). In the cold plate test (C), • indicates significant differences when compared sciatic nerve-injured NOS1-KO mice vs. sham-operated NOS1-KO mice (• $p<0.05$, •• $p<0.01$, one-way ANOVA followed by Scheffe test). Results are shown as mean values ± SEM; n = 10–12 animals per experimental group.
doi:10.1371/journal.pone.0014321.g001

group. Thus, a marked and long-lasting decrease of the paw withdrawal latencies was observed in the ipsilateral paw of WT mice exposed to sciatic nerve injury from day 1 to day 21 ($p<0.001$) after surgery (one-way ANOVA vs. WT sham-operated animals). In contrast, a significant decrease of thermal hyperalgesia was observed in sciatic nerve-injured NOS1-KO mice from day 1 to day 21 ($p<0.001$) when compared to the WT group (one-way ANOVA).

The possible development of thermal hyperalgesia in NOS2-KO mice after sciatic nerve injury has been also evaluated. The two-way ANOVA also revealed a significant effect of the genotype ($p<0.001$), surgery ($p<0.001$) and their interaction ($p<0.007$) from day 1 to day 21 after surgery in the ipsilateral paw of NOS2-KO mice as compared to WT group. Indeed, a significant decrease of thermal hyperalgesia was observed in sciatic nerve-injured NOS2-KO mice from day 1 to day 21 ($p<0.001$) when compared to the WT group (one-way ANOVA).

Withdrawal latencies of the contralateral paw were not modified in any of the experimental groups (data not shown).

Thermal allodynia. Sciatic nerve ligature enhanced the score values during the cold thermal stimulation in WT mice and this response was significantly attenuated or abolished in NOS1-KO and NOS2-KO mice (Fig. 1C), respectively. Baseline score values were similar in all genotypes. Sham operation did not produce any modification of thermal nociceptive responses neither in WT nor in KO mice for the whole duration of the experiment.

For NOS1-KO mice, the two-way ANOVA only revealed a significant effect of the genotype ($p<0.029$) at days 14 and 21 after surgery, a significant effect of the surgery ($p<0.006$) on days 4, 7, 10, 14 and 21 and a significant interaction between theme at days 1 and 21 ($p<0.036$) after surgery in the ipsilateral paw of NOS1-KO mice as compared to the WT group. Thus, WT mice exposed to sciatic nerve injury significantly increased the score values after surgery, revealing the development of thermal allodynia on day 1 ($p<0.046$), day 4 ($p<0.028$), day 7 ($p<0.001$), day 10 ($p<0.004$), day 14 ($p<0.001$) and day 21 ($p<0.001$) after surgery (one-way ANOVA vs. WT sham-operated animals). In NOS1-KO mice exposed to sciatic nerve injury only showed significant thermal allodynia on day 7 ($p<0.008$), day 10 ($p<0.017$), day 14 ($p<0.050$) and day 21 ($p<0.011$) after surgery (one-way ANOVA vs. sham operated animals), although a significant decrease of this thermal allodynia was observed when compared sciatic nerve-injured NOS1-KO vs. WT mice on days 14 ($p<0.017$) and 21 ($p<0.001$) after surgery (one-way ANOVA).

The possible development of thermal allodynia in NOS2-KO mice after sciatic nerve injury has been also evaluated. The two-way ANOVA revealed a significant effect of the genotype ($p<0.011$) from days 1 to 21 after surgery, a significant effect of surgery ($p<0.001$) on days 7, 10, 14 and 21 and a significant interaction between genotype and surgery from day 7 to day 21 ($p<0.001$) after surgery in the ipsilateral paw of NOS2-KO mice as compared to the WT group. Thus, thermal allodynia was not developed in NOS2-KO mice. Indeed, a significant decrease of thermal allodynia was observed when compared sciatic nerve-injured NOS2-KO mice on day 1 ($p<0.049$), day 4 ($p<0.033$), day 7 ($p<0.001$), day 10 ($p<0.001$), day 14 ($p<0.001$) and day 21 ($p<0.001$) with WT mice (one-way ANOVA).

Expression of NOS1 in the spinal cord of WT and NOS2-KO mice

The relative mRNA levels of NOS1 gene in the spinal cord from sham-operated and sciatic nerve-injured WT and NOS2-KO mice are shown in Fig. 2A. Our results showed that although the two way ANOVA did not show a significant effect of the genotype or surgery, a significant interaction between theme was demonstrated ($p<0.005$). Thus, sciatic nerve ligation significantly increased the NOS1 gene expression in WT but not in NOS2-KO mice, when comparing sham-operated vs. sciatic nerve-injured animals (one way ANOVA, $p<0.016$). Our results did not show any significant differences between genotypes as comparing the expression of NOS1 mRNA among theme in sham-operated mice.

The protein expression of NOS1 in the spinal cord from sham-operated and sciatic nerve-injured WT and NOS2-KO mice are

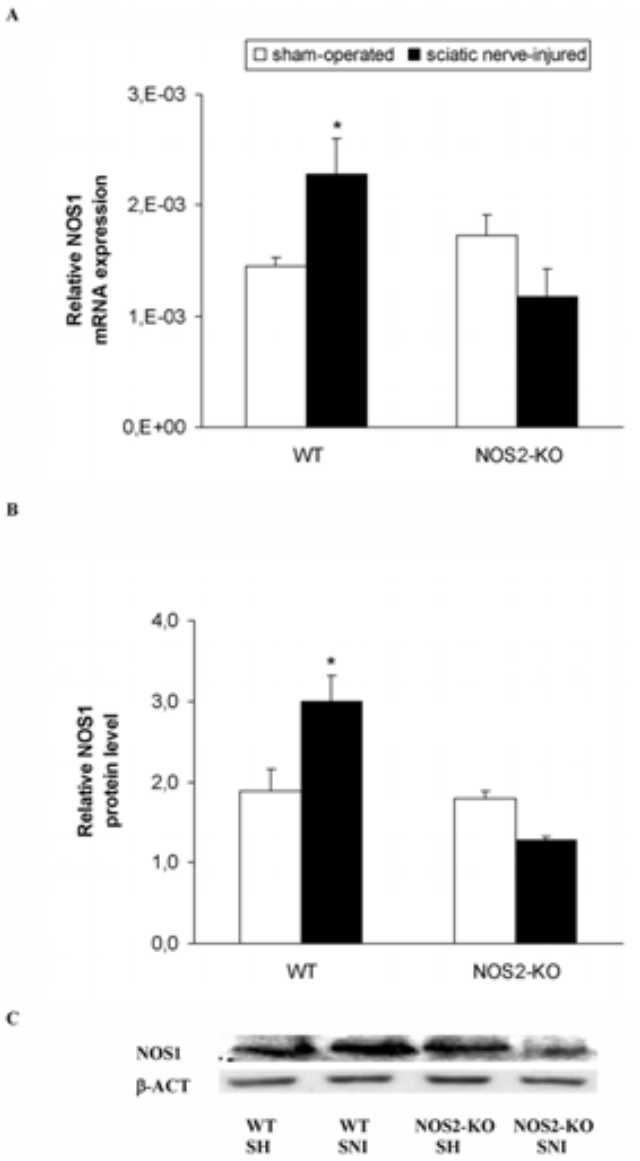

Figure 2. Spinal cord expression of NOS1 in WT and NOS2-KO mice. The relative NOS1 mRNA (**A**) and protein (**B**) expression in the ipsilateral site of the lumbar section of the spinal cord from sham-operated (SH) and sciatic nerve-injured (SNI) WT and NOS2-KO mice at 21 days after sciatic nerve ligation are represented. In both figures, * indicates significant differences when compared sciatic nerve-injured WT mice vs. sham-operated WT animals (* $p<0.05$, one-way ANOVA followed by Student-Newman-Keuls test). A representative example of Western blots for NOS1 protein (155 kDa) in which β-actin (43 kDa) was used as a loading control is shown in **C**. Data are expressed as mean values ± SEM; n = 4–5 samples per group.
doi:10.1371/journal.pone.0014321.g002

shown in Fig. 2B. The two way ANOVA revealed a significant effect of the genotype ($p<0.001$) and a significant interaction between genotype and surgery ($p<0.003$). Thus and according to that occurs with the mRNA levels, while sciatic nerve ligation significantly increases the NOS1 protein levels in WT mice it did not change their expression in NOS2-KO animals, when comparing sham-operated vs. sciatic nerve-injured animals (one way ANOVA, $p<0.001$). Our results did not show any significant differences between genotypes when comparing the expression of NOS1 protein among theme in sham-operated mice.

The mRNA and protein levels of NOS1 gene in the spinal cord from sham-operated and sciatic nerve-injured NOS1-KO were undetectable (data not shown).

Expression of NOS2 in the spinal cord of WT and NOS1-KO mice

The relative mRNA levels of NOS2 gene in the spinal cord from sham-operated and sciatic nerve-injured WT and NOS1-KO mice are shown in Fig. 3A. For NOS2 although the two way ANOVA did not show any effect of the genotype or surgery, a significant interaction between theme was demonstrated ($p<0.011$). Indeed, sciatic nerve ligation did not alter the NOS2 expression in the spinal cord of WT mice, but significantly increased their expression in NOS1-KO mice, when comparing sham-operated vs. sciatic nerve-injured animals (one way ANOVA, $p<0.021$). Non significant differences were found between genotypes as compared the expression of NOS2 mRNA among theme in sham-operated mice.

The protein expression of NOS2 in the spinal cord from sham-operated and sciatic nerve-injured WT and NOS1-KO mice are shown in Fig. 3B. The two way ANOVA did not show any effect of the genotype or surgery but a significant interaction between theme was demonstrated ($p<0.019$). Indeed, sciatic nerve injury did not alter the NOS2 protein levels in the spinal cord of WT mice but significantly increased their expression in NOS1-KO mice when comparing sham-operated vs. sciatic nerve-injured animals (one way ANOVA, $p<0.028$). Non significant differences were found between genotypes when compared the expression of NOS2 protein among theme in sham-operated mice.

The mRNA and protein levels of NOS2 gene in the spinal cord from sham-operated and sciatic nerve-injured NOS2-KO were undetectable (data not shown).

Expression of NOS3 in the spinal cord of WT, NOS2-KO and NOS1-KO mice

The relative mRNA levels of NOS3 gene in the spinal cord from sham-operated and sciatic nerve-injured WT, NOS2-KO and NOS1-KO mice are shown in Fig. 4A. The two way ANOVA showed a significant effect of the genotype ($p<0.001$) and a significant interaction between genotype and surgery ($p<0.016$). Indeed, our results showed that while the spinal cord expression of NOS3 in sham-operated NOS1-KO mice was significantly higher than those presented in WT and NOS2-KO mice (one way ANOVA, $p<0.005$), non significant differences between genotypes were found at 21 days after nerve injury.

The protein levels of NOS3 in the spinal cord from sham-operated and sciatic nerve-injured WT, NOS2-KO and NOS1-KO mice are shown in Fig. 4B. The two way ANOVA showed a significant effect of the genotype ($p<0.015$) and surgery ($p<0.034$) and according to what occurs with the mRNA expression, while the NOS3 expression in sham-operated NOS1-KO mice was significantly higher to than those observed in sham-operated WT or NOS2-KO mice (one way ANOVA, $p<0.006$), these differences between genotypes disappear after sciatic nerve ligation.

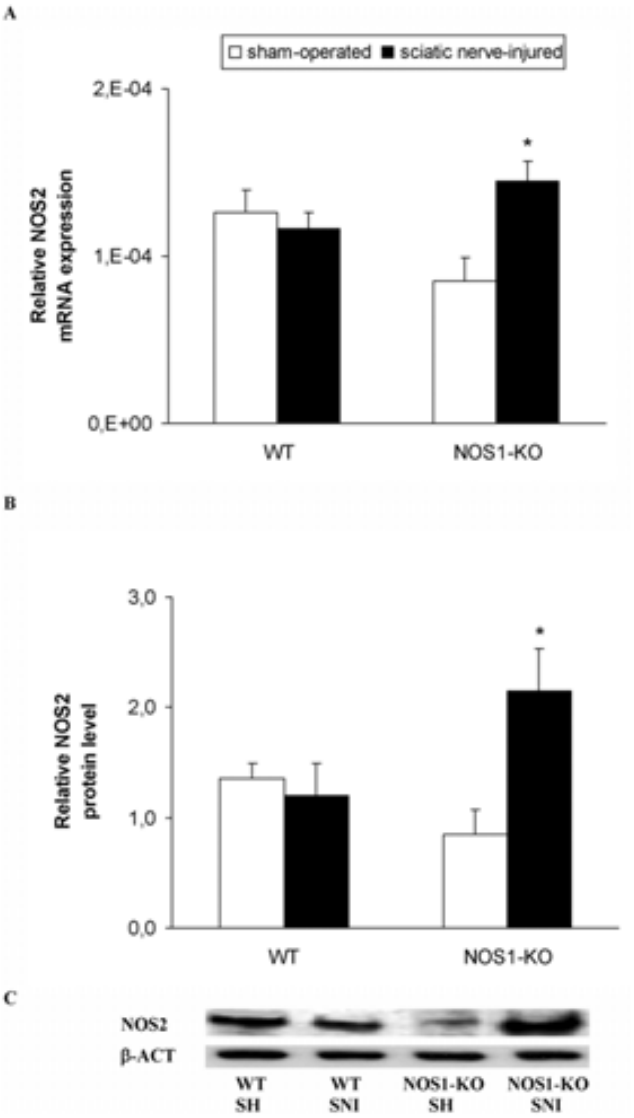

Figure 3. Spinal cord expression of NOS2 in WT and NOS1-KO mice. The relative NOS2 mRNA (**A**) and protein (**B**) expression in the ipsilateral site of the lumbar section of the spinal cord from sham-operated (SH) and sciatic nerve-injured (SNI) WT and NOS1-KO mice at 21 days after sciatic nerve ligation are represented. In both figures, * indicates significant differences when compared sciatic nerve-injured NOS1-KO mice vs. sham-operated NOS1-KO animals (* $p<0.05$, one-way ANOVA followed by Student-Newman-Keuls test). A representative example of Western blots for NOS2 protein (130 kDa) in which β-actin (43 kDa) was used as a loading control is shown in **C**. Data are expressed as mean values ± SEM; n = 4–5 samples per group.
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Discussion

The present study demonstrates for first time the participation of NOS1 and NOS2 in the development and maintenance of thermal hyperalgesia and thermal allodynia induced by the chronic constriction of sciatic nerve and suggests that the increased spinal cord expression of NOS1 is regulated by NOS2, and might be responsible for the maintenance of chronic peripheral neuropathic pain in mice. Our data also indicate that the

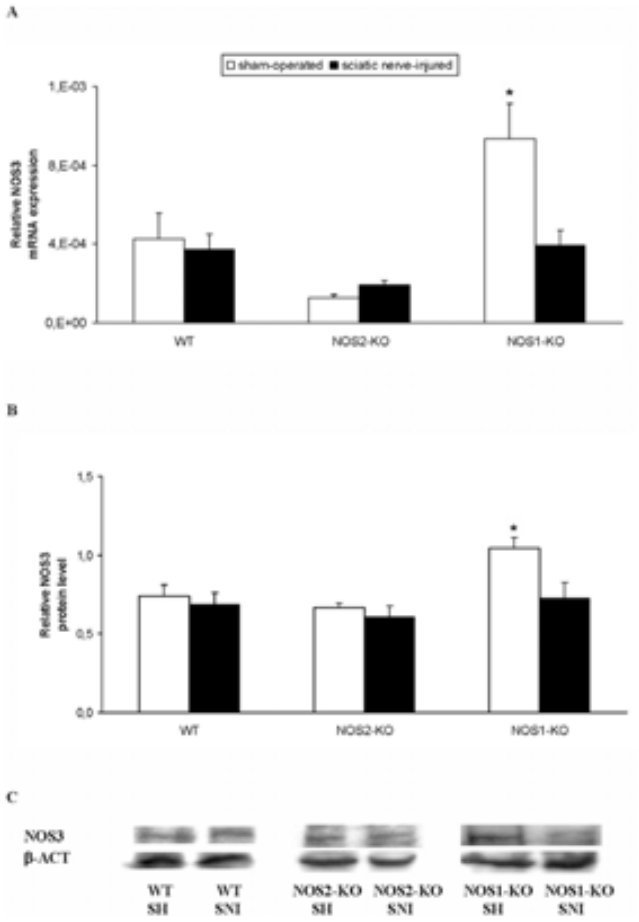

Figure 4. Spinal cord expression of NOS3 in WT, NOS2-KO and NOS1-KO mice. The relative NOS3 mRNA (**A**) and protein (**B**) expression in the ipsilateral site of the lumbar section of the spinal cord from sham-operated (SH) and sciatic nerve-injured (SNI) WT, NOS2-KO and NOS1-KO mice at 21 days after sciatic nerve ligation are represented. In both figures, * indicates significant differences when compared sham-operated NOS1-KO animals vs. the other groups. (* $p<0.05$, one-way ANOVA followed by Student-Newman-Keuls test). A representative example of Western blot for NOS3 protein (140 kDa) in which β-actin (43 kDa) was used as a loading control is shown in **C**. Data are expressed as mean values ± SEM; n = 4–5 samples per group.
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enhanced spinal cord expression of NOS3 in NOS1-KO mice might compensates for the lack of NOS1 under basal conditions but not after sciatic nerve ligation.

Our results showed that the mechanical allodynia and thermal hyperalgesia induced by sciatic nerve injury was completely abolished in both NOS knockout mice, while thermal allodynia was significantly attenuated and completely abolished in NOS1-KO and NOS2-KO animals, respectively. In accordance to our results, the pharmacological inhibition of NOS1 or NOS2 isoenzymes as well as the genetic deletion of NOS1 both attenuate the mechanical hypersensitivity observed after the spinal or sciatic nerve injury in mice [4–5,15]. Despite that, our study extended these findings and provides the first evidence that the targeted disruption of NOS1 or NOS2 genes also abolishes or diminishes the thermal hyperalgesia and thermal allodynia, the other major behavioral manifestations of neuropathic pain observed in WT mice from days 1 to 21 following sciatic nerve ligation. Before surgery, similar baseline withdrawal thresholds to mechanical and thermal stimuli were found in WT and both knockout mice. Responses of sham-operated mice also remained unchanged in NOS1 or NOS2 knockout mice as compared to WT. These results indicate that in the absence of nerve injury, the nitric oxide synthesized by NOS1 or NOS2 do not seems to tonically modulate the mechanical and thermal nociceptive sensitivity [13,16]. Summing up, our data demonstrated that nitric oxide synthesized by both NOS1 and NOS2 isoenzymes is involved in the development and maintenance of sciatic nerve injury-induced neuropathic pain.

It is well known that nitric oxide generated by NOS1 or NOS2 also contributes to the processing of nociceptive signals induced by other types of chronic pain such as inflammatory [17]. In accordance, our and other previous studies have revealed the different roles played by nitric oxide synthesized by NOS1 or NOS2 in the maintenance of mechanical allodynia and thermal hyperalgesia induced by inflammatory pain [16,18]. Surprisingly, the behavioral data of this study show that both enzymes (NOS1 and NOS2) have a similar participation in the expression of sciatic nerve injury-induced mechanical allodynia and thermal hyperalgesia. Thus, it is only in the maintenance of sciatic nerve injury-induced thermal allodynia where NOS2 seems to be more implicated than NOS1. These results support the hypothesis that the involvement of each NOS enzyme varies according to the type of nociceptive stimulus [3,5,19].

The molecular mechanisms implicated in the neuropathic pain states are not clear. However, one consequence of nerve injuries is the manifestation of adaptive changes in the expression of diverse receptors, channels and enzymes, such as NOS in the dorsal root ganglia and the spinal cord of nerve injured animals. At present, the possible effects of chronic sciatic nerve ligation on the induction of NOS enzymes in the spinal cord are not completely elucidated. Our data showed that peripheral neuropathic pain increases the transcription and expression of NOS1 in the spinal cord of WT mice, indicating that NOS1 might be the main responsible for the maintenance of peripheral neuropathic pain after the total sciatic nerve ligation. In accordance to our results, an increase in the spinal cord expression of NOS1 at 14 days after nerve injury have been also demonstrated although the expression of this isoenzyme on day 7^{th} after injury remains unaltered [5,7–8]. These findings indicate that the NOS1 spinal cord changes induced by nerve injury could be related to the time point after surgery, suggesting that NOS1 seems to be more involved in the late (14 and 21 days) than in the early stages of sciatic nerve lesion.

The possible alterations in NOS2 and NOS3 expression induced by neuropathic pain have been also evaluated in this study. Our results did not show significant changes in the expression of NOS2 and NOS3, mRNA and protein, on day 21^{th} after sciatic nerve injury. In accordance to our findings, DeAlba et al. [4] also demonstrated that the spinal cord protein levels of NOS2 did not change at 26 days after total sciatic nerve ligation, although an increase in the protein levels of NOS2 has been also demonstrated in the spinal cord of WT mice at 7 or 14 days after nerve injury [8]. These data suggest that spinal NOS2 seems to be more involved in the early (7 and 14 days) than in the late (21 and 26 days) stages of nerve injury-induced neuropathic pain

In contrast to NOS1 and NOS2, the non significant changes in the spinal cord expression of NOS3 in the early (7 and 14 days) or late (21 days) phases of nerve injury suggest that this isoenzyme did not play an essential role in the development or maintenance of neuropathic pain [5,11].

The possible compensatory changes in the expression of other NOS isoforms that might occurs in the spinal cord of NOS1 and

NOS2 knockout mice at 21 days after sciatic nerve ligation were also evaluated. Our results demonstrate that in contrast to WT mice, the spinal cord expression of NOS1 in NOS2-KO animals was not altered by sciatic nerve injury. Therefore, we hypothesized that the unaltered changes in NOS1 expression observed in NOS2-KO mice and the genetic lack of NOS2 in these KO mice might explain the absence of mechanical and thermal allodynia as well as thermal hyperalgesia in their behavior responses after sciatic nerve ligation.

Our results also show, for first time, that the expression of NOS2 is up-regulated in the spinal cord of NOS1-KO mice during chronic sciatic nerve injury-induced neuropathic pain. This compensatory up-regulation of NOS2 in NOS1-KO mice suggests that the increased expression of this isoenzyme might be responsible for the thermal allodynia observed in NOS1-KO mice at 21 days after surgery. This hypothesis was supported by the absence of thermal allodynia observed in NOS2-KO mice at this time point after sciatic nerve ligation. In addition, our data also indicate that the presence of NOS2 is required for the increased expression of NOS1 in the spinal cord whereas an enhanced expression of NOS1 might avoid an up-regulation of NOS2 in the spinal cord of sciatic nerve-injured mice.

In the present study, we also demonstrated that no compensatory changes in the spinal cord expression of NOS3 take place in sciatic nerve-injured NOS1 and NOS2 knockout mice as well as in sham-operated NOS2-KO mice [12]. In addition, no compensatory changes in the spinal cord expression of NOS1 gene in sham-operated NOS2-KO mice have been also established. However, while the disruption of NOS1 gene did not alter the mRNA or protein levels of NOS2 in sham-operated mice a significant increase in the spinal cord expression of NOS3 was further demonstrated in sham-operated NOS1-KO mice [18]. These results indicated that the compensatory changes in the spinal cord expression of NOS3 in NOS1-KO under basal conditions does not fully compensate for NOS1 function in neuropathic pain, which is mainly produced by NOS2.

In summary, our results demonstrate the participation of nitric oxide synthesized by NOS1 or NOS2 in the development and maintenance of mechanical and thermal allodynia as well as the thermal hyperalgesia induced by the total constriction of sciatic nerve and propose these enzymes as interesting therapeutic targets for the treatment of chronic peripheral neuropathic pain. Our data also indicate that: i) the increased spinal cord expression of NOS1 plays a critical role in the maintenance of peripheral neuropathic pain, ii) a compensatory up-regulation of NOS2 isoform takes place in the spinal cord of sciatic nerve-injured NOS1-KO mice, and iii) the up-regulation of NOS1 in the spinal cord of sciatic nerve-injured WT mice at 21 days after surgery is modulated by NOS2.

Materials 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) and wild type (WT) mice with the same genetic background (C57BL/6J) were acquired from Harlan Laboratories (Barcelona, Spain). All mice weighing 21 to 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 conducted between 9:00 AM and 5:00 PM. Animal procedures were conducted in accordance with the guidelines of the European Communities, Directive 86/609/EEC regulating animal research and approved by the local ethical committee of our Institution (Comissió d'Etica en l'Experimentació Animal i Humana de la Universitat Autònoma de Barcelona, #00691).

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 [20]. 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 WT and KO mice have been used as controls.

The development of mechanical and thermal allodynia, and thermal hyperalgesia was evaluated by using the von Frey filaments, cold plate and plantar tests, respectively. All genotypes were tested in each paradigm on days 0, 1, 4, 7, 10, 14 and 21 after sciatic nerve ligation or sham induction.

Nociceptive behavioural 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. [21]. 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.

Thermal hyperalgesia was assessed as previously reported by Hargreaves et al. [22]. 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 ×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 10 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.

Thermal allodynia to cold stimulus was assessed by using the hot/cold-plate analgesia meter (Ugo Basile, Italy), previously described by Bennett and Xie [20]. The number of elevations of each hind paw was recorded in the mice exposed to the cold plate (4±0.5°C) for 5 minutes. For each animal, a score was calculated as the difference of number of elevations between ipsilateral and contralateral paw.

Molecular experiments

Tissue isolation. Sham-operated and sciatic nerve-injured WT, NOS1-KO and NOS2-KO mice were sacrificed at 21 days after surgery by cervical dislocation. Tissues from the ipsilateral site of the lumbar section of the spinal cord were removed immediately after sacrifice, frozen in liquid nitrogen and stored at −80°C until assay. Because of the small size of the unilateral lumbar section of the spinal cord, tissues from two animals were pooled together to obtain enough RNA or protein levels for performing the real time-PCR or Western blot analysis.

Total RNA extraction and reverse transcription. 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 was ≥1.9) was determined by spectrophotometry. 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 NOS1, NOS2 and NOS3 was determined by relative real-time PCR using pre-developed mice TaqMan gene expression assays (Applied Biosystems, CA, USA) for these genes: Mm0435189_m1 for NOS1, Mm01309902_m1 for NOS2 and Mm00435217_m1 for NOS3. A probe against GAPDH (Mm 99999915_g1) was used as endogenous control. PCR reactions were set up in 96-well plates containing the corresponding cDNA, 2× universal master mix (Applied Biosystems, CA, USA), the forward and reverse primers and the TaqMan probe. The PCR reaction mixture also contained 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. Water controls were included to ensure specificity. All samples were assayed in duplicate. Relative expression of the target genes was calculated by means of the comparative threshold cycle (CT) method [23].

Western blot analysis. The NOS1, NOS2 and NOS3 protein levels in the lumbar section of the spinal cord were analyzed by Western blot. Tissues were homogenized in buffer (50 mM Tris-Base, 150 nM NaCl, 1% NP-40, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.5 Triton X-100, 0.1% SDS, 1 mM Na_3VO_4, 25 mM NaF, 0.5% protease inhibitor cocktail, 1% phosphatise inhibitor cocktail). All reactive were purchased at Sigma (St. Louis, MO, USA) with the exception of NP-40 from Calbiochem (Biosciences, La Jolla, CA, USA). The crude homogenate was solubilised 1 hour at 4°C, sonicated for 10 seconds and centrifugated at 4°C for 15 min at 700×g. The supernatants (100 µg of total protein) were mixed with 4× laemmli loading buffer and then loaded onto 4% stacking/5% separating SDS-polyacrylamide gels. The proteins were electrophoretically transferred onto PVDF membrane overnight, blocked with PBST +2% nonfat dry milk, and subsequently incubated overnight at 4°C with a polyclonal rabbit anti-NOS1 antibody (1:150, BD Transduction Laboratories, San Diego, CA, USA), a polyclonal rabbit anti-NOS2 antibody (1:200, Chemicon, Millipore), a polyclonal rabbit anti-NOS3 antibody (1:100, BD Transduction Laboratories, San Diego, CA, USA), or a monoclonal rabbit anti-β-actin antibody (1:10.000, Sigma, St. Louis, MO, USA). β-actin was used as a loading control. The proteins were detected by an horseradish peroxidase-conjugated anti-rabbit secondary antibody (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and visualized by chemiluminescence reagents provided with the ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and exposure onto hyperfilm (GE, Healthcare). The intensity of blots was quantified by densitometry.

Experimental protocol. In a first set of experiments, we assessed the influence of NOS1 or NOS2 deletion in the development and expression of peripheral neuropathic pain. Then, WT, NOS1-KO and NOS2-KO 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. All animals were tested in each paradigm on days 1, 4, 7, 10, 14 and 21 after the chronic constriction of the sciatic nerve by using the same sequence as for baseline responses. We used sham-operated WT, NOS1-KO and NOS2-KO mice as controls.

In a second set of experiments, we examined the mRNA and protein levels of NOS1, NOS2 and NOS3 in the ipsilateral site of the lumbar section of the spinal cord from sciatic nerve-injured and sham-operated WT, NOS1-KO and NOS2-KO mice at 21 days after surgery by using real time PCR and Western blot, respectively.

Statistical analysis. Data are expressed as mean ± standard error of the mean (SEM). For each knockout mice and experimental day, data obtained in the von Frey filament stimulation model, plantar and cold plate tests from sciatic nerve-injured or sham-operated mice were compared by using a two-way ANOVA repeated measures (genotype and surgery as between factors of variation), followed by a one-way ANOVA or the corresponding Student's t test when required. Changes in the expression (mRNA and protein levels) of NOS1, NOS2 and NOS3 in the lumbar section of the spinal cord from sciatic nerve-injured or sham-operated WT, NOS1-KO and NOS2-KO mice at 21 days after surgery were analyzed by using a two-way ANOVA (genotype and surgery as between factors of variation), followed by a one-way ANOVA when required. A value of $p<0.05$ was considered significant.

Author Contributions

Conceived and designed the experiments: AH OP. Performed the experiments: AH RN SL JMMC. Analyzed the data: AH RN SL JMMC. Wrote the paper: AH OP.

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5.2. *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.*

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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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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 whether 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, we evaluated the effects of [D-Pen^2,D-Pen^5]-enkephalin (DPDPE); (2-methyl-1-propyl-1*H*-indol-3-yl)-1-naphthalenylmethanone (JWH-015); and a NOS1 [*N*-[(4*S*)-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 [1*H*-[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] 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-knockout (KO), and NOS2-KO mice, also was 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, and not in NOS1-KO mice. Results suggest that inactivation of the 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 studies 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 after nerve injury are 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,

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

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, to study whether 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-1*H*-indol-3-yl)-1-naphthalenylmethanone; JWH-015] agonist; 2) the reversibility of these effects by their coadministration with a specific DOPr (naltrindole) or a CB2R ([6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl](4-methoxyphenyl)methanone; AM630) antagonist; and 3) the antiallodynic and antihyperalgesic effects produced by selective NOS1 [*N*-[(4*S*)-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 [1*H*-[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 also was evaluated.

Materials and Methods

Animals

Male NOS1-knockout mice (C57BL/6J background) and NOS2-knockout mice (C57BL/6J background) were purchased from The Jackson Laboratory (Bar Harbor, ME), whereas WT mice with the same genetic background (C57BL/6J) were acquired from Harlan Laboratories (Barcelona, Spain). All mice weighing 21 to 25 g were housed under 12:12-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 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 National Institutes of Health (Bethesda, MD).

Induction of Neuropathic Pain

Neuropathic pain was induced by the chronic constriction of the sciatic nerve. In brief, 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 hind limb, 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 nonoperated (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. 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 in height, 9 cm in diameter) with a wire grid bottom through which the von Frey filaments (bending force range from 0.008 to 3.5 g; North Coast Medical, Inc., San Jose, CA) were applied by using a modified version of the up-down paradigm, as reported by Chaplan et al. (1994). The 0.4-g filament 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 and 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 to allow an appropriate behavioral immobility. The baseline values were between 1.3 and 1.5 g.

Thermal Hyperalgesia. Thermal hyperalgesia was assessed as reported by Hargreaves et al. (1988). Paw withdrawal latency in response to radiant heat was measured using the plantar test apparatus (Ugo Basile, Comerio, Italy). In brief, mice were placed in Plexiglas boxes (20 cm in height × 9 cm in 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 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. The baseline values were between 8.4 and 9.0 s.

Thermal Allodynia. Thermal allodynia to cold stimulus was assessed by using the hot/cold-plate analgesia meter (Ugo Basile) as 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°C) for 5 min. The baseline values were between zero and one 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 −80°C until assay. Samples from two or three animals were pooled into one experimental sample for each spinal cord or dorsal root ganglia tissue, respectively. All tissues were homogenized in ice-cold TRIzol reagent (Invitrogen, Renfrewshire, UK) with a homogenizer (Ultra-Turf, T8; Ika Werke, Staufen, Germany), and the total RNA was extracted. 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) 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. The expression of DOPr and CB2R was determined by real-time PCR using predeveloped mice TaqMan gene expression assays (Applied Biosystems, Foster City, CA) for the following genes: GenBank numbers Mm00443063_m1 (*DOPr*) and Mm00438286_m1 (*CB2R*). A probe against *GAPDH* (GenBank number Mm99999915_g1) was used as endogenous control, and reactions without RNA were included as negative controls to ensure the specificity. PCRs were set up in 96-well plates containing the corresponding cDNA, 0.9 μM each forward and reverse primer, 0.25 μM TaqMan MGB probe, and a final concentration of 1× universal master mix (Applied Biosystems), which provides the PCR buffer, $MgCl_2$, dNTPs, and the thermal stable AmpliTaq Gold DNA polymerase. The assay was conducted using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). 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 described previously, 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 nonoperated (naive) mice as controls. However, because 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 the specific DOPr agonist DPDPE (38.7–232.3 nmol; Clark et al., 1986), the 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 also were evaluated. Animals were tested in each paradigm pre- and postdrug administration using the same sequence as mentioned above.

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 coadministration of a 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 also were 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. Then, the effects of the subplantar coadministration 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 also were evaluated. The doses of all tested drugs were selected according to previous experiments as the doses that 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 also were evaluated.

In all experiments, antinociception in Von Frey filaments and plantar test are expressed as the percentage maximal possible effect, where the test latencies pre- (baseline) and postdrug administration are compared and calculated according to the following equation: percentage maximal possible effect = [(drug − baseline)/(cut-off − baseline)] × 100.

In the cold-plate test, the inhibitory effects were calculated according to the following equation: percentage inhibition = [(paw elevations number at baseline − paw elevations number after drug)/paw elevations number at baseline] × 100. For each drug and test, the ED_{50} value, defined as the dose that produces a 50% effect based on the E_{max} estimated from the double reciprocal plot, also was 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, also was evaluated by using real-time PCR.

Drugs

JWH-015, AM630, and L-NIL were acquired from Tocris Bioscience (Ellisville, MI). DPDPE, naltrindole hydrochloride, NANT, ODQ, and Rp-8-pCPT-cGMPs were purchased from Sigma-Aldrich. 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 ± S.E.M. 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 versus 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 five to six 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 these animals compared with naive (nonoperated) 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, nonsignificant changes were observed in the contralateral paw compared sciatic nerve injured, sham-operated, or naive mice.

Effects of Subplantar Administration of Specific DOPr and CB2R Agonists Alone or Coadministered 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, although the thermal antiallodynic effects of JWH-015 were significantly higher than those obtained in vehicle-treated mice ($p < 0.05$; Student's *t* test; JWH-015 versus vehicle), the thermal antiallodynic effects of DPDPE in the ipsilateral paw were only modestly improved compared with the effects produced by vehicle in the same paw. Moreover, by 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 and 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 either in the contralateral paw of sciatic nerve-injured mice or in the ipsilateral or contralateral paw of naive animals (data not shown).

In all tests, the antiallodynic and antihyperalgesic effects produced by DPDPE (Table 3) or JWH-015 (Table 3) in the ipsilateral paw of sciatic nerve-injured WT mice were completely reversed by the subplantar coadministration 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 D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2, 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 In-

TABLE 1

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

Values are means ± S.E.M., with $n = 8$ to 10 animals per experimental group.

Test	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, s)	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, no.)	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*

* $p < 0.05$ denotes significant differences vs. naive or sham-operated mice (one-way ANOVA, followed by the Student-Newman-Keuls test) for each test and paw.

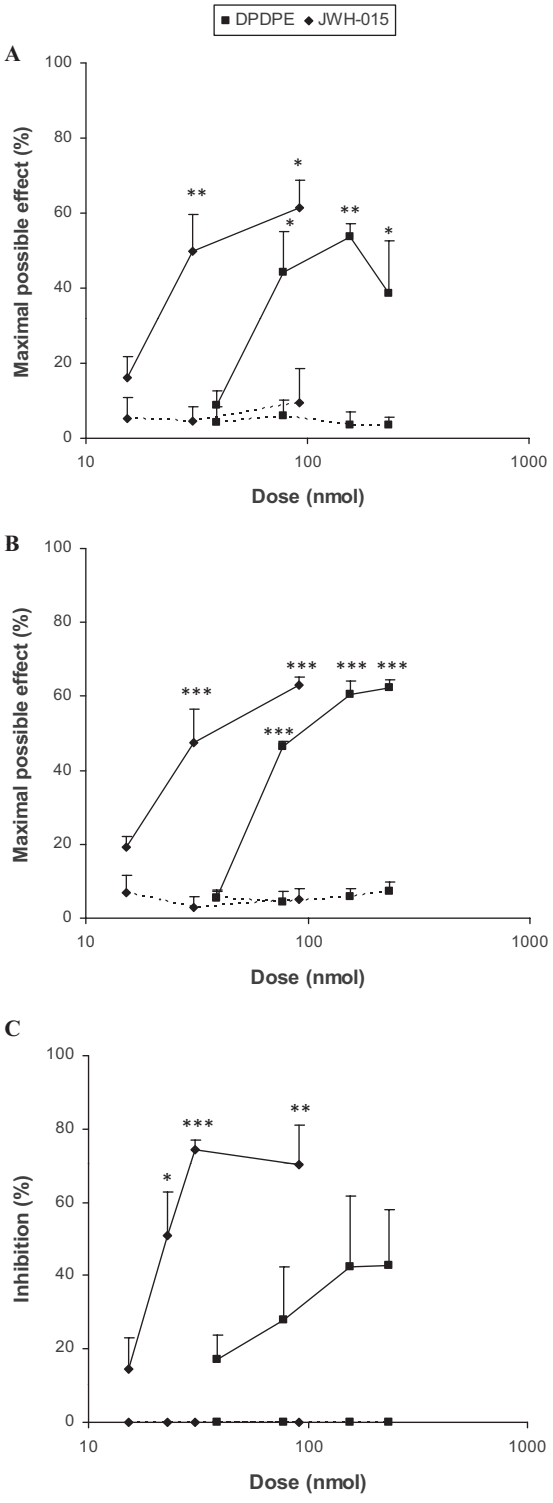

Fig. 1. Effects of the subplantar administration of different doses (logarithmic axis) of a specific DOPr agonist (DPDPE), a specific CB2R agonist (JWH-015), and their corresponding vehicle (dotted lines) in mechanical allodynia (A), thermal hyperalgesia (B), and thermal allodynia (C) induced by CCI 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 ± S.E.M. (five to six animals for each dose and drug tested). In all tests, for each drug and dose, *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ denote significant differences compared with each agonist versus their corresponding vehicle-treated group (Student's t test).

jury 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, by analyzing the ED_{50} values of NOS1, NOS2, L-guanylate cyclase, 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 and 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 and 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 JWH-015 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 JWH-015 during neuropathic pain was assessed by evaluating the effects of the coadministration of DPDPE (38.7 nmol) or JWH-015 (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 coadministration 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 coadministration 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 ($p < 0.001$, one-way ANOVA followed by Student-Newman-Keuls test).

The local coadministration of DPDPE or JWH-015 plus vehicle, NANT, L-NIL, ODQ, or Rp-8-pCPT-cGMPs did not have any significant effect in either the contralateral paw of sciatic nerve-injured WT mice or in the ipsilateral or contralateral paw of naive animals (data not shown).

TABLE 2

Comparison of the potencies (ED_{50}) of the subplantar administration of DPDPE or JHW-015 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

Data are expressed as ED_{50} values (nanomoles) with 95% confidence limits (in parentheses) determined on the quantal data of five to six animals per dose. For each test, the ratio of the ED_{50} values between agonists also is indicated.

Test	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

TABLE 3

Reversal of the effects of DPDPE (154.8 nmol) and JWH-015 (91.6 nmol) 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

Effects of the subplantar administration of vehicle, naltrindole, or AM630 alone also are shown. Values are means ± S.E.M., with $n = 5$ to 7 animals per group.

	Mechanical Allodynia (MPE)	Thermal Hyperalgesia (MPE)	Thermal Allodynia (Inhibition)
		%	
Effects of DPDPE 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	3.6 ± 1.7	8.3 ± 8.3
Effects of JWH-015 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

MPE, maximal possible effect.
* $p < 0.05$, significant differences compared with other groups (one-way ANOVA, followed by the Student-Newman-Keuls test) for each test.

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. A 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 it significantly increased their expression in NOS1-KO mice ($p < 0.037$, Student's t test; compared with their respective naive mice). Our results did not show any significant difference between genotypes compared with the expression of DOPr mRNA among them in naive or sciatic nerve-injured mice.

In the dorsal root ganglia, a 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, compared with their respective naive mice (Fig. 5B). Nonsignificant differences were found between genotypes compared with the expression of DOPr mRNA among them 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. A two-way ANOVA showed a significant effect of the surgery ($p < 0.001$) and a marginally 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, compared with 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, a two-way ANOVA only showed a significant effect of the surgery ($p < 0.012$). Thus, although sciatic nerve injury significantly increased the expression of CB2R in the dorsal root ganglia of WT ($p < 0.029$, Student's t test; versus naive WT) and NOS2-KO mice ($p < 0.040$, Student's t test; versus naive NOS2-KO), nonsignificant changes were observed in NOS1-KO mice (Fig. 5D). Our results did not show any significant difference between genotypes compared with 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. It is interesting to note that the local antiallodynic and antihyperalgesic effects of DPDPE and JWH-015 were significantly enhanced by their coadministration 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. More-

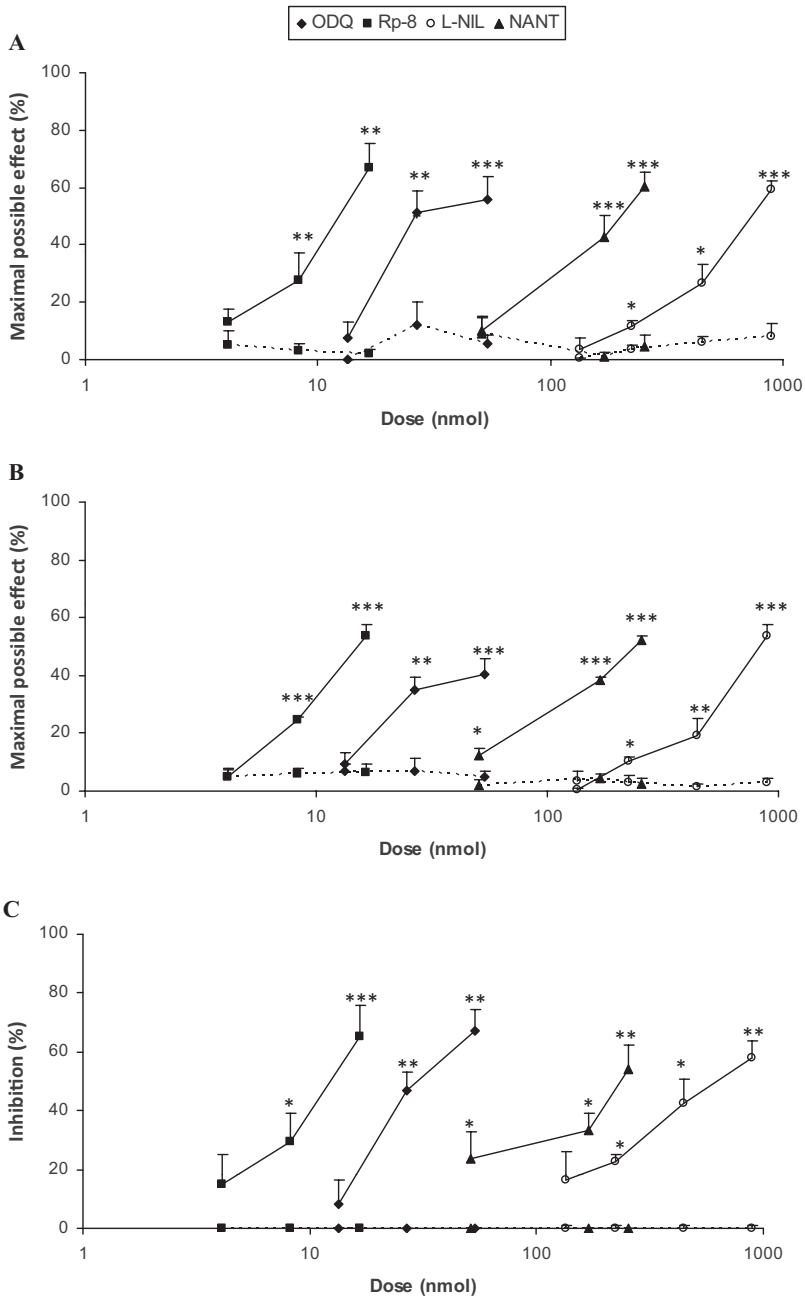

Fig. 2. Effects of the subplantar administration of different doses (logarithmic axis) of a specific NOS1 (NANT), NOS2 (L-NIL), guanylate cyclase (ODQ), and PKG (Rp-8-pCPT-cGMPs; Rp-8) inhibitor and their corresponding vehicle (dotted lines) in mechanical allodynia (A), thermal hyperalgesia (B), and 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 ± S.E.M. (five to six 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 compared with each inhibitor versus their corresponding vehicle-treated group (Student's t test).

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

Data are expressed as ED_{50} values (nanomoles) with 95% confidence limits determined on the quantal data of five to six animals per dose.

Test	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

over, although 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. By comparing the ED_{50} values of DPDPE and JWH-015, our data revealed that the DOPr agonist is effective at doses 3.6 to 4.1 times higher than the CB2R agonist in reversing the mechanical allodynia and thermal hyperalgesia induced by sciatic nerve injury. It is curious that a higher relative efficacy of JWH-015 than DPDPE was observed on the inhibition of thermal allodynia, in which 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 ago-

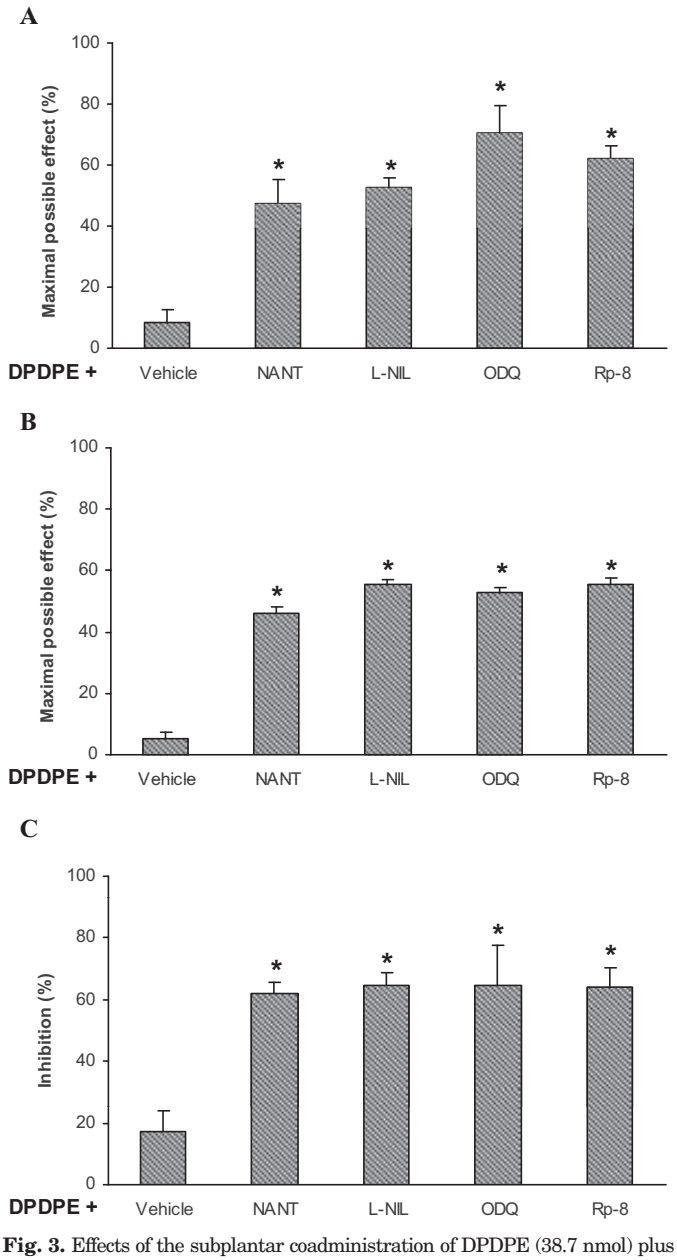

Fig. 3. Effects of the subplantar coadministration of DPDPE (38.7 nmol) plus vehicle, NANT (50.9 nmol), L-NIL (223.5 nmol), ODQ (13.4 nmol), or Rp-8-pCPT-cGMPs (4.1 nmol; Rp-8) in the ipsilateral paw of sciatic nerve-injured WT mice at 21 days after CCI. All drugs were coadministered 20 min before starting 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) ± S.E.M. (five to six animals per group). For each behavioral test, *, $p < 0.05$ denotes significant differences versus group treated with DPDPE + vehicle (one-way ANOVA followed by Student-Newman-Keuls test).

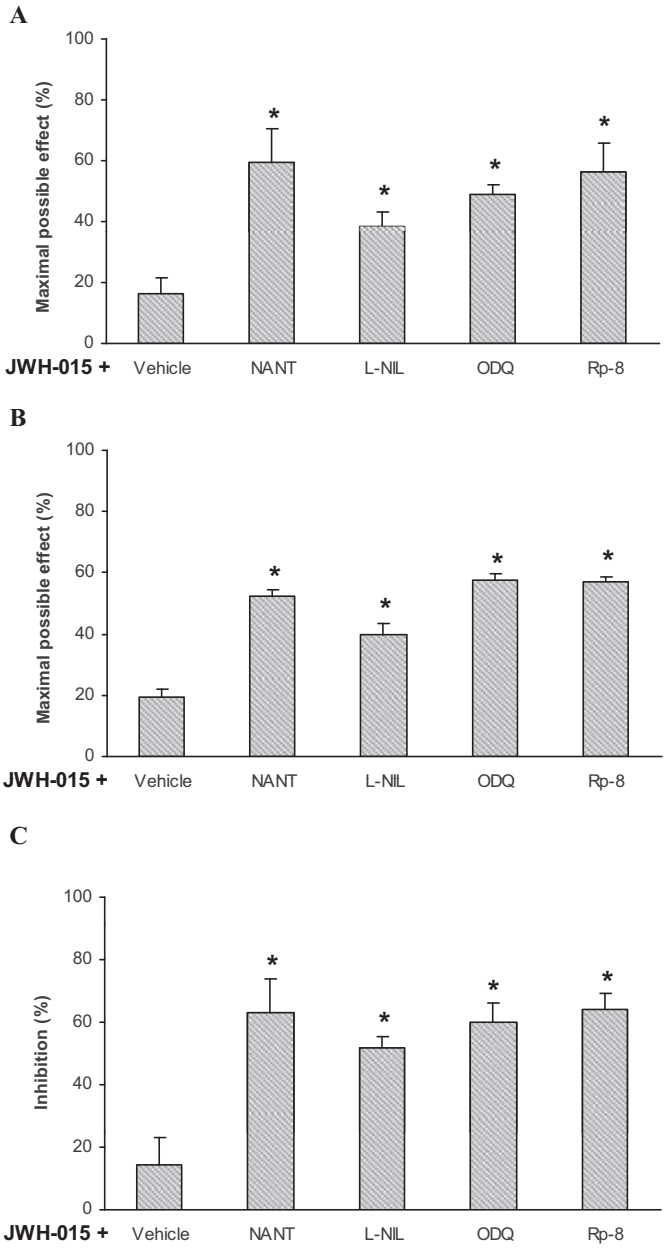

Fig. 4. Effects of the subplantar coadministration of JWH-015 (15.3 nmol) plus vehicle, NANT (50.9 nmol), L-NIL (223.5 nmol), ODQ (13.4 nmol), or Rp-8-pCPT-cGMPs (4.1 nmol; Rp-8) in the ipsilateral paw of WT mice at 21 days after CCI. All drugs were coadministered 20 min before starting behavioral testing. 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) ± S.E.M. (five to six animals per group). For each behavioral test, *, $p < 0.05$ denotes significant differences versus group treated with JWH-015 + vehicle (one-way ANOVA followed by Student-Newman-Keuls test).

nists after sciatic nerve injury was demonstrated by the complete reversion of their effects with selective antagonists that 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 studies 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, in which 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). In addition, the peripheral involvement of this nitric oxide signaling 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

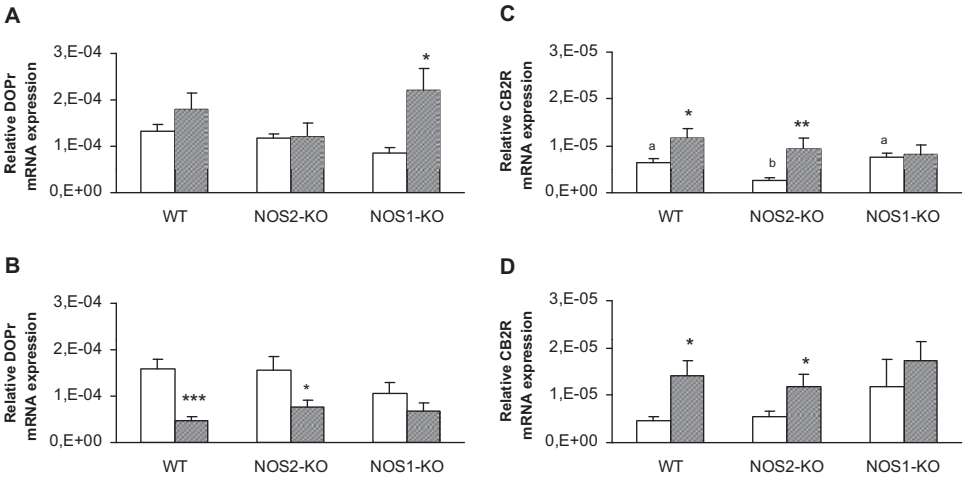

Fig. 5. Relative DOPr mRNA expression in the spinal cord (A) and dorsal root ganglia (B) of naive mice (white columns) and total sciatic nerve-ligated (striped columns) WT, NOS2-KO, and NOS1-KO mice. The figure also shows the relative CB2R mRNA expression in the spinal cord (C) and dorsal root ganglia (D) of naive mice (white columns) and total sciatic nerve-ligated (striped columns) WT, NOS2-KO, and NOS1-KO mice. Data are expressed as mean values ± S.E.M. (five to six samples per group). For each genotype, *, $p < 0.05$; **, $p < 0.001$; and ***, $p < 0.0001$ denote 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).

well as soluble guanylate cyclase or PKG blockers generates potent dose-dependent antiallodynic and antihyperalgesic effects after peripheral nerve injury, in which 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 peripheral nitric oxide-cGMP-PKG pathway activation.

In other pain models, such as acute and inflammatory models, a clear relationship between the antinociceptive effects of opioids and 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 NOS, inducible NOS, or both are inhibited, either pharmacologically or by using knockout mice for these enzymes (Li and Clark, 2001; Pol, 2007; Leánez et al., 2009). Moreover, the coadministration 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 also was investigated. It is interesting that 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 coadministration 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 also has 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 versus CCI) than the postinjury 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 after 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 also have been demonstrated by other studies (Zhang et al., 2003; Wotherspoon et al., 2005). In summary and taking account that DOPr is 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 principals responsible for the decreased and increased synthesis of peripheral DOPr and CB2R that leads to the lower peripheral potency of DPDPE compared with 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, in contrast to NOS1-KO mice and similar to WT mice, nerve injury also decreased DOPr and increased CB2R expression in the dorsal root ganglia and did not alter (DOPr) or enhance (CB2R) their expression in the spinal cord of NOS2-KO animals. 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, because it is implicated in the decreased or not-altered DOPr mRNA expression as well as in the increased transcription of CB2R that takes place in the peripheral and central nervous system of animals with neuropathic pain.

In summary, our data demonstrate that the inactivation of the 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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5.3. *Peripheral effects of morphine and expression of μ-opioid receptors in the dorsal root ganglia during neuropathic pain: nitric oxide signaling.*

Hervera A, Negrete R, Leánez S, Martín-Campos JM, Pol O.

Mol Pain. **2011**;7:25.

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Peripheral effects of morphine and expression of μ-opioid receptors in the dorsal root ganglia during neuropathic pain: nitric oxide signaling

Arnau Hervera[1], Roger Negrete[1], Sergi Leánez[1], Jesús M Martín-Campos[2] and Olga Pol[1*]

Abstract

Background: The local administration of μ-opioid receptor (MOR) agonists attenuates neuropathic pain but the precise mechanism implicated in this effect is not completely elucidated. We investigated if nitric oxide synthesized by neuronal (NOS1) or inducible (NOS2) nitric oxide synthases could modulate the local antiallodynic effects of morphine through the peripheral nitric oxide-cGMP-protein kinase G (PKG)-ATP-sensitive K^+ (KATP) channels signaling pathway activation and affect the dorsal root ganglia MOR expression during neuropathic pain.

Results: In wild type (WT) mice, the subplantar administration of morphine dose-dependently decreased the mechanical and thermal allodynia induced by the chronic constriction of the sciatic nerve (CCI), which effects were significantly diminished after their co-administration with different subanalgesic doses of a 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), L-guanylate cyclase (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; ODQ), PKG ((Rp)-8-(para-chlorophenylthio)guanosine-3',5'-cyclic monophosphorothioate; Rp-8-pCPT-cGMPs) inhibitor or a KATP channel blocker (glibenclamide). The evaluation of the expression of MOR in the dorsal root ganglia from sham-operated and sciatic nerve-injured WT, NOS1 knockout (KO) and NOS2-KO mice at 21 days after surgery demonstrated that, although the basal mRNA and protein levels of MOR were similar between WT and both NOS-KO animals, nerve injury only decreased their expression in WT mice.

Conclusions: These results suggest that the peripheral nitric oxide-cGMP-PKG-KATP signaling pathway activation participates in the local antiallodynic effects of morphine after sciatic nerve injury and that nitric oxide, synthesized by NOS1 and NOS2, is implicated in the dorsal root ganglia down-regulation of MOR during neuropathic pain.

Background

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 studies have demonstrated that the peripheral administration of μ-opioid receptor (MOR) agonists elicits antinociception in different models of neuropathic pain [1,2] and that their expression decreases after nerve injury [2,3]. Even so, the precise mechanisms implicated in the peripheral actions of morphine as well as in the expression of MOR during neuropathic pain are not completely elucidated.

Several studies have shown that nitric oxide, synthesized by neuronal (NOS1) or inducible (NOS2) nitric oxide synthases, mediates numerous neuropathic pain symptoms via central and peripheral nitric oxide-cGMP-PKG pathway activation [4-6]. Accordingly, the expression of NOS1 and NOS2 is up-regulated in the spinal cord and dorsal root ganglia of animals with neuropathic pain [7,8]. Moreover, the mechanical and thermal allodynia induced by nerve injury was reversed by the administration of selective NOS, guanylate cyclase o PKG inhibitors and attenuated or abolished in NOS1 and NOS2 knockout (KO) animals [4,6,8-10].

It is well known that the peripheral nitric oxide-cGMP-protein kinase G (PKG)-ATP-sensitive K^+

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(KATP) channels signaling pathway activation plays a critical role in the local antinociceptive effects of morphine during inflammatory pain [11-13] but not in the peripheral antinociceptive effects of δ-opioid receptor (DOR) agonists during neuropathic pain [6]. In addition, several studies also show that nitric oxide regulates the expression of MOR and DOR under several pain conditions [6,14,15] but the exact role of nitric oxide in the peripheral antinociceptive actions of morphine and expression of MOR during neuropathic pain is not known.

Thus, to study if the nitric oxide-cGMP-PKG-KATP peripheral pathway activation, triggered by NOS1 and NOS2, could modulate the local effects of morphine in nerve-injured wild type (WT) mice, at 21 days after the chronic constriction of the sciatic nerve (CCI), we evaluated: 1) the mechanical and thermal antiallodynic effects of the subplantar administration of morphine; 2) the reversibility of these effects by their local co-administration with a selective MOR antagonist, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2 (CTAP) or a peripheral non-selective opioid receptor antagonist, naloxone methiodide (NX-ME); 3) the mechanical and thermal antiallodynic effects of a high dose of morphine co-administered with different subanalgesic doses of a 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 (1*H*-[1,2,4]oxadiazolo [4,3-*a*]quinoxalin-1-one; ODQ), PKG ((Rp)-8-(para-chlorophenylthio)guanosine-3',5'-cyclic monophosphorothioate; Rp-8-pCPT-cGMPs) inhibitor or a KATP channel blocker (glibenclamide).

To evaluate the role played by nitric oxide, synthesized by NOS1 and NOS2, in the peripheral expression of MOR during neuropathic pain, the mRNA and protein levels of MOR in the dorsal root ganglia of sciatic nerve-injured WT, NOS1-KO and NOS2-KO mice, at 21 days after surgery, were also assessed.

Results

Expression of neuropathic pain in WT mice

In accordance to our previous reports [6,8], the total sciatic nerve ligation produced unilateral mechanical allodynia and thermal allodynia at 21 days after surgery. Thus, sciatic nerve injury led to a significant decrease in the percentage of the basal response of the threshold for evoking paw withdrawal to a mechanical stimulus in the ipsilateral paw of sciatic nerve-injured animals (37.4 ± 3.5) as compared to their contralateral paw (100.0 ± 6.3) as well as to the contralateral (104.5 ± 4.7) and ipsilateral (93.5 ± 9.1) paws of sham-operated mice (P < 0.001; one-way ANOVA followed by the Student Newman Keuls test). Similar results has been obtained for thermal allodynia where a significant increase in the number of paw elevations to cold thermal stimulus in the ipsilateral paw of sciatic nerve-injured animals (5.7 ± 0.6) as compared to their contralateral paw (0.2 ± 0.2) as well as to the contralateral (0.3 ± 0.2) and ipsilateral (0.2 ± 0.2) paws of sham-operated mice, has been also demonstrated (P < 0.001; one-way ANOVA followed by the Student Newman Keuls test).

Effects of the subplantar administration of morphine in the mechanical and thermal allodynia induced by sciatic nerve injury in WT mice and reversal of their effects by CTAP or NX-ME

The subplantar administration of morphine into the ipsilateral paw dose-dependently inhibited the mechanical (Figure 1A) and thermal (Figure 1B) allodynia induced by the chronic constriction of the sciatic nerve. Thus, the mechanical and thermal antiallodynic effects produced by high doses of morphine 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, analyzing the ED_{50} values our data showed that the potency of morphine on the inhibition of mechanical, 194.9 nmol (148.7-255.9) and thermal sensitivity, 225.9 nmol (191.0-267.1) induced by sciatic nerve injury was very analogous.

The subplantar administration of morphine or vehicle did not elicit any significant antinociceptive effect neither in the contralateral paw of sciatic nerve-injured mice nor in the ipsilateral or contralateral paw of sham-operated mice (data not shown).

The mechanical (Figure 2A) and thermal (Figure 2B) antiallodynic effects produced by morphine (400 nmol) in the ipsilateral paw of sciatic nerve-injured WT mice were completely reversed by the subplantar co-administration with a selective MOR (CTAP, 108.7 nmol) or the non-selective peripherally acting opioid receptor (NX-ME, 42.6 nmol) antagonist (P <0.001; one way ANOVA followed by the Student Newman Keuls test). The subplantar administration of vehicle, CTAP or NX-ME alone in sciatic nerve-injured and sham-operated WT mice did not show any significant effect on the two different nociceptive responses evaluated in this study.

Involvement of the peripheral nitric oxide-cGMP-PKG-KATP signaling pathway triggered by NOS1 and NOS2 in local antiallodynic effects produced by morphine after the sciatic nerve injury in WT mice

The role of the peripheral nitric oxide-cGMP-PKG-KATP signaling pathway, activated by NOS1 and NOS2, in the local mechanical and thermal antiallodynic effects produced by morphine during neuropathic pain was assessed by evaluating the effects produced by a high

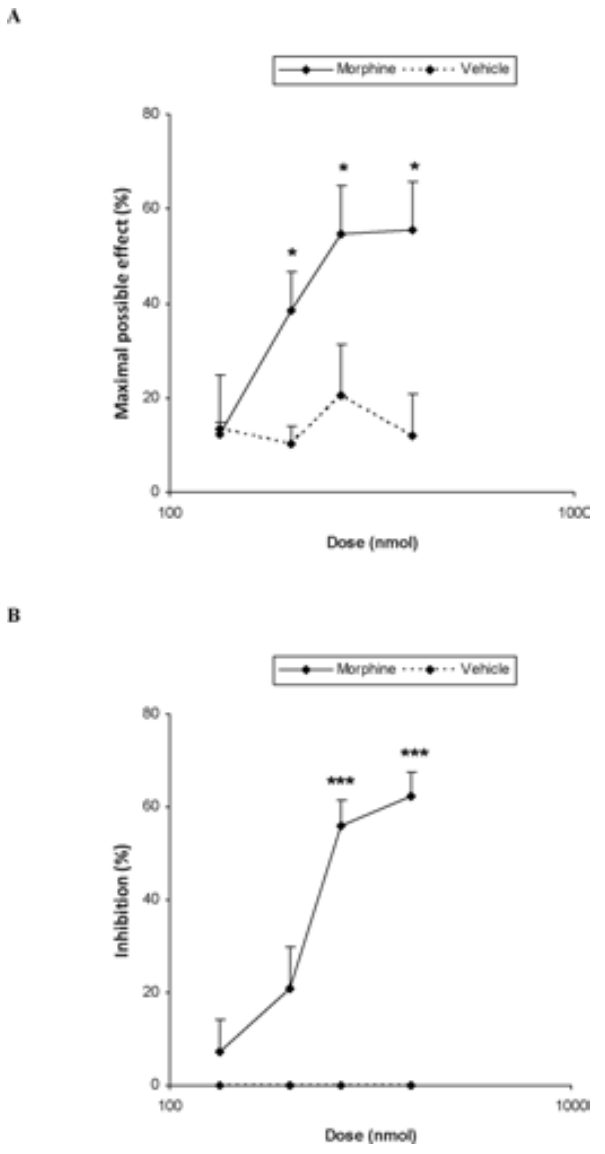

Figure 1 Antiallodynic effects of morphine. Effects of the subplantar administration of different doses (logarithmic axis) of morphine or vehicle on the mechanical (A) and thermal allodynia (B) induced by CCI in the ipsilateral paw of WT mice at 21 days after surgery. Morphine was administered 20 min before starting behavioral testing. Data are expressed as mean values of maximal possible effect (%) for mechanical allodynia and inhibition (%) for thermal allodynia ± SEM (5-6 animals for dose). In both tests, for each dose, * P <0.05 and *** P <0.001 denote significant differences between morphine and vehicle treated animals (Student's t test).

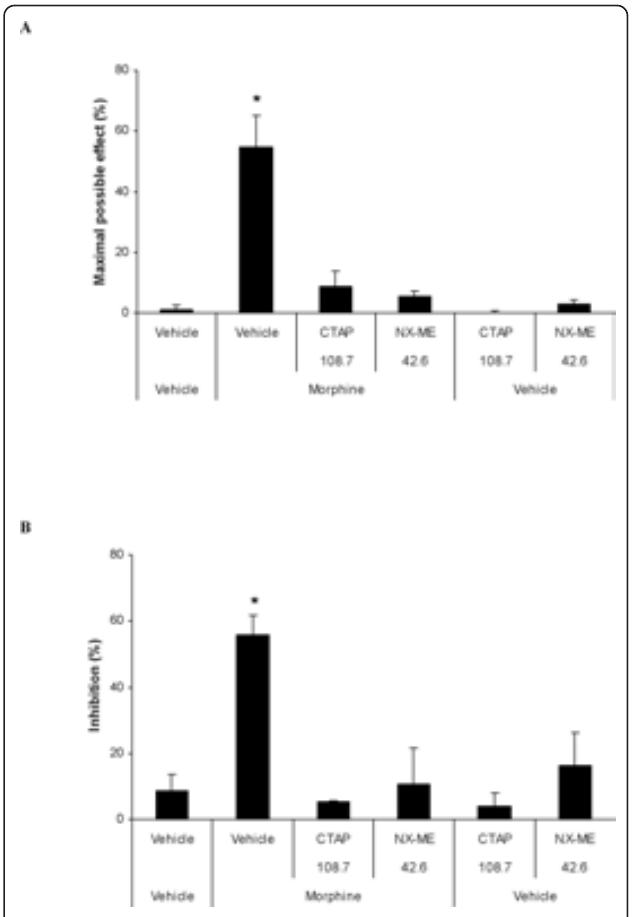

Figure 2 Reversion of the antiallodynic effects of morphine. Reversal of the effects of morphine (400 nmol) on the mechanical (A) and thermal (B) allodynia induced by CCI in the ipsilateral paw of WT mice, at 21 days after CCI, by the subplantar co-administration of a selective MOR antagonist (CTAP; 108.7 nmol) or a peripheral non-selective opioid receptor antagonist (NX-ME; 42.6 nmol). The effects of the subplantar administration of vehicle, CTAP (108.7 nmol) or NX-ME (42.6 nmol) administered alone are also shown. Data are expressed as mean values of maximal possible effect (%) for mechanical allodynia and inhibition (%) for thermal allodynia ± SEM (5-6 animals for each group). For each test, * represents significant differences compared to the other groups (P <0.05; one way ANOVA, followed by the Student Newman Keuls test).

dose of morphine (400 nmol) co-administered with different dose of NANT, L-NIL, ODQ, Rp-8-pCPT-cGMPs, glibenclamide or vehicle in sciatic nerve-injured WT mice at 21 days after surgery.

Our results showed that the local mechanical and thermal antiallodynic effects of morphine in the ipsilateral paw of sciatic nerve-injured WT mice were inhibited by their peripheral co-administration with NANT or L-NIL (Figure 3) as well as with ODQ, Rp-8-pCPT-cGMPs or glibenclamide (Figure 4) in a dose-dependent manner (P <0.001, one way ANOVA followed by Student Newman Keuls test). Moreover, the local co-administration of morphine plus NANT, L-NIL, ODQ, Rp-8-pCPT-cGMPs or glibenclamide did not have any significant effect neither on the contralateral paw of sciatic nerve-injured mice nor in the ipsilateral or contralateral paw of sham-operated animals (data not shown).

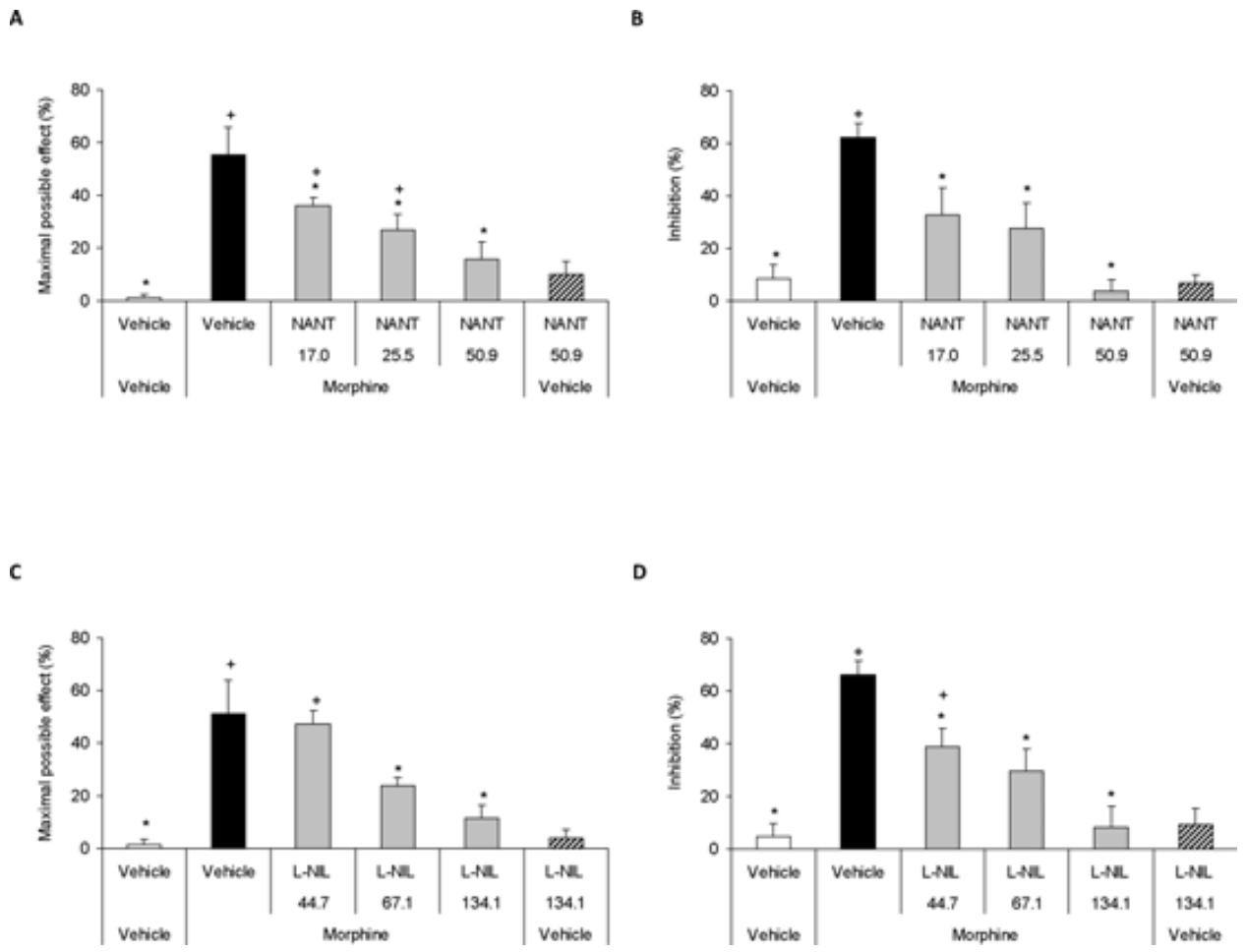

Figure 3 Role of the peripheral nitric oxide synthesized by NOS1 and NOS2 in the antiallodynic effects of morphine. Mechanical (A, C) and thermal (B, D) antiallodynic effects of the subplantar co-administration of morphine (400 nmol) plus vehicle or different doses of NANT (17.0 - 50.9 nmol; A, B) or L-NIL (44.7 - 134.1 nmol; C, D) in the ipsilateral paw of sciatic nerve-injured WT mice at 21 days after surgery. The effects of the subplantar administration of vehicle and the maximal doses of NANT (50.9 nmol) or L-NIL (134.1 nmol) injected alone are also shown. All drugs were administered 20 min before starting behavioral testing. Data are expressed as mean values of the maximal possible effect (%) for mechanical allodynia and as inhibition (%) for thermal allodynia ± SEM (5-6 animals per group). For each behavioral test and selective inhibitor assayed, * P <0.05 denotes significant differences vs. group treated with morphine plus vehicle (one way ANOVA followed by Student Newman Keuls test) and + P <0.05 denotes significant differences vs. group treated with vehicle (one way ANOVA followed by the Student Newman Keuls test).

Our results also indicated that the subplantar administration of the highest doses of NANT (50.9 nmol) or L-NIL (134.1 nmol; Figure 3) as well as of ODQ (13.3 nmol), Rp-8-pCPT-cGMP (4.1 nmol) or glibenclamide (60.7 nmol; Figure 4) administered alone did not produce any significant antiallodynic effect on the ipsilateral paws of sciatic nerve-injured WT mice as compared to vehicle group. Moreover, the subplantar administration of these doses of NANT, L-NIL, ODQ, Rp-8-pCPT-cGMPs or glibenclamide as well as of 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 sham-operated animals (data not shown).

The expression of MOR in the dorsal root ganglia of sciatic nerve-injured WT, NOS1-KO and NOS2-KO mice

The mRNA and protein levels of MOR in the dorsal root ganglia of WT and both NOS-KO mice are shown in Figure 5A and 5B, respectively. Although the two way ANOVA did not show any effect of the genotype or surgery, a significant interaction between theme was demonstrated for mRNA (P <0.037) and protein (P <0.029) expression. Thus, while sciatic nerve injury significantly decreases the MOR mRNA (P <0.043, Student's t test) and protein (Student's t test, P <0.002) levels in WT mice, it did not change their expression in both KO mice when comparing sciatic nerve-injured vs. sham-operated animals.

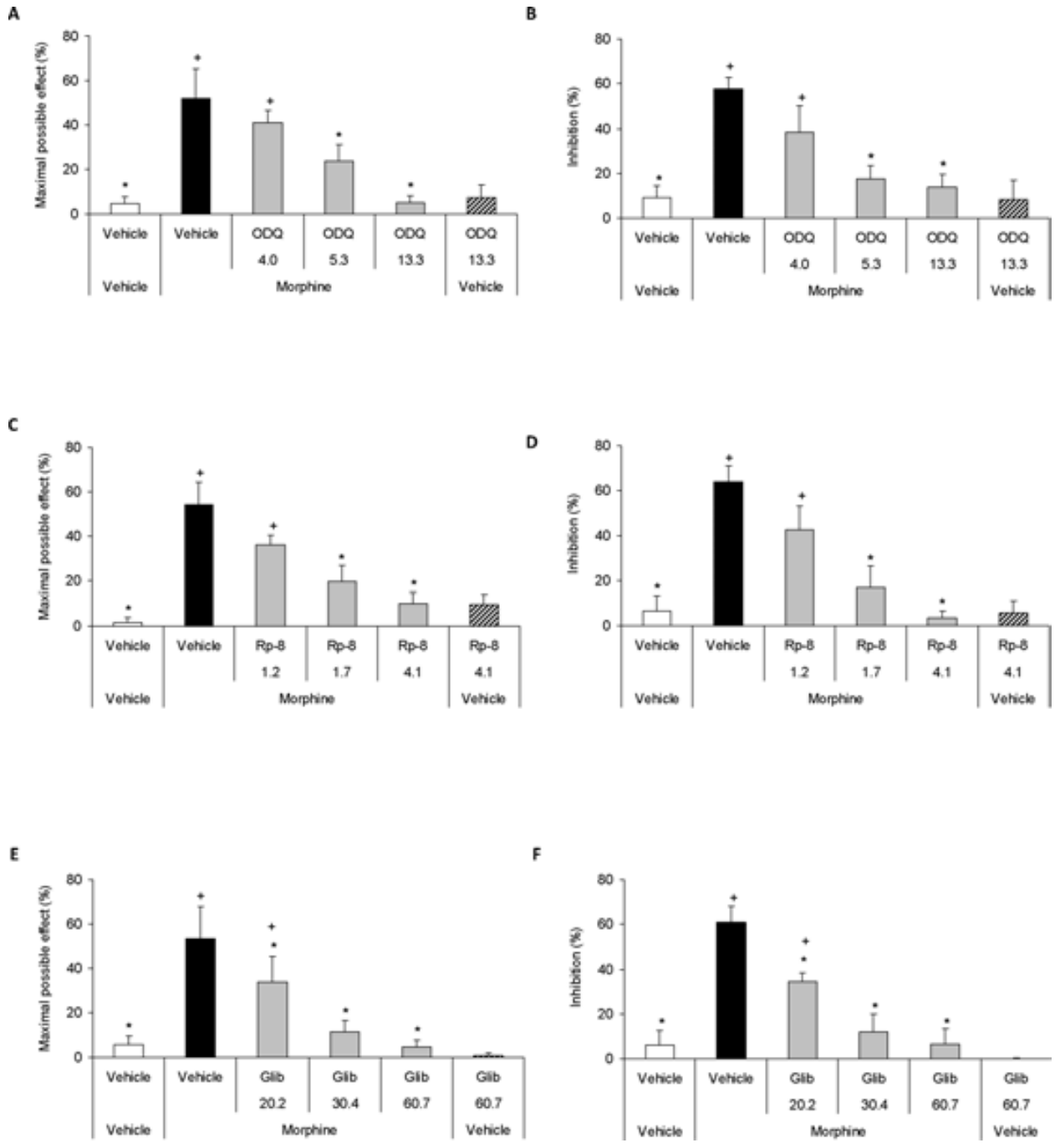

Figure 4 Role of the peripheral nitric oxide-cGMP-PKG-KATP signaling pathway in the antiallodynic effects of morphine. Mechanical (A, C, E) and thermal (B, D, F) antiallodynic effects of the subplantar co-administration of morphine (400 nmol) plus vehicle or different doses of ODQ (4.0 - 13.3 nmol; A, B), Rp-8 (1.2 - 4.1 nmol; C, D) or glibenclamide (Glib; 20.2 - 60.7 nmol; E,F) in the ipsilateral paw of sciatic nerve-injured WT mice at 21 days after surgery. The effects of the subplantar administration of vehicle and the maximal doses of ODQ (13.3 nmol), Rp-8 (4.1 nmol) or glibenclamide (60.7 nmol) injected alone are also shown. All drugs were administered 20 min before starting behavioral testing. Data are expressed as mean values of the maximal possible effect (%) for mechanical allodynia and as inhibition (%) for thermal allodynia ± SEM (5-6 animals per group). For each behavioral test and selective inhibitor assayed, * P <0.05 denotes significant differences vs. group treated with morphine plus vehicle (one way ANOVA followed by the Student Newman Keuls test) and + P <0.05 denotes significant differences vs. group treated with vehicle (one way ANOVA followed by Student Newman Keuls test).

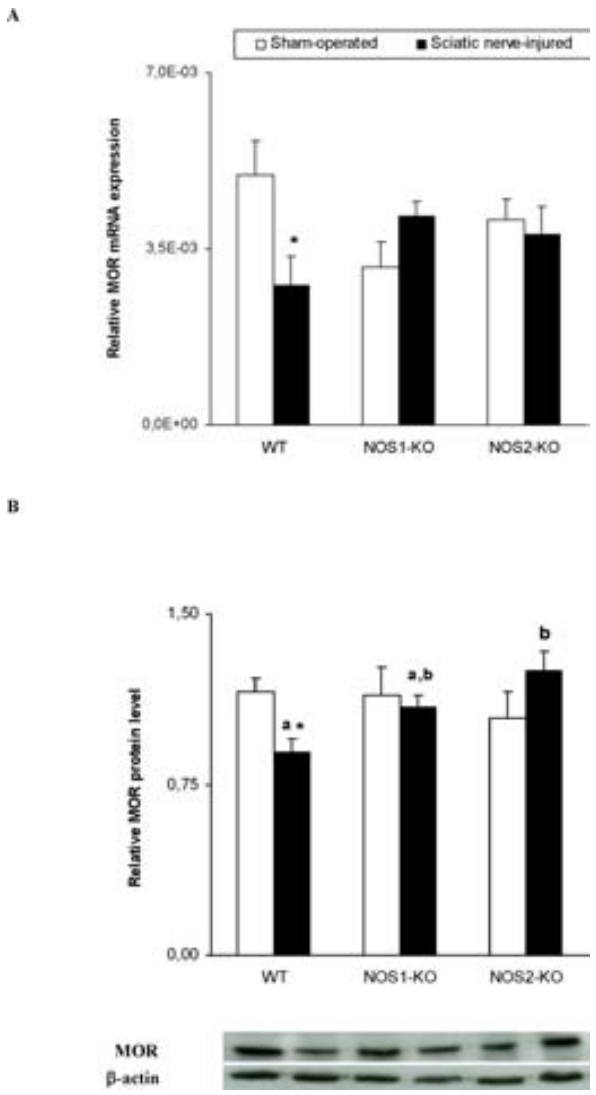

Figure 5 Dorsal root ganglia expression of MOR in WT, NOS1-KO and NOS2-KO mice. Relative mRNA (A) and protein (B) expression of MOR in the ipsilateral dorsal root ganglia from sham-operated or sciatic nerve-injured WT, NOS1-KO and NOS2-KO mice. Data are expressed as mean values ± SEM (4-5 samples per group). For each genotype, * P <0.05 denotes significant differences between sciatic nerve-injured and sham-operated mice (Student's t test). For each experimental group, different letters (a, b) denote significant differences between genotypes (P <0.05; one way ANOVA followed by the Student Newman Keuls test).

In addition, non significant differences were found between genotypes when compared the expression of MOR mRNA among theme in sham-operated or sciatic nerve-injured mice, although a significant increase in the MOR protein levels were observed in sciatic nerve-injured mice when compared NOS2-KO vs. WT animals (one way ANOVA, P <0.014).

Discussion

The present results demonstrate for first time that the local administration of morphine dose-dependently inhibited the mechanical and thermal allodynia induced by sciatic nerve injury through the activation of the peripheral nitric oxide-cGMP-PKG-KATP signaling pathway. This study also shows that nitric oxide, synthesized by NOS1 and NOS2, is implicated in the peripheral down-regulation of MOR during neuropathic pain.

In a model of CCI-induced neuropathic pain, our results confirmed the mechanical antiallodynic effects of MOR agonists locally administered [1,2] and further demonstrated the thermal antiallodynic effects produced by morphine in these experimental conditions with a similar effectiveness. The specificity and the peripheral antiallodynic effects of morphine after sciatic nerve injury was demonstrated by the complete reversion of their effects with a selective MOR antagonist (CTAP) and a non-selective peripherally acting opioid receptor antagonist (NX-ME), which did not have any effect when were administered alone. In addition, the highest dose of morphine did not produce any significant effect in the contralateral paw of sciatic nerve-injured mice, supporting the peripheral site of action of this drug.

A clear relationship between the antinociceptive effects of MOR agonists and the peripheral nitric oxide-cGMP-PKG-KATP signaling pathway activation during inflammatory pain has been extensively demonstrated [11-13,16]. Our results show for first time, that the local administration of morphine alleviates the mechanical and thermal allodynia induced by sciatic nerve injury through the activation of the peripheral nitric oxide-cGMP-PKG signaling pathway, triggered by NOS1 and NOS2, which culminate in an increased activation of KATP channels causing the hyperpolarization of nociceptive neurons. Indeed, the local mechanical and thermal antiallodynic effects produced by a high dose of morphine in sciatic nerve-injured WT mice were dose-dependently diminished with their co-administration with subanalgesic doses of L-NIL, NANT, ODQ, Rp-8-pCPT-cGMPs or glibenclamide. These results are in contrast to the reduced nociceptive responses induced by morphine after their intrathecal co-administration with several nitric oxide-cGMP-PKG-MAPKs inhibitors [17]. These findings support the idea that the nitric oxide-cGMP pathway may play different roles depending on the site of action, whereas peripheral activation produces antinociception, their central activation should be causing of nociceptive behaviors [18]. In addition, while the peripheral administration of morphine causes antinociception by the activation of the peripheral nitric oxide-cGMP signaling pathway which culminate in an increased activation of KATP channels causing the hyperpolarization of nociceptive neurons [13], their

intrathecal administration produces nociception by the activation of the spinal nitric oxide-cGMP signaling pathway that culminate in an increased activation of MAPKs which increases membrane excitability and induces spinal neuronal sensitization [19]. Moreover, the results of the present study are also in contrast to the enhanced antinociceptive effects of a DOR agonist after their co-administration with peripheral nitric oxide synthases or cGMP-PKG pathway blockers in sciatic nerve-injured animals [6]. Therefore, our findings demonstrate that while MOR agonists use the same mechanism of action to produce peripheral antinociception during inflammatory and neuropathic pain with different effectiveness, DOR agonists did not active the same way to produce peripheral antinociception in both types of pain, although a comparable potency was maintained [2,6]. Thus, a possible explanation for the reduced effectiveness of locally administered MOR agonists during neuropathic pain as compared to inflammatory, apart from the different alterations in the expression of MOR that occurs after peripheral inflammation (increases) or nerve injury (decreases) [2], might be also related to the drastic reduction in the peripheral KATP channels described in nerve-injured animals [20].

Several studies have demonstrated the involvement of nitric oxide in the regulation of opioid receptor gene transcription after peripheral inflammation and nerve injury [6,21,22]. In this report, we have investigated the role played by nitric oxide, synthesized by NOS1 and NOS2, in the decreased expression of MOR after neuropathic pain by using knockout mice for these enzymes. Our results showed that, although the basal dorsal root ganglia mRNA and protein levels of MOR were similar between WT and NOS-KO animals, nerve injury only decreased the MOR expression in WT mice. These findings suggest that nitric oxide, derived from NOS1 and NOS2, is implicated in the peripheral down-regulation of MOR after sciatic nerve-injury. Therefore and according to what occurs with the peripheral actions of morphine during inflammatory and neuropathic pain, these molecular data also support the evidence of the dual role played by nitric oxide in the modulation of the expression of MOR in both pain models. That is, while nitric oxide increases the peripheral expression of MOR during inflammation, it decreases their expression after nerve injury.

In summary, our data demonstrate that the activation of the nitric oxide-cGMP-PKG-KATP signaling peripheral pathway participates in the local antiallodynic effects produced by morphine during sciatic nerve injury and that nitric oxide, synthesized by NOS1 and NOS2, is involved in the decreased expression of MOR during neuropathic pain.

Conclusions

The present study demonstrates for first time that morphine can effectively attenuate neuropathic pain through the activation of the peripheral nitric oxide-cGMP-PKG-KATP signaling pathway and the decreased expression of MOR after sciatic nerve injury is regulated by nitric oxide. These data contribute to a better comprehension of the mechanism through peripheral MOR agonists produce antinociception after nerve injury and provide new insights into the development of novel therapeutic approaches for alleviating neuropathic pain.

Methods

Animals

Male NOS1-KO (C57BL/6J background) and NOS2-KO 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 (Barcelona, Spain). All mice weighing 21 to 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.

Animal procedures were conducted in accordance with the guidelines of the UK Animals Act 1986 (Scientific Procedures) and the guidelines of the European Communities Directive 86/609/EEC regulating animal research. The study protocol was approved by the local Committee of Animal Use and Care of the Autonomous University of Barcelona. All experiments were performed under blind conditions.

Induction of neuropathic pain

Neuropathic pain was induced by the chronic constriction of the sciatic nerve [6,8]. 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 [23]. 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 over-tightening 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 controls.

The development of mechanical and thermal allodynia was evaluated by using the von Frey filaments and cold plate 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 [24]. 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 drug administration in order to allow an appropriate behavioral immobility.

Thermal allodynia to cold stimulus was assessed by using the hot/cold-plate analgesia meter (Ugo Basile, Italy), previously described by Bennett and Xie [23]. The number of elevations of each hind paw was recorded in the mice exposed to the cold plate (4 ± 0.5°C) for 5 minutes.

Molecular experiments

Tissue isolation

Sham-operated and sciatic nerve-injured WT, NOS1-KO and NOS2-KO mice were sacrificed at 21 days after surgery by cervical dislocation. Three dorsal root ganglia from the ipsilateral lumbar section (L3 to L5) were collected from each animal. They were removed immediately after sacrifice, frozen in liquid nitrogen and stored at -80°C until assay. Because of the small size of the unilateral dorsal root ganglia, tissues from three to five animals were pooled together to obtain enough RNA or protein levels for performing the real time-PCR or Western blot analysis, respectively.

Total RNA extraction and reverse transcription

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. 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 MOR mRNA was determined by real-time PCR using a designed mice TaqMan® gene expression assay (Applied Biosystems, CA, USA) for this gene (MU-OR1-E2E3). 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 1× 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 gene was calculated by means of the comparative threshold cycle (CT) method [25].

Western blot analysis

The MOR protein levels were analyzed by Western blot. Tissues were homogenized in ice-cold lysis buffer (50 mM Tris·Base, 150 nM NaCl, 1% NP-40, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.5 Triton X-100, 0.1% SDS, 1 mM Na_3VO_4, 25 mM NaF, 0.5% protease inhibitor cocktail, 1% phosphatase inhibitor cocktail). All reagents were purchased at Sigma (St. Louis, MO, USA) with the exception of NP-40 from Calbiochem. The crude homogenate was solubilised 1 hour at 4°C, sonicated for 10 seconds and centrifugated at 4°C for 15 min at 700 × g. The supernatants (80 μg of total protein) were mixed with 4 × laemmli loading buffer and then loaded onto 4% stacking/10% separating SDS-polyacrylamide gels. The proteins were electrophoretically transferred onto PVDF membrane for 90 minutes, blocked with PBST + 5% nonfat dry milk, and subsequently incubated overnight at 4°C with a polyclonal rabbit anti-MOR antibody (1:1.000, Chemicon, Millipore). The proteins were detected by a horseradish peroxidase-conjugated anti-rabbit secondary antibody (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and visualized by chemiluminescence reagents provided with the ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and exposure onto hyperfilm (GE, Healthcare). The intensity of blots was quantified by densitometry. The membrane was stripped and reapplied with a monoclonal rabbit anti-β-actin antibody (1:10.000, Sigma, St. Louis, MO, USA) 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 CCI previously used by us [6,8]. After the habituation period, baseline responses were established in the following

sequence: von Frey filaments and cold plate tests. After that, neuropathic pain was induced and animals were again tested in each paradigm at 21 days after surgery before and after drug administration. Sham-operated mice were used as controls.

Due to the lack of mechanical and thermal allodynia induced by CCI in NOS1-KO and NOS2-KO mice, as we have previously demonstrated [8], the mechanical and thermal antiallodynic effects produced by the subplantar administration of different doses of morphine (133-400 nmol) or saline in the ipsilateral and contralateral paws of sciatic nerve-injured and sham-operated animals, were only evaluated in WT mice.

In another set of experiments, the specificity of the mechanical and thermal antiallodynic effects produced by a high dose of morphine (400 nmol) in sciatic nerve-injured WT mice at 21 days after surgery, was assessed by evaluating the reversibility of their effects with the peripheral co-administration with a selective MOR antagonist (CTAP; 108.7 nmol) or a peripheral non-selective opioid receptor antagonist (NX-ME; 42.6 nmol). The effects of these antagonists administered alone were also tested in sciatic nerve-injured and sham-operated WT mice at 21 days after surgery.

The possible involvement of the peripheral nitric oxide-cGMP-PKG- KATP signaling pathway, activated by NOS1 and NOS2, in the local mechanical and thermal antiallodynic effects of a MOR agonist has been evaluated in an extra group of WT mice. For this purpose, the local effects produced by different doses of NANT (17.0-50.9 nmol) a selective NOS1 inhibitor [26], L-NIL (44.7-134.1 nmol) a selective NOS2 inhibitor [27], ODQ (4.0-13.3 nmol) a selective soluble guanylyl cyclase inhibitor [28], Rp-8-pCPT-cGMPs (1.2-4.1 nmol) a PKG inhibitor [29], glibenclamide (20.2-60.7 nmol) a KATP channel blocker [30] or vehicle administered alone or combined with a high dose of morphine (400 nmol) in the ipsilateral and contralateral paws of sciatic nerve-injured and sham-operated WT mice at 21 days after surgery, were also evaluated. The doses of all tested inhibitors were selected according to our previous experiments as the ones which did not produce a significant antiallodynic effect after CCI-induced neuropathic pain [6].

In all experiments, antinociception in Von Frey filaments is 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:

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

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

$$\text{Percentage inhibition} = [(\text{paw elevations number at baseline} - \text{paw elevations number after drug}) / \text{paw elevations number at baseline}] \times 100.$$

Finally, the mRNA and protein levels of MOR in the ipsilateral site of the dorsal root ganglia from sciatic nerve-injured or sham-operated WT, NOS1-KO and NOS2-KO mice at 21 after surgery were also evaluated by using real time PCR and western blot, respectively.

Drugs

Morphine-HCl was obtained from Alcaiber S.A. (Madrid, Spain) and L-NIL from Tocris (Ellisville, MI). CTAP, NX-ME, NANT, ODQ, Rp-8-pCPT-cGMPs and glibenclamide were purchase from Sigma-Aldrich (St. Louis, MO). Morphine, CTAP, NX-ME, NANT, L-NIL and Rp-8-pCPT-cGMPs were dissolved in saline solution (0.9% NaCl) while ODQ and glibenclamide in dimethyl sulfoxide (DMSO; 10% and 50% solution in saline, respectively). All drug combinations were diluted in the highest required concentration of DMSO. All drugs 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 dose, the comparison of the effects produced by morphine vs. the effects produced by vehicle in the contralateral and ipsilateral paw of nerve-injured or sham-operated 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 mechanical and thermal antiallodynic effects produced by morphine with CTAP or NX-ME and the effects produced by these antagonists administered alone in the ipsilateral paw of sciatic nerve-injured and sham operated WT mice were analyzed by using a one way ANOVA followed by the Student Newman Keuls test.

The comparison between the mechanical and thermal antiallodynic effects produced by a high dose of morphine subplantarly administered alone or combined with different doses of specific inhibitors (NANT, L-NIL,

ODQ, Rp-8-pCPT-cGMPs or glibenclamide) in the ipsilateral paw of sciatic nerve-injured and sham-operated WT mice was performed by using a one way ANOVA followed by the Student Newman Keuls test.

Changes in the expression of MOR (mRNA or protein) in the dorsal root ganglia of sciatic nerve-injured and sham-operated WT, NOS1-KO and NOS2-KO mice at 21 after surgery, 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.

Abbreviations

CCI: chronic constriction of the sciatic nerve; cGMP: guanosine 3',5'-cyclic monophosphate; CTAP: D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2; DMSO: dimethyl sulfoxide; DOR: δ-opioid receptor; KATP: ATP-sensitive K^+ channels; KO: knockout; L-NIL: L-N(6)-(1-iminoethyl)-lysine; MOR: μ-opioid receptor; 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; NX-ME: naloxone methiodide; ODQ: 1*H*-[124]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; SEM: standard error of the mean; WT: wild type.

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Authors' contributions

AH and OP conceived and designed the experiments. AH RN SL and JMMC performed the experiments. AH RN SL and JMMC analyzed the data. AH and OP wrote the manuscript. All authors have read and approve the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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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

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ABSTRACT

Tolerance to the local antiallodynic effects of morphine, DPDPE ([D-Pen(2),D-Pen(5)]-Enkephalin) or JWH-015 ((2-methyl-1-propyl-1H-indol-3-yl)-1-naphthalenylmethanone) after their repeated administration during neuropathic pain was evaluated. The role of the nitric oxide–cGMP–protein kinase G (PKG)–c-Jun N-terminal kinase (JNK) signaling pathway on the peripheral morphine-induced tolerance after the chronic constriction of sciatic nerve in mice was also assessed. The mechanical and thermal antiallodynic effects produced by a high dose of morphine, DPDPE or JWH-015 subplantarly administered daily from days 10 to 20 after nerve injury were estimated with the von Frey filaments and cold plate tests. The antiallodynic effects of the repeated administration of morphine combined with a sub-analgesic dose of a selective inducible nitric oxide synthase (NOS2) (L-N(6)-(1-iminoethyl)-lysine; L-NIL), L-guanylate cyclase (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; ODQ), PKG ((Rp)-8-(para-chlorophenylthio)guanosine-3′,5′-cyclic monophosphorothioate; Rp-8-pCPT-cGMPs) or JNK (anthra[1,9-cd]pyrazol-6(2H)-one; SP600125) inhibitor from days 10 to 20 after injury were also evaluated. The repeated administration of morphine, but not DPDPE or JWH-015, produced a rapid development of tolerance to its mechanical and thermal antiallodynic effects in sciatic nerve-injured mice. The co-administration of morphine with L-NIL, ODQ, Rp-8-pCPT-cGMPs or SP600125 avoided the development of morphine antiallodynic tolerance after nerve injury. These findings reveal that the repeated local administration of DPDPE or JWH-015 did not induce antinociceptive tolerance after sciatic nerve injury-induced neuropathic pain. Our data also indicate that the peripheral nitric oxide–cGMP–PKG–JNK signaling pathway participates in the development of morphine tolerance after nerve injury and propose the inactivation of this pathway as a promising strategy to avoid morphine tolerance during neuropathic pain.

1. Introduction

Recent studies demonstrated that the peripheral single administration of μ- and δ-opioid as well as cannabinoid 2 (CB_2) receptor agonists elicited antinociception after the chronic constriction of sciatic nerve-induced neuropathic pain (Hervera et al., 2010a, 2011; Obara et al., 2009). It is also well known that while the prolonged subcutaneous or spinal cord administration of μ-opioid receptor agonists induce the development of antinociceptive tolerance under neuropathic pain conditions (Al-Hasani and Bruchas, 2011; Ossipov et al., 1995), it did not occurs with the repeated systemic administration of CB_2 receptor agonists (Codd et al., 2009; Hama and Sagen, 2009; Romero-Sandoval et al., 2008). Nevertheless, the possible development of antinociceptive tolerance induced by the repeated subplantar administration of μ- and δ-opioid as well as CB_2 receptor agonists in sciatic nerve-injured mice and the mechanisms implicated in this process remain unclear.

Several studies demonstrated that the activation of the nitric oxide–cGMP–protein kinase G (PKG)–c-Jun N-terminal kinase (JNK) signaling pathway is involved in numerous neuropathic pain symptoms (Ji et al., 2009) and in the development of opioid tolerance (Guo et al., 2009; Ozdemir et al., 2011; Santamarta et al., 2005). Thus, the expression of the inducible nitric oxide synthase (NOS2) is up-regulated in the spinal cord and dorsal root ganglia from nerve-injured and morphine tolerance animals (De Alba et al., 2006; Levy et al., 1999). Chronic pain and morphine treatment also increase the JNK phosphorylation in the spinal cord (Guo et al., 2009). Indeed the local administration of selective NOS2 (L-N(6)-(1-iminoethyl)-lysine; L-NIL), L-guanylate cyclase (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; ODQ), PKG ((Rp)-8-(para-chlorophenylthio)guanosine-3′,5′-cyclic monophosphorothioate; Rp-8-pCPT-cGMPs) or JNK (anthra

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[1,9-cd]pyrazol-6(2H)-one; SP600125) inhibitors reverses the hypersensitivity to pain induced by the spinal or sciatic nerve injury (De Alba et al., 2006; Guan et al., 2007; LaBuda et al., 2006) and avoids the development of morphine tolerance under basal conditions, without pain (Guo et al., 2009; Ozdemir et al., 2011; Santamarta et al., 2005). Moreover, the genetic deletion of the neuronal nitric oxide synthase (NOS1) and NOS2 genes attenuated the development of morphine tolerance under basal and inflammatory pain states, whereas the co-administration of L-arginine with morphine resulted in the development of functional tolerance to their analgesic effects (Heinzen and Pollack, 2004a; Liu et al., 2006; Romero et al., 2010). However, the possible involvement of the nitric oxide–cGMP–PKG–JNK signaling pathway in the development of morphine antiallodynic tolerance during neuropathic pain has not been yet evaluated.

In a neuropathic pain model induced by sciatic nerve injury we evaluated: 1) the mechanical and thermal antiallodynic effects of the subplantar administration of different doses of a specific μ- (morphine) and δ-opioid (DPDPE) as well as a CB_2 receptor (JWH-015) agonist; 2) the reversibility of these effects by their co-administration with specific and unspecific antagonists; 3) the development of antiallodynic tolerance to the repeated subplantar administration of a high dose of morphine, DPDPE or JWH-015 and 4) the antiallodynic effects of the repeated local co-administration of a high dose of morphine with sub-analgesic doses of selective NOS2 (L-NIL), soluble guanylate cyclase (ODQ), PKG (Rp-8-pCPT-cGMPs) or JNK (SP600125) inhibitors.

2. Material and methods

2.1. Animals

Male C57BL/6J mice were purchased from Harlan Laboratories (Barcelona, Spain). All mice weighing 21 to 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 conducted between 9:00 AM and 5:00 PM. All experiments were carried out according to the Ethical Guidelines of the International Association for the Study of Pain and approved by the local ethical committee of our Institution (Comissió d'Ètica en l'Experimentació Animal i Humana de la Universitat Autònoma de Barcelona)

2.2. Induction of neuropathic pain

Neuropathic pain was induced by the chronic constriction of the sciatic nerve (Hervera et al., 2010b). 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 prevented over-tightening 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 controls.

The development of mechanical and thermal allodynia was evaluated by using the von Frey filaments and cold plate tests, respectively. All animals were tested in each paradigm before surgery and from days 9 to 20 after nerve injury.

2.3. Nociceptive behavioral tests

2.3.1. Mechanical allodynia

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 utilized 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.

2.3.2. Thermal allodynia

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 °C) for 5 min.

2.4. Experimental protocol

In a first set of experiments we assessed the expression of neuropathic pain by using the mouse model of sciatic nerve injury previously used by us (Hervera et al., 2010b). After the habituation period, baseline responses were established in the following sequence: von Frey filaments and cold plate tests. After that, neuropathic pain was induced and animals were again tested in each paradigm from days 9 to 20 after surgery. Sham-operated mice were used as controls.

In a second set of experiments, we investigated the mechanical and thermal antiallodynic effects produced by the single subplantar administration of different doses of morphine (a μ-opioid receptor agonist), DPDPE (a δ-opioid receptor agonist) or JWH-015 (a CB_2 receptor agonist) and their respective vehicles in the ipsilateral and contralateral paws of sciatic nerve-injured and sham-operated animals. The specificity of the peripheral mechanical and thermal antiallodynic effects produced by a high dose of morphine (400 nmol), DPDPE (155 nmol) and JWH-015 (92 nmol) in sciatic nerve-injured mice was also assessed by evaluating the reversibility of their effects with the peripheral co-administration with a selective μ-opioid (CTAP; 108.7 nmol), δ-opioid (naltrindole; 110.9 nmol) or CB_2 receptor (AM630; 59.5 nmol) antagonist as well as with a peripheral non-selective receptor antagonist (naloxone methiodide; 42.6 nmol) or a selective cannabinoid 1 (CB_1) receptor antagonist (AM251; 270.1 nmol). The effects of these antagonists administered alone were also tested in sciatic nerve-injured and sham-operated mice. The doses of the antagonists were selected according to our previous studies (Hervera et al., 2010a, 2011). In another set of experiments, we evaluated the effect of the repeated subplantar administration of a high dose of morphine (400 nmol), DPDPE (155 nmol) or JWH-015 (92 nmol) and their respective vehicles in the ipsilateral and contralateral paws of sciatic nerve-injured and sham-operated animals from days 10 to 20 after surgery.

Finally, the involvement of the nitric oxide–cGMP–PKG–JNK signaling pathway in the antinociceptive tolerance induced by the repeated subplantar administration of morphine has been also studied by evaluating the local mechanical and thermal antiallodynic effects produced by the repeated co-administration of 400 nmol of morphine with a sub-analgesic dose of selective NOS2 (L-NIL; 44.7 nmol), L-guanylate cyclase (ODQ; 4 nmol), PKG (Rp-8-pCPT-cGMPs; 1.2 nmol) or JNK (SP600125; 100 nmol) inhibitors in the ipsilateral and contralateral paws of sciatic nerve-injured and sham-

operated animals from days 10 to 20 after surgery. In these experiments the doses of all tested inhibitors were selected according to our previous studies as the ones which produce a sub-analgesic effect under neuropathic pain conditions (Hervera et al., 2010a, 2011).

Animals were tested in each paradigm after drug administration using the same sequence as mentioned in the first paragraph.

2.5. Drugs

Morphine–HCl was obtained from Alcaliber S.A. (Madrid, Spain). JWH-015, AM630, AM251 and L-NIL were purchased from Tocris (Ellisville, MI). DPDPE ([D-Pen 2,5]-enkephalin), CTAP, naltrindole, naloxone methiodide, ODQ and Rp-8-pCPT-cGMPs were acquired from Sigma-Aldrich (St. Louis, MO). SP600125 was purchased from Calbiochem (Darmstadt, Germany).

Morphine–HCl, DPDPE, CTAP, naloxone methiodide, naltrindole, L-NIL and Rp-8-pCPT-cGMPs were dissolved in saline solution (0.9% NaCl), while AM630, AM251, ODQ, JWH-015 and SP600125 in dimethyl sulfoxide (DMSO; 50% solution in saline). All drug combinations were diluted in the highest required concentration of DMSO. All drugs, 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 30 min before behavioral testing with the exception of SP600125 which was administered subplantarly 3 h 30 min before testing (Gao et al., 2009). For each group treated with a drug the respective control group received the same volume of vehicle.

2.6. Statistical analysis

Data are expressed as mean ± standard error of the mean (S.E.M.). For each test and dose, the comparison of the effects produced by the single administration of each drug in the ipsilateral paw of nerve-injured mice vs. the effects produced by their corresponding vehicle in the ipsilateral paw of nerve-injured mice or those produced by the same drug in the ipsilateral and contralateral paw of sham-operated mice was evaluated by using a Student's *t* test.

For each test, the reversion of the mechanical and thermal antiallodynic effects produced by morphine, DPDPE or JWH-015 with their respective antagonists and the effects produced by these antagonists administered alone in the ipsilateral paw of sciatic nerve-injured mice were analyzed by using a one way ANOVA followed by the Student–Newman–Keuls test.

The comparison of the effects produced by the repeated subplantar administration of morphine, DPDPE, JWH-015 or their respective vehicle along the different time tested in the von Frey filament and cold plate tests, in the contralateral and ipsilateral paw of nerve-injured or sham-operated mice, were performed by using a two way ANOVA repeated measures. For each test and day, the comparison of the effects produced by each drug in the ipsilateral paw of nerve-injured mice vs. the effects produced by their corresponding vehicle in the ipsilateral paw of nerve-injured mice and those produced by the same drug in the ipsilateral and contralateral paw of sham-operated mice was evaluated by using a one way ANOVA followed by the Student–Newman–Keuls test.

The comparison of the mechanical and thermal antiallodynic effects produced by a high dose of morphine subplantarly administered alone or combined with a specific inhibitor (L-NIL, ODQ, Rp-8-pCPT-cGMPs or SP600125), in the ipsilateral and contralateral paw of sciatic nerve-injured and sham-operated mice along the different time tested, was performed by using a two way ANOVA repeated measures. For each test and day, the comparison of the effects produced by morphine alone or combined in the ipsilateral paw of nerve-injured mice vs. the effects produced by their corresponding vehicle in the ipsilateral paw of nerve-injured mice and those produced by the same drug combination in the ipsilateral and contralateral paw of sham-operated mice was evaluated by using a one way ANOVA followed by the Student–Newman–Keuls test. A value of $P<0.05$ was considered as a significant.

3. Results

3.1. Expression of neuropathic pain induced by sciatic nerve injury

In accordance to our previous reports, the total sciatic nerve ligation led to a significant decrease of the threshold for evoking paw withdrawal to a mechanical stimulus on the injury side. Mechanical allodynia was observed from days 9 to 20 after sciatic nerve ligation ($P<0.02$ vs. their respective sham vehicle treated group, Student's *t* test; Figs. 1 and 2). Withdrawal latencies of the ipsilateral paw from sham-operated mice (Figs. 1 and 2) as well as of the contralateral paw from sciatic nerve-injured (2.51 ± 0.09) and sham-operated mice (2.54 ± 0.08) were not modified.

Sciatic nerve ligation also enhanced the number of paw lifts after cold thermal stimulation on the injured site. This thermal allodynia was observed at day 9 after sciatic nerve injury and persisted for more than 20 days after ligation ($P<0.04$ vs. their respective sham vehicle treated group, Student's *t* test; Figs. 1 and 2). The number of paw lifts of the ipsilateral paw from sham-operated mice (Figs. 1 and 2) as well as of the contralateral paw from sciatic nerve-injured (0.13 ± 0.13) and sham-operated mice (0.00 ± 0.00) was not modified.

3.2. Effects of the single subplantar administration of morphine, DPDPE or JWH015 on the mechanical and thermal allodynia induced by sciatic nerve injury

The subplantar administration of morphine, DPDPE or JWH-015 into the ipsilateral paw dose-dependently inhibited the mechanical and thermal allodynia induced by nerve injury at 10 days after surgery (Fig. 1).

The mechanical antiallodynic effects produced by morphine (133, 200 and 400 nmol), DPDPE (77 and 155 nmol) and JWH-015 (31 and 92 nmol) in the ipsilateral paw of sciatic nerve-injured mice were significantly higher than those obtained in their corresponding sciatic nerve-injured vehicle treated group ($P<0.02$; Student's *t* test; Fig. 1A, C and E).

The thermal antiallodynic effects produced by high doses of morphine (200 and 400 nmol) and JWH-015 (31 and 92 nmol), but not of DPDPE, in the ipsilateral paw of sciatic nerve-injured mice were also significantly higher than those obtained in their corresponding sciatic nerve-injured vehicle treated group ($P<0.05$; Student's *t* test; Fig. 1B, D and F). The maximal inhibitory effect produced by each drug corresponds to produce by a dose of 400 nmol for morphine, 155 nmol for DPDPE and 92 nmol for JWH-015.

Moreover, the subplantar administration of different doses of morphine, DPDPE, JWH-015 or vehicle did not produce any mechanical and thermal antiallodynic effect neither in the ipsilateral paw of sham-operated mice (Fig. 1) nor in the contralateral paw of sciatic nerve-injured or sham-operated mice (Table 1).

3.3. Reversion of the antiallodynic effects of morphine, DPDPE and JWH-015 by specific and unspecific antagonists after sciatic nerve injury

The mechanical (Fig. 2A) and thermal (Fig. 2B) antiallodynic effects produced by a high dose of morphine in the ipsilateral paw of sciatic nerve-injured mice were completely reversed by its subplantar co-administration with a selective μ-opioid receptor (CTAP) or a peripheral opioid receptor (naloxone methioide) antagonist ($P<0.043$; one way ANOVA, followed by the Student–Newman–Keuls test). In a similar way, the mechanical (Fig. 2C) and thermal (Fig. 2D) antiallodynic effects produced by a high dose of DPDPE in

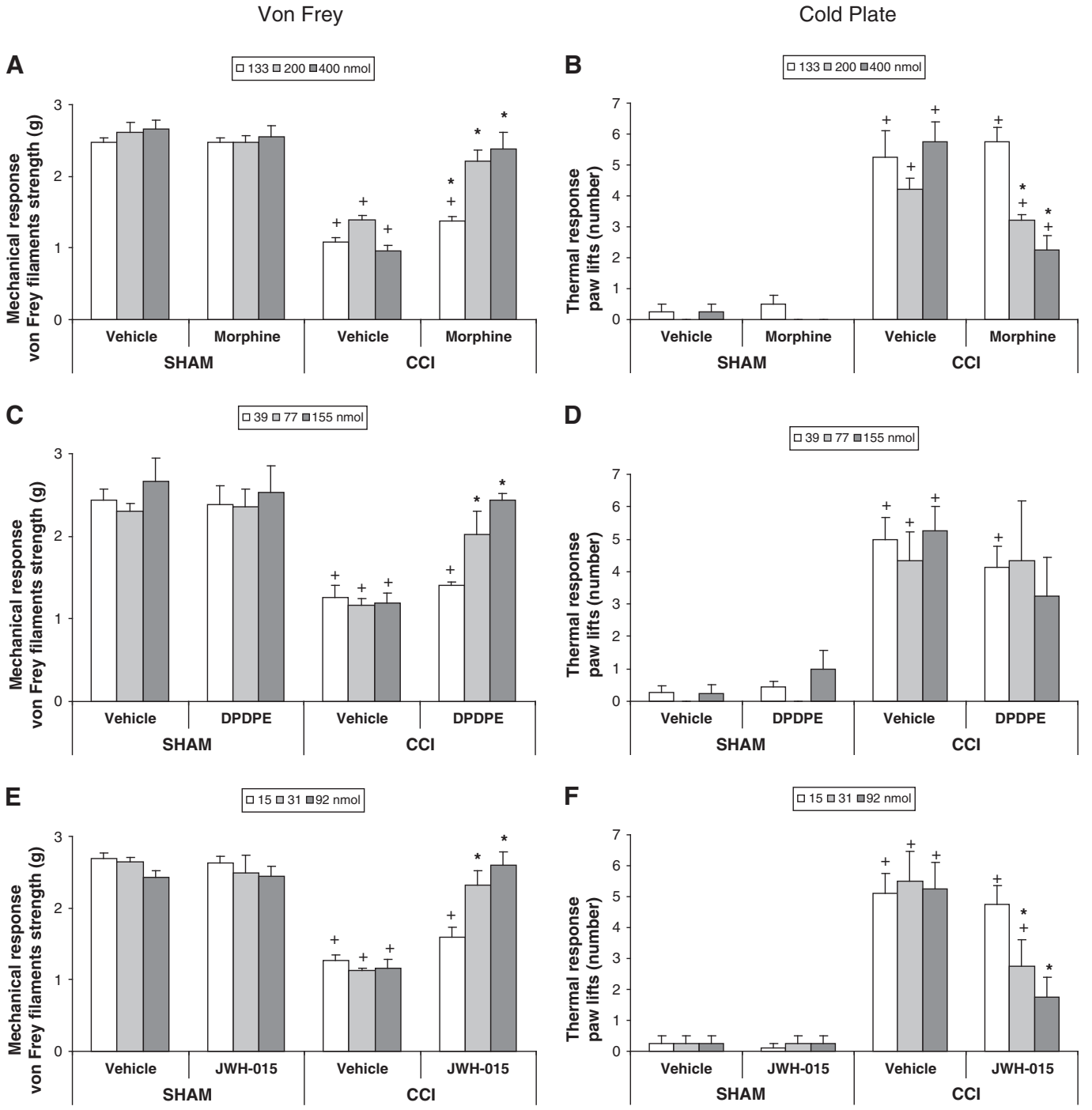

Fig. 1. Effects of the single subplantar administration of different doses of morphine (A, B), DPDPE (C, D) or JWH-015 (E, F) on the mechanical (A, C, E) and thermal (B, D, F) responses in the ipsilateral paw of sciatic nerve-injured (CCI) and sham-operated (sham) mice. For each test, drug and dose tested, * indicates significant differences vs. their respective vehicle treated mice (*$P<0.05$, Student's *t* test) and + when compared vs. their respective sham-operated mice (+$P<0.05$, Student's *t* test). Results are shown as mean values ± S.E.M.; n = 5–7 animals per experimental group.

the ipsilateral paw of sciatic nerve-injured mice were completely reversed by its subplantar co-administration with a selective δ-opioid receptor (naltrindole) or a peripheral opioid receptor (naloxone methioide) antagonist ($P<0.048$; one way ANOVA, followed by the Student–Newman–Keuls test). Finally, the mechanical (Fig. 2E) and thermal (Fig. 2F) antiallodynic effects produced by a high dose of JWH015 in the ipsilateral paw of sciatic nerve-injured mice were completely reversed by its subplantar co-administration with a selective CB_2 receptor antagonist (AM630; $P<0.035$; one way ANOVA, followed by the Student–Newman–Keuls test). The subplantar administration of AM251 (a selective CB_1 receptor antagonist) was unable to revert the local antiallodynic effects produced by JWH-015.

The subplantar injection of the different antagonists administered alone in sciatic nerve-injured mice did not have any significant effect on the two different nociceptive responses evaluated in this study. In addition, the subplantar administration of agonists combined with their respective antagonists did not produce any significant effect in the contralateral and ipsilateral paw of sham-operated mice.

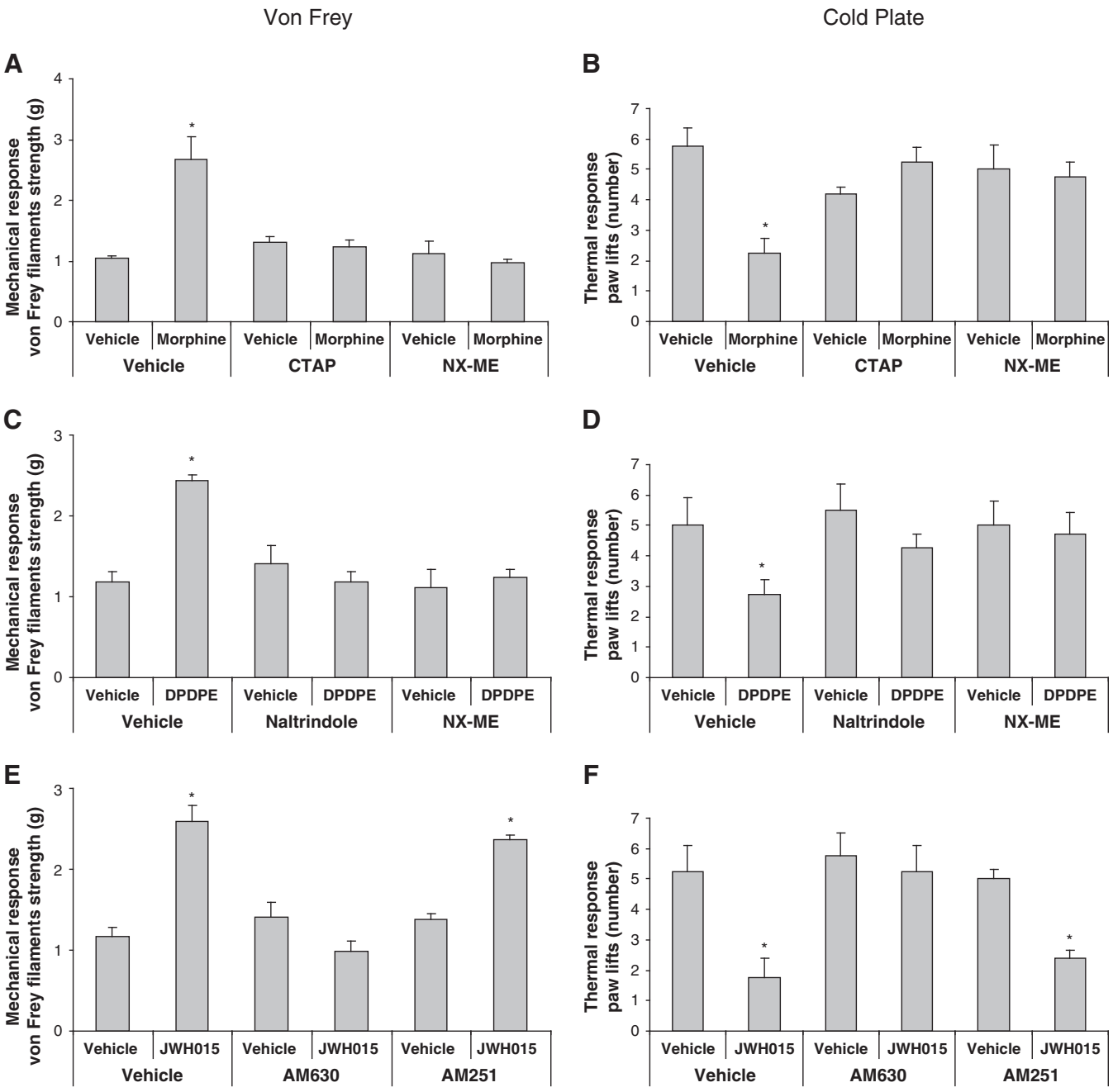

Fig. 2. Effects of the subplantar administration of a high dose of morphine (400 nmol; A, B), DPDPE (155 nmol; C, D) or JWH-015 (92 nmol; E, F) alone or combined with CTAP (108.7 nmol), naltrindole (110.9 nmol), naloxone methiodide (NX-ME; 42.6 mol), AM630 (59.5 nmol) or AM251 (270.1 nmol) on the mechanical and thermal responses in the ipsilateral paw of sciatic nerve-injured mice. For each test and drug, * indicates significant differences as compared to the other groups ($P<0.05$, one way ANOVA followed by the Student–Newman–Keuls test). Results are shown as mean values ± S.E.M.; n = 5–7 animals per experimental group.

3.4. Effects of the repeated subplantar administration of morphine, DPDPE or JWH015 on the mechanical and thermal allodynia induced by sciatic nerve injury

The effects of the repeated subplantar administration of 400 nmol of morphine, 155 nmol of DPDPE, 92 nmol of JWH-015 or their corresponding vehicle on the inhibition of the mechanical and thermal allodynia induced by nerve injury are shown in Fig. 3. In both behavioral tests, the two way ANOVA repeated measures reveal a significant effect of the treatment ($P<0.001$), time ($P<0.001$) and their interaction ($P<0.032$) in the ipsilateral paw of sciatic nerve-injured mice.

As a consequence, while the mechanical antiallodynic effects produced by the repeated administration of DPDPE and JWH-015 were significantly different than those produced by their respective vehicle treated group from days 10 to 20 after nerve injury, the inhibitory effects produced by morphine were only significantly higher than those produced by their respective vehicle treated group from days 10 to 12 after nerve injury induction ($P<0.03$; one way ANOVA followed by the Student–Newman–Keuls test; Fig. 3A). Moreover, the antiallodynic effects produced by the repeated subplantar administration of morphine are significantly lower to those produced by this drug in sham-operated mice from days 11 to 20 after nerve injury ($P<0.001$; one way ANOVA followed by the Student–Newman–Keuls test; Fig. 3B).

Table 1
Effects of the single administration of different doses of morphine, DPDPE, JWH015 or their respective vehicles on the mechanical and thermal responses induced by surgery in the contralateral paws of sham-operated (sham) or sciatic nerve-injured (CCI) mice. Results are shown as mean values ± S.E.M.; n = 5–7 animals per experimental group.

		Mechanical response Von Frey filaments strength (g)			Thermal response Paw lifts (number)		
Doses (nmol)		133	200	400	133	200	400
Sham	Vehicle	2.70 ± 0.13	2.49 ± 0.24	2.55 ± 0.16	0.25 ± 0.25	0.33 ± 0.21	0.13 ± 0.13
	Morphine	2.55 ± 0.20	2.81 ± 0.08	2.62 ± 0.10	0.33 ± 0.21	0.25 ± 0.25	0.33 ± 0.21
CCI	Vehicle	2.48 ± 0.05	2.33 ± 0.19	2.85 ± 0.18	0.50 ± 0.29	0.13 ± 0.13	0.33 ± 0.21
	Morphine	2.31 ± 0.24	2.41 ± 0.14	2.61 ± 0.07	0.13 ± 0.13	0.50 ± 0.29	0.25 ± 0.25
Doses (nmol)		39	77	155	39	77	155
Sham	Vehicle	2.45 ± 0.16	2.55 ± 0.08	2.58 ± 0.22	0.50 ± 0.29	0.25 ± 0.25	0.50 ± 0.29
	DPDPE	2.44 ± 0.13	2.30 ± 0.10	2.55 ± 0.46	0.00 ± 0.00	0.13 ± 0.13	0.17 ± 0.17
CCI	Vehicle	2.38 ± 0.21	2.60 ± 0.09	2.70 ± 0.16	0.25 ± 0.25	0.17 ± 0.17	0.25 ± 0.25
	DPDPE	2.38 ± 0.23	2.35 ± 0.22	2.67 ± 0.34	0.33 ± 0.21	0.14 ± 0.14	0.33 ± 0.21
Doses (nmol)		15	31	92	15	31	92
Sham	Vehicle	2.65 ± 0.23	2.64 ± 0.10	2.66 ± 0.16	0.25 ± 0.25	0.13 ± 0.13	0.50 ± 0.29
	JWH-015	2.46 ± 0.21	2.64 ± 0.07	2.44 ± 0.08	0.25 ± 0.25	0.25 ± 0.25	0.13 ± 0.13
CCI	Vehicle	2.69 ± 0.09	2.69 ± 0.09	2.52 ± 0.09	0.13 ± 0.13	0.13 ± 0.13	0.33 ± 0.21
	JWH-015	2.62 ± 0.10	2.49 ± 0.24	2.72 ± 0.22	0.17 ± 0.17	0.25 ± 0.25	0.25 ± 0.25

Sciatic nerve-injured mice treated with vehicle showed significantly lower thresholds than those obtained in sham-operated mice from days 10 to 20 after surgery ($P<0.001$; one way ANOVA followed by the Student–Newman–Keuls test).

For thermal allodynia, while the antiallodynic effects produced by the repeated administration of DPDPE and JWH-015 were significantly higher than those produced by their respective vehicle treated group from days 10 to 20 after nerve injury, the inhibitory effects

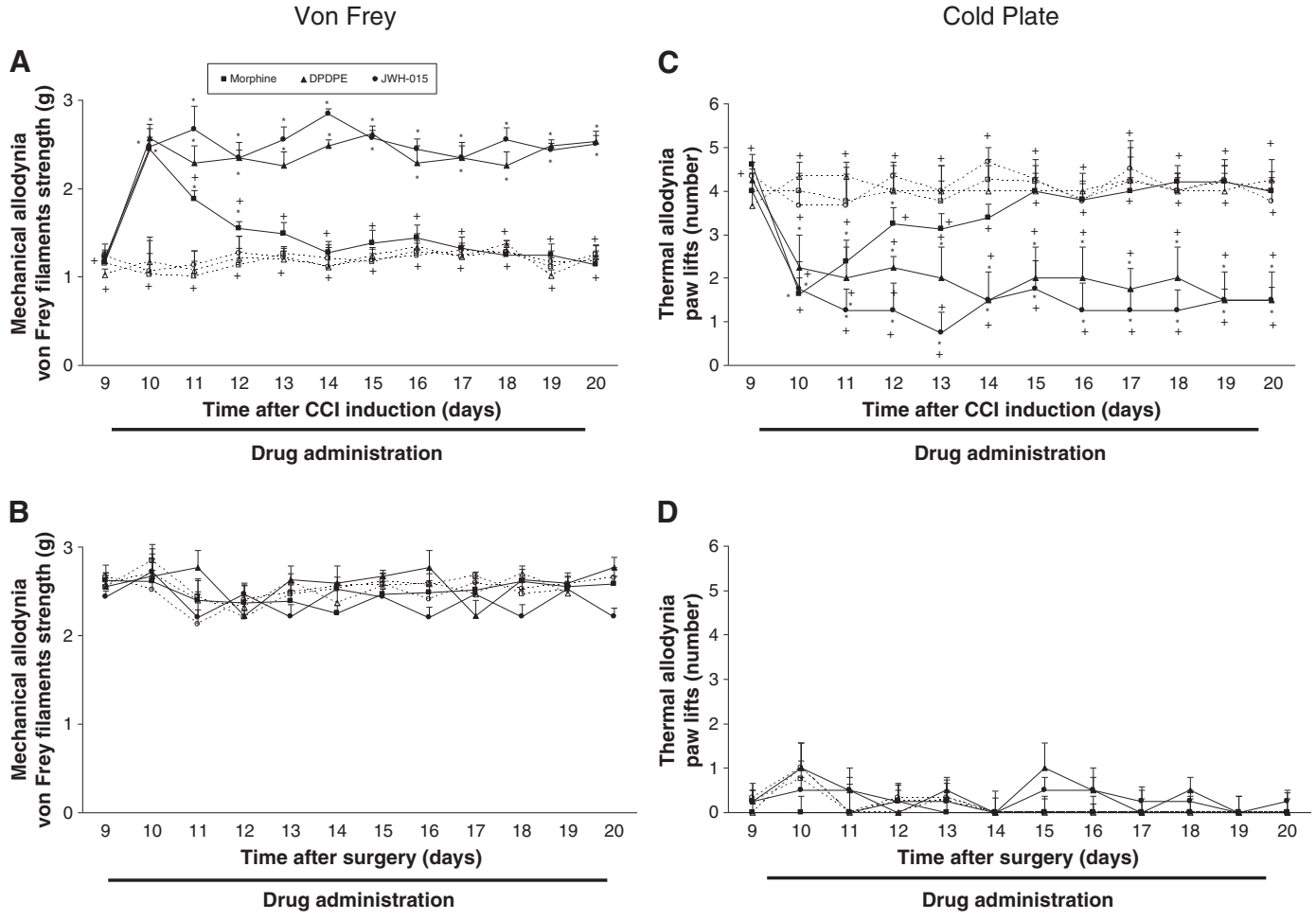

Fig. 3. Effects of the repeated subplantar administration of morphine (400 nmol), DPDPE (155 nmol) or JWH-015 (92 nmol) (continuous lines) or their respective vehicles (discontinuous lines) on the mechanical (A, B) and thermal (C, D) responses in the ipsilateral paw of sciatic nerve-injured (A and C) and sham-operated (B and D) mice from days 10 to 20 after surgery. For each test, drug and day tested, * indicates significant differences vs. their respective vehicle treated mice and + when compared vs. their respective sham-operated mice ($P<0.05$, one way ANOVA followed by the Student–Newman–Keuls test). Results are shown as mean values ± S.E.M.; n = 5–7 animals per experimental group.

produced by morphine were only significantly higher than those produced by their respective vehicle treated group from days 10 to 12 after nerve injury induction ($P<0.05$; one way ANOVA followed by the Student–Newman–Keuls test; Fig. 3C). In addition, the thermal antiallodynic effects produced by the repeated subplantar administration of morphine, DPDPE and JWH-015 were significantly lower to those produced by these drugs in sham-operated mice from days 10 to 20 after nerve injury ($P<0.001$; one way ANOVA followed by the Student–Newman–Keuls test; Fig. 3D). Sciatic nerve-injured mice treated with vehicle showed significantly higher paw lift values than those in sham-operated mice from days 10 to 20 after nerve surgery ($P<0.001$; one way ANOVA followed by the Student–Newman–Keuls test).

Moreover, the repeated subplantar administration of morphine, DPDPE, JWH-015 or vehicle did not produce any thermal or mechanical antiallodynic effect neither in the ipsilateral paw of sham-operated mice (Fig. 3B and D) nor in the contralateral paw of sciatic nerve-injured or sham-operated mice (data not shown).

These results indicated that the mechanical and thermal allodynia induced by nerve injury was completely reversed by the repeated subplantar administration of DPDPE or JWH-015, once a day for 10 consecutive days, while the antiallodynic effects produced by the single subplantar administration of morphine were drastically diminished at 3 days after their repeated injection.

3.5. Involvement of the nitric oxide–cGMP–PKG–JNK pathway in morphine-induced antiallodynic tolerance during neuropathic pain

The role of the nitric oxide–cGMP–PKG–JNK pathway in morphine-induced antiallodynic tolerance during neuropathic pain was assessed by evaluating the mechanical and thermal antiallodynic effects produced by the repeated co-administration of a high dose of morphine (400 nmol) with a sub-analgesic dose of L-NIL (44.7 nmol), ODQ (4 nmol), Rp-8-pCPT-cGMPs (1.2 nmol) or SP600125 (100 nmol) from days 10 to 20 after sciatic nerve injury induction (Fig. 4).

In both behavioral tests, the two way ANOVA repeated measures reveals a significant effect of the treatment ($P<0.001$), time ($P<0.001$) and their interaction ($P<0.001$) in the ipsilateral paw of sciatic nerve-injured mice. Indeed, our results showed that the co-administration of morphine plus L-NIL, ODQ, Rp-8-pCPT-cGMPs or SP600125 avoids the development of morphine-induced tolerance on the inhibition of the mechanical and thermal allodynia induced by nerve injury.

As a consequence, while the local mechanical antiallodynic effects (Fig. 4A and B) produced by morphine combined with L-NIL, ODQ, Rp-8-pCPT-cGMPs or SP600125 from days 10 to 20 after nerve injury were significantly higher than those produced by their respective vehicle treated groups, the inhibitory effects produced by morphine

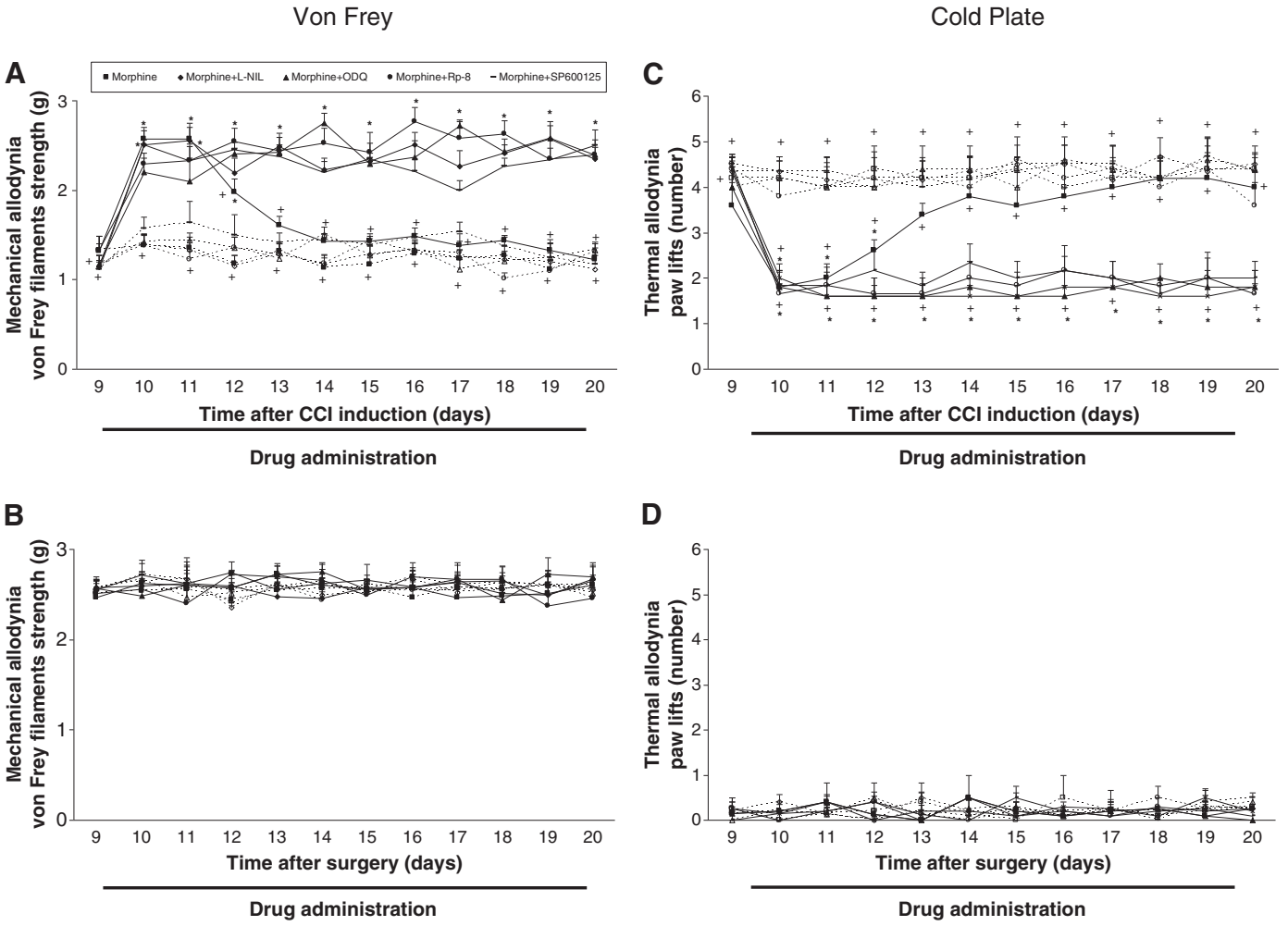

Fig. 4. Effects of the repeated subplantar administration of morphine (400 nmol) alone or combined with L-NIL (44.7 nmol), ODQ (4 nmol), Rp-8-pCPT-cGMPs (Rp-8; 1.2 nmol) or SP600125 (100 nmol) (continuous lines) or their respective vehicles (discontinuous lines), on the mechanical (A, B) and thermal (C, D) responses in the ipsilateral paw of sciatic nerve-injured (A and C) and sham-operated (B and D) mice from days 10 to 20 after surgery. For each test, drug and day tested, * indicates significant differences vs. their respective vehicle treated mice and + when compared vs. their respective sham-operated mice ($P<0.05$, one way ANOVA followed by the Student–Newman–Keuls test). Results are shown as mean values ± S.E.M.; n = 5–7 animals per experimental group.

alone were only significantly different to those produced by their respective vehicle treated group from days 10 to 12 after nerve injury induction ($P<0.05$, one way ANOVA followed by the Student–Newman–Keuls test). In addition, the mechanical antiallodynic effects produced by morphine in sciatic nerve-injured mice were significantly lower to that produced by this drug in sham-operated mice from days 12 to 20 after nerve injury ($P<0.005$, one way ANOVA followed by the Student–Newman–Keuls test). Sciatic nerve-injured mice treated with vehicle showed significantly lower thresholds than those in sham-operated mice from days 10 to 20 after surgery ($P<0.001$; one way ANOVA followed by the Student–Newman–Keuls test).

For thermal allodynia, while the local thermal antiallodynic effects (Fig. 4C and D) produced by morphine combined with L-NIL, ODQ, Rp-8-pCPT-cGMPs or SP600125 were significantly higher than those produced by their respective vehicle treated groups from days 10 to 20 after nerve injury, the inhibitory effects produced by morphine alone were only significantly different to those produced by their respective vehicle treated group from days 10 to 12 after nerve injury induction ($P<0.02$, one way ANOVA followed by the Student–Newman–Keuls test). Sciatic nerve-injured mice treated with vehicle showed significantly higher paw lift values than those observed in sham-operated mice from days 10 to 20 after surgery ($P<0.001$; one way ANOVA followed by the Student–Newman–Keuls test).

Moreover, the repeated peripheral administration of morphine alone or combined with L-NIL, ODQ, Rp-8-pCPT-cGMPs or SP600125 did not have any significant effect in the ipsilateral paw of sham-operated mice (Fig. 4B and D) or in the contralateral paw of sciatic nerve-injured or sham-operated mice (data not shown).

Thus, the inhibition of the nitric oxide–cGMP–PKG–JNK signaling pathway preserved the antiallodynic effects produced by the single subplantar administration of morphine in sciatic nerve-injured mice during their repeated treatment.

4. Discussion

The present study revealed for first time that in contrast to morphine, the repeated subplantar administration of DPDPE and JWH-015 did not induce antiallodynic tolerance in sciatic nerve-injured mice. Moreover, the repeated subplantar co-administration of morphine with specific inhibitors of the peripheral nitric oxide–cGMP–PKG–JNK signaling pathway avoids the development of morphine antiallodynic tolerance during neuropathic pain. These data demonstrate the participation of the peripheral nitric oxide–cGMP–PKG–JNK signaling pathway in the development of morphine-induced tolerance after sciatic nerve injury and propose the inactivation of this pathway as a promising strategy to avoid morphine tolerance during neuropathic pain (Fig. 5).

In a model of sciatic nerve injury-induced neuropathic pain, our results confirmed the mechanical and thermal antiallodynic effects produced by the subplantar administration of μ- and δ-opioid as well as CB_2 receptor agonists in sciatic nerve-injured animals at 21 days after surgery (Hervera et al., 2010a, 2011) and further demonstrated the dose–response antiallodynic effects produced by these drugs at 10 days after sciatic nerve injury. The specificity and the peripheral antiallodynic effects induced by morphine and DPDPE after sciatic nerve injury were demonstrated with the complete reversion of their effects by their local co-administration with selective antagonists (CTAP and naltrindole) or the non-selective peripherally acting opioid receptor antagonist (naloxone methiodide), which did not have any effect when were administered alone. The specificity of the antiallodynic effects of JWH-015 after sciatic nerve injury was also demonstrated by the complete reversion of their effects with their local co-administration with a selective CB_2 (AM630), but not CB_1 (AM251), receptor antagonist. In addition, the fact that highest doses of morphine, DPDPE or JWH-015 did not produce any significant effect in the contralateral paw of sciatic nerve-injured mice also validates a peripheral site of action of these drugs under neuropathic pain conditions.

Our results also demonstrated that the mechanical and thermal antiallodynic effects produced by the single subplantar administration of a high dose of morphine were drastically decreased after 3 days of daily repeated administration, while the inhibitory effects produced by the single injection of a high dose of DPDPE or JWH-015 were preserved following their repeated subplantar administration. These findings provide the first evidence that the consecutive subplantar administration of morphine, but not DPDPE or JWH-015, induces the development of tolerance to its antiallodynic effects during neuropathic pain. In accordance to our results, others studies have reported that the chronic subcutaneous or intrathecal administration of μ-opioid receptor agonists induced antinociceptive tolerance under neuropathic pain conditions (Ossipov et al., 1995). However and in contrast to our results, other works demonstrated that the repeated subcutaneous administration of morphine did not induce antihyperalgesic and antiallodynic tolerance after the partial sciatic nerve injury-induced neuropathic pain (Narita et al., 2011; Sounvoravong et al., 2004). These discrepancies could be related to the type of neuropathic pain model (partial sciatic nerve injury vs. chronic constriction injury) as well as to the route of drug administration.

Relative to DPDPE and JWH-015, our findings supported the absence of antinociceptive tolerance following the repeated subcutaneous and/or spinal cord administration of δ-opioid and CB_2 receptor agonists in animals with chronic pain (Beaudry et al., 2009; Chattopadhyay et al., 2008; Codd et al., 2009; Hama and Sagen, 2009; Romero-Sandoval et al., 2008) and further demonstrated that the repeated subplantar administration of these drugs, besides to be potent analgesic agents to treat neuropathic pain, are devoid of antiallodynic tolerance. The absence of tolerance of DPDPE or JWH-015 during neuropathic pain, contrarily to morphine, could be mainly explained by the different signaling pathways activated by these drugs after nerve injury. Thus, while morphine activates the NO–cGMP–PKG signaling pathway to produce antinociception (Hervera et al., 2011) the activation of this pathway blocks the inhibitory effects produced by DPDPE and JWH-015 under neuropathic pain conditions (Hervera et al., 2010a). These results support the idea proposed by other authors, that the repeated activation of the peripheral δ-opioid receptors reduces the principal symptoms of neuropathic pain without tolerance by reducing the voltage-gated sodium channels (Nav1.7 and Nav1.8) through the activation of protein kinase C (Chattopadhyay et al., 2008; Gaveriaux-Ruff et al., 2011). In a similar way, the continuous activation of spinal CB_2 receptors could also reduce neuropathic pain without tolerance by inhibiting the activated microglia induced by nerve injury (Romero-Sandoval et al., 2008).

The mechanisms underlying the development of tolerance to the antinociceptive action of morphine are not clear. Many researchers have suggested that, one of the multiple systems involved in the development of morphine tolerance, is the activation of the nitric oxide–cGMP–PKG–JNK signaling pathway (Guo et al., 2009; Heinzen and Pollack, 2004b; Joharchi and Jorjani, 2007; Ozdemir et al., 2011). Indeed, the co-administration of morphine with specific NOS or cGMP inhibitors (Guo et al., 2009; Ozdemir et al., 2011; Santamarta et al., 2005) as well as the genetic deletion of NOS1 and NOS2 genes attenuated the development of morphine tolerance under basal and inflammatory pain states (Heinzen and Pollack, 2004b; Romero et al., 2010). Moreover, the co-administration of a nitric oxide donor with morphine increases the development of functional tolerance to its analgesic effects under basal conditions (Ozdemir et al., 2011). Even so, the role of the nitric oxide–cGMP–PKG–JNK signaling pathway in the development of peripheral morphine tolerance under neuropathic pain conditions remained unclear.

In this study, the possible involvement of the nitric oxide–cGMP–PKG–JNK signaling pathway in the development of antiallodynic

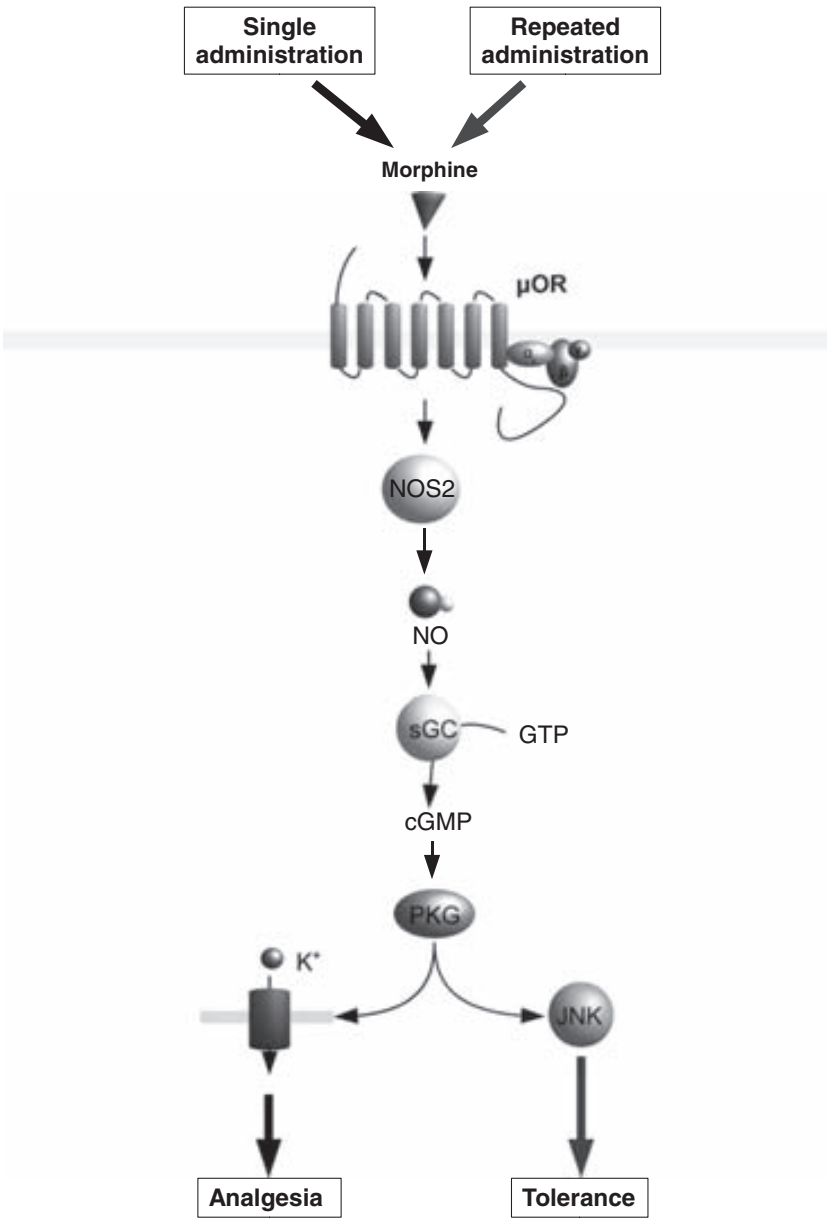

Fig. 5. Schematic representation of the nitric oxide–cGMP–PKG signaling pathway activated by the single or repeated subplantar administration of morphine during neuropathic pain in mice.

morphine tolerance in animals, with a well established chronic sciatic nerve injury-induced neuropathic pain, was assessed by evaluating the mechanical and thermal antiallodynic effects produced by the repeated subplantar co-administration of a high dose of morphine combined with a sub-analgesic dose of a selective NOS2, soluble guanylate cyclase, PKG or JNK inhibitor during 10 consecutive days. Our findings show that the local administration of morphine with L-NIL, ODQ, Rp-8-pCPT-cGMPs or SP600125 avoided the development of morphine tolerance by preserving the antiallodynic effects produced by the single administration of morphine during neuropathic pain. These data show for first time, that the peripheral nitric oxide–cGMP–PKG–JNK signaling pathway, triggered by NOS2, plays a critical role in the development of morphine antiallodynic tolerance in sciatic nerve injury-induced neuropathic pain.

Therefore, while the single subplantar administration of morphine inhibited the mechanical and thermal allodynia induced by sciatic nerve injury through the activation of the peripheral nitric oxide–cGMP–PKG–ATP-sensitive $K+$ (KATP) channels signaling pathway which culminates in the hyperpolarization of nociceptive neurons inducing analgesia (Hervera et al., 2011), the repeated subplantar administration of morphine-induced antiallodynic tolerance by the activation of the peripheral nitric oxide–cGMP–PKG–JNK signaling pathway which culminates in a neuronal sensitization causing morphine tolerance. These data support the idea that nitric oxide, derived from NOS2, seems to be required for the peripheral acute antiallodynic effects of morphine as well as to the development of morphine antiallodynic tolerance during neuropathic pain, where the PKG activation of the KATP channels or JNK pathway makes the different effects produced by the acute and chronic peripheral administration of morphine.

In summary, our data indicate that the use of the local administration of δ-opioid and CB_2 receptor agonists may be a well alternative for the management of peripheral neuropathic pain due to the lack of antinociceptive tolerance after their repeated administration. Our results also demonstrate the participation of the peripheral nitric oxide–cGMP–PKG–JNK signaling pathway in the development

of morphine antiallodynic tolerance after sciatic nerve injury and propose the inactivation of this pathway as a promising strategy to avoid morphine tolerance under a neuropathic pain state.

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5.5. *Carbon monoxide reduces neuropathic pain and spinal microglial activation by inhibiting nitric oxide synthesis in mice*

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Carbon Monoxide Reduces Neuropathic Pain and Spinal Microglial Activation by Inhibiting Nitric Oxide Synthesis in Mice

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Abstract

Background: Carbon monoxide (CO) synthesized by heme oxygenase 1 (HO-1) exerts antinociceptive effects during inflammation but its role during neuropathic pain remains unknown. Our objective is to investigate the exact contribution of CO derived from HO-1 in the modulation of neuropathic pain and the mechanisms implicated.

Methodology/Principal Findings: We evaluated the antiallodynic and antihyperalgesic effects of CO following sciatic nerve injury in wild type (WT) or inducible nitric oxide synthase knockout (NOS2-KO) mice using two carbon monoxide-releasing molecules (CORM-2 and CORM-3) and an HO-1 inducer (cobalt protoporphyrin IX, CoPP) daily administered from days 10 to 20 after injury. The effects of CORM-2 and CoPP on the expression of HO-1, heme oxygenase 2 (HO-2), neuronal nitric oxide synthase (NOS1) and NOS2 as well as a microglial marker (CD11b/c) were also assessed at day 20 after surgery in WT and NOS2-KO mice. In WT mice, the main neuropathic pain symptoms induced by nerve injury were significantly reduced in a time-dependent manner by treatment with CO-RMs or CoPP. Both CORM-2 and CoPP treatments increased HO-1 expression in WT mice, but only CoPP stimulated HO-1 in NOS2-KO animals. The increased expression of HO-2 induced by nerve injury in WT, but not in NOS2-KO mice, remains unaltered by CORM-2 or CoPP treatments. In contrast, the over-expression of CD11b/c, NOS1 and NOS2 induced by nerve injury in WT, but not in NOS2-KO mice, were significantly decreased by both CORM-2 and CoPP treatments. These data indicate that CO alleviates neuropathic pain through the reduction of spinal microglial activation and NOS1/NOS2 over-expression.

Conclusions/Significance: This study reports that an interaction between the CO and nitric oxide (NO) systems is taking place following sciatic nerve injury and reveals that increasing the exogenous (CO-RMs) or endogenous (CoPP) production of CO may represent a novel strategy for the treatment of neuropathic pain.

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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. The mechanisms contributing to this syndrome involve local inflammatory responses, changes in the plasticity of neuronal nociceptive pathways and activation of spinal microglia [1].

Nitric oxide (NO) synthesized either by neuronal (NOS1) or inducible (NOS2) nitric oxide synthase mediates numerous neuropathic pain symptoms via cGMP-PKG pathway activation [2–4]. Accordingly, the expression of both enzymes is up-regulated in the spinal cord and dorsal root ganglia of animals with neuropathic pain [5–7]. The hypersensitivity effects induced by nerve injury are significantly diminished or absent in NOS1 (NOS1-KO) and NOS2 (NOS2-KO) knockout animals [6,8] or reversed by the administration of selective NOS, guanylate cyclase or PKG inhibitors [4,5,9]. Moreover, the intraperitoneal administration of a NO donor potentiates the mechanical and thermal hypersensitivity induced by neuropathic pain [10].

Carbon monoxide (CO) synthesized by heme oxygenases-1 (HO-1) or 2 (HO-2), is another gaseous neurotransmitter implicated in the modulation of nociceptive pathways. However, while HO-2 exerts a pronociceptive effect during neuropathic pain [11], HO-1 plays an important role in the modulation of acute inflammatory pain [12,13]. Consequently, the expression of HO-2 increases after nerve injury and the mechanical and thermal hypersensitivity to pain induced by nerve injury has been shown to be markedly decreased in HO-2-KO mice [14,15]. In contrast, the over-expression of HO-1 is associated with potent anti-inflammatory and antinociceptive effects during inflammatory pain [11,12]. However, the exact contribution of CO synthesized by HO-1 in the modulation of the main symptoms of neuropathic pain induced by sciatic nerve injury remains unknown.

It is interesting to note that, similarly to NO, CO also activates the cGMP-PKG pathway. As a result, a cross-talk has been reported between these two gases in several *in vitro* and *in vivo* models. For instance, NOS2-derived NO as well as NO donors contribute to induction of HO-1 by cGMP-PKG-dependent pathway activation [16], indicating that NO is an important regulator of CO produced by HO-1 [17–19]. Studies *in vivo* also demonstrated that while the antihyperalgesic effects induced by CO depend on the integrity of the NOS pathways, the antinociceptive responses produced by NO are independent of CO [13]. Nevertheless, the possible interaction between the NO and CO systems during neuropathic pain has not been investigated.

Carbon monoxide-releasing molecules (CO-RMs) are a new class of chemical agents able to reproduce numerous biological effects of HO-1-derived CO [20–23]. Several authors has shown that CO-RMs and HO-1 induction using cobalt protoporphyrin IX (CoPP) exert potent anti-inflammatory effects in vivo [11,24]. However, the possible antiallodynic and antihyperalgesic effects produced by these compounds during neuropathic pain have not been evaluated.

It is well known that microglia modulates several neuronal changes occurring during the development and maintenance of numerous chronic states, including neuropathic pain [1,25]. Interestingly, the administration of inhibitors of microglial cells activation significantly reduces the behavioral symptoms of neuropathic pain [25]. Thus, we investigated the potential role of CO and HO-1-derived CO in the modulation of neuropathic pain as well as the possible mechanisms involved in this action. Specifically, in sciatic nerve-injured WT and NOS2-KO mice we evaluated: 1) the mechanical antiallodynic, thermal antihyperalgesic and thermal antiallodynic effects produced by the administration of two CO-RMs, tricarbonyldichlororuthrnium(II) dimer (CORM-2) and tricarbonylchloro (glycinate)ruthenium (II) (CORM-3) as well as a classical HO-1 inducer (CoPP) and 2) the effect of these treatments on the expression of HO and NOS isoforms as well as CD11b/c, a marker of microglia activation, in the dorsal root ganglia and spinal cord of these animals.

Results

Effect of CORM-2, CORM-3 and CoPP Treatments in WT and NOS2-KO Sciatic Nerve-injured Mice

Animals were intraperitoneally administered twice daily with CORM-2 (5 mg/kg), CORM-3 (5 mg/kg), CoPP (2.5 mg/kg) or vehicle for a period of 11 days after surgery. On days 1, 5 and 11 of treatment mice were sequentially assessed for mechanical allodynia, thermal hyperalgesia and thermal allodynia.

Sciatic nerve injury led to a significant decrease of the threshold for evoking hind paw withdrawal to a mechanical stimulus in WT animals (Fig. 1A) which response was abolished in NOS2-KO mice (Fig. 1D). That is, mechanical allodynia was developed in vehicle treated WT mice exposed to sciatic nerve injury from days 10 to 20 after surgery when compared to sham-operated mice ($p<0.001$; one way ANOVA). This mechanical allodynia was significantly attenuated in nerve-injured WT mice repeatedly treated with CORM-2, CORM-3 or CoPP (Fig. 1A). The three-way ANOVA revealed a significant effect of the surgery, treatment and time ($p<0.001$) and a significant interaction between treatment and time ($p<0.001$), surgery and treatment ($p<0.001$), surgery and time ($p<0.001$) and between surgery, treatment and time ($p<0.001$). Indeed, mechanical allodynia was equally reduced on day 1 in CORM-2 and CORM-3, but not in CoPP, treated mice ($p<0.001$; one way ANOVA vs. vehicle nerve-injured treated mice), although the antiallodynic efficacy of all of them increased progressively on days 5 and 11 of treatment ($p<0.001$; one way ANOVA vs. vehicle nerve-injured treated mice). In sham-operated WT mice CORM-2, CORM-3 and CoPP treatments did not produce any effect as compared to sham-operated vehicle treated WT mice for the whole duration of the experiment.

The effects of CORM-2, CORM-3 or CoPP treatments in NOS2-KO mice exposed to sciatic nerve injury have been also evaluated. The three-way ANOVA did not reveal any significant effect of the surgery, treatment and time and non significant interaction between theme was demonstrated. Mechanical allodynia was not developed in NOS2-KO mice and the systemic administration of CORM-2, CORM-3 or CoPP did not alter the lack of mechanical allodynia observed in these nerve-injured animals (Fig. 1D). Sham operation did not produce any effect neither in CORM-2, CORM-3 or CoPP nor in vehicle treated NOS2-KO mice for the whole duration of the experiment.

Sciatic nerve injury led to a significant decrease of the threshold for evoking paw withdrawal to a thermal stimulus in WT mice from days 10 to 20 after surgery as compared to sham-operated mice ($p<0.001$; one way ANOVA). This thermal hyperalgesia was significantly attenuated in nerve-injured WT mice repeatedly treated with CORM-2, CORM-3 or CoPP (Fig. 1B). The three-way ANOVA revealed a significant effect of the surgery, treatment and time ($p<0.001$) and a significant interaction between treatment and time ($p<0.001$), surgery and treatment ($p<0.001$), surgery and time ($p<0.001$) as well as between surgery, treatment and time ($p<0.001$). Indeed, thermal hyperalgesia was completely blocked on day 1 in CORM-2 and CORM-3 treated WT mice ($p<0.001$; one way ANOVA vs. vehicle nerve-injured treated mice) and this level of efficacy was similarly maintained for both compounds on days 5 and 11 of treatment ($p<0.001$; one way ANOVA vs. vehicle nerve-injured treated mice). In contrast, thermal hyperalgesia was only significantly reduced by CoPP on day 5 ($p<0.001$; one way ANOVA vs. vehicle nerve-injured treated mice) and its antihyperalgesic efficacy increased progressively on day 11 of treatment ($p<0.001$; one way ANOVA vs. vehicle nerve-injured treated mice). In sham-operated WT mice CORM-2, CORM-3 and CoPP treatments did not produce any effect as compared to sham-operated vehicle treated WT mice for the whole duration of the experiment.

The effects of CORM-2, CORM-3 or CoPP treatments in NOS2-KO mice after sciatic nerve injury have been also evaluated. The three-way ANOVA did not reveal any significant effect of the surgery, treatment and time and non significant interaction between theme was demonstrated. Thermal hyperalgesia was not developed in NOS2-KO mice and the systemic administration of CO-RM's or CoPP did not alter the absence of thermal hypersensitivy observed in these nerve-injured NOS2-KO animals (Fig. 1E). Sham operation did not produce any effect neither in CORM-2, CORM-3 or CoPP nor in vehicle treated NOS2-KO mice for the whole duration of the experiment.

Sciatic nerve injury increased the number of ipsilateral paw lifts during cold thermal stimulation in WT mice from days 10 to 20 after surgery as compared to sham-operated WT mice ($p<0.001$; one way ANOVA). This thermal allodynia was significantly attenuated in nerve-injured WT mice repeatedly treated with CORM-2, CORM-3 or CoPP (Fig. 1C). The three-way ANOVA revealed a significant effect of the surgery ($p<0.001$), treatment ($p<0.001$) and time ($p<0.010$) as well as a significant interaction between treatment and time ($p<0.013$), surgery and treatment ($p<0.022$), surgery and time ($p<0.001$) and the triple interaction between surgery, treatment and time ($p<0.014$). Indeed, although thermal allodynia was similarly reduced on days 1 and 5 in

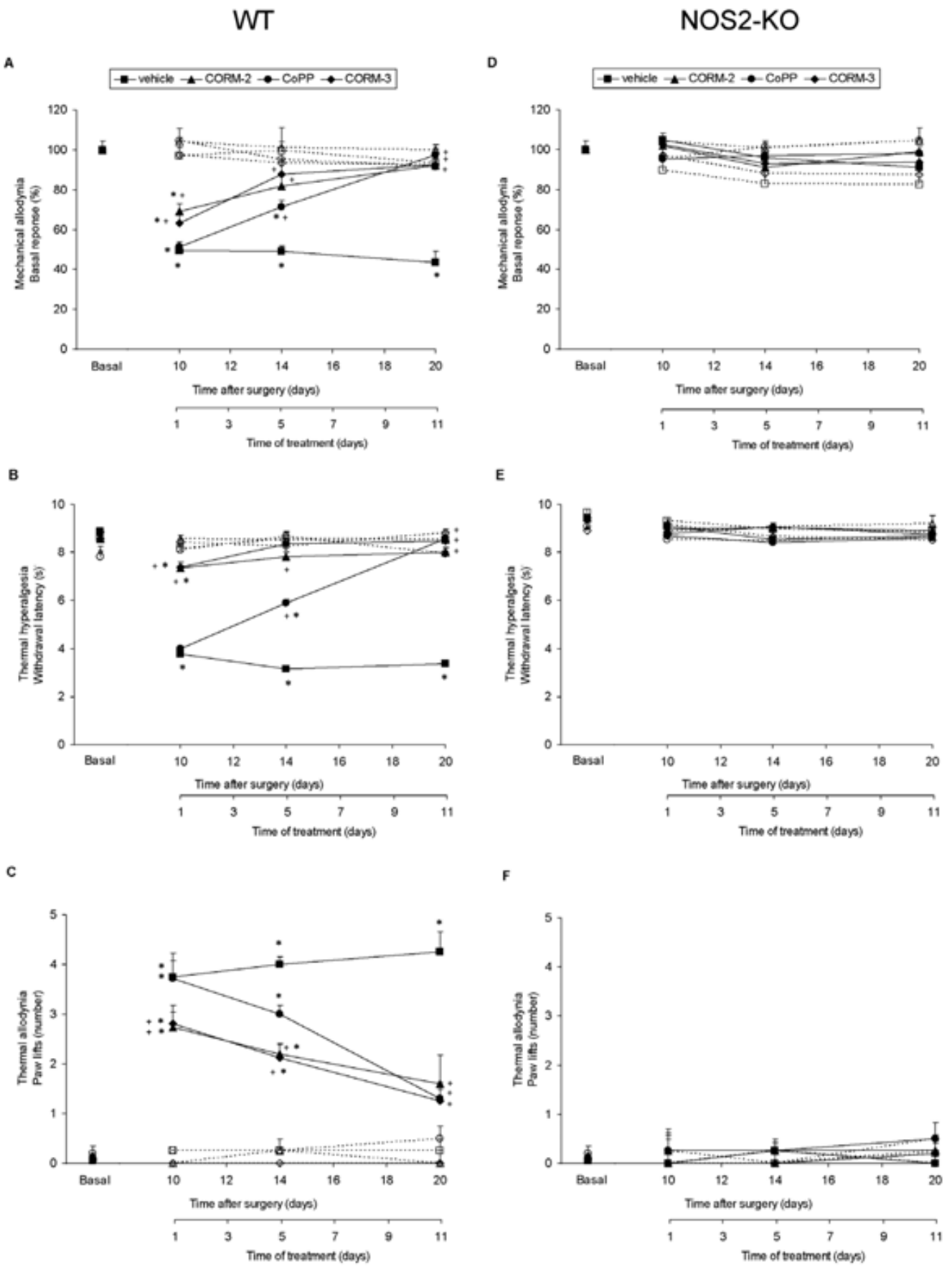
WT
NOS2-KO
A
B
C
D
E
F
vehicle
CORM-2
CoPP
CORM-3
Mechanical allodynia
Basal reponse (%)
Thermal hyperalgesia
Withdrawal latency (s)
Thermal allodynia
Paw lifts (number)
Basal
Time after surgery (days)
Time of treatment (days)

Figure 1. Effect of CORM-2, CORM-3 and CoPP on sciatic nerve-injured WT and NOS2-KO mice. The development of the mechanical allodynia (A and D), thermal hyperalgesia (B and E) and thermal allodynia (C and F) in sciatic nerve-injured (continuous lines) and sham-operated (discontinuous lines) WT or NOS2-KO mice treated during 11 consecutive days with vehicle, CORM-2, CORM-3 or CoPP at 10, 14 and 20 days after surgery is shown. For each genotype, test, day and drug evaluated, *indicates significant differences when compared vs. vehicle sham-operated group ($p<0.001$, one-way ANOVA followed by the Student Newman Keuls test) and +indicates significant differences when compared vs. vehicle nerve-injured group ($p<0.001$, one-way ANOVA followed by the Student Newman Keuls test). Results are shown as mean values ± SEM; n = 7 animals per experimental group.
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CORM-2 and CORM-3, but not in CoPP, treated WT mice ($p<0.001$; one way ANOVA vs. vehicle nerve-injured treated mice) the antiallodynic efficacy of all treatments increased progressively on day 11, where thermal allodynia was completely blocked by CORM-2, CORM-3 or CoPP treatments ($p<0.001$; one way ANOVA vs. vehicle nerve-injured treated mice). In sham-operated WT mice CORM-2, CORM-3 or CoPP treatments did not produce any effect as compared to sham-operated vehicle treated WT animals for the whole duration of the experiment.

The effects of CORM-2, CORM-3 or CoPP treatments in NOS2-KO mice after sciatic nerve injury have been also evaluated. The three-way ANOVA did not reveal any significant effect of the surgery, treatment and time and non significant interaction between theme was demonstrated. Thermal allodynia was not developed in NOS2-KO mice and the systemic administration of CORM-2, CORM-3 or CoPP did not alter the absence of thermal allodynia observed in these NOS2-KO nerve-injured animals (Fig. 1F). Sham operation did not produce any effect neither in CORM-2, CORM-3 or CoPP nor in vehicle treated NOS2-KO mice for the whole duration of the experiment.

In all experiments, CORM-2, CORM-3 or CoPP treatments did not have any significant effect in the contralateral paw of sciatic nerve-injured or sham-operated WT or NOS2-KO animals (data not shown).

Effect of CORM-2 and CoPP on HO-1 and HO-2 Protein Expression in the Dorsal Root Ganglia and Spinal Cord from WT and NOS2-KO Sciatic Nerve-injured Mice

The protein levels of HO-1 in the dorsal root ganglia (A) and spinal cord (B) from sciatic nerve-injured WT or NOS2-KO mice treated with vehicle, CORM-2 or CoPP are shown in Fig. 2. For each tissue, the expression of HO-1 from sham-operated WT or NOS2-KO vehicle treated mice has been also shown. In both tissues, non-significant differences were found between genotypes as compared to the expression of HO-1 among them in vehicle sham-operated or vehicle sciatic nerve-injured mice treated with vehicle. However, while in sciatic nerve-injured WT mice the dorsal root ganglia and spinal cord expression of HO-1 was significantly increased by CORM-2 or CoPP treatments ($p<0.001$; one-way ANOVA vs. sham-operated and nerve-injured vehicle treated WT mice), in NOS2-KO mice the expression of this enzyme was only enhanced by CoPP ($p<0.001$; one-way ANOVA vs. to the other groups). In addition, the enhanced

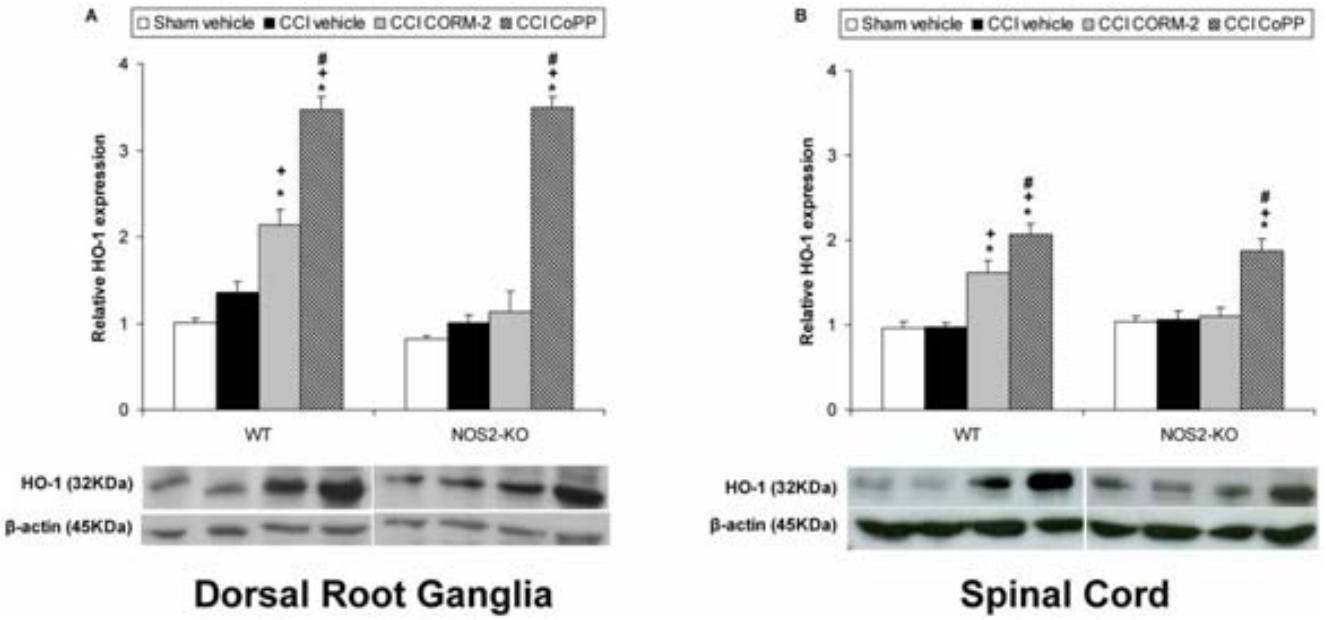

Figure 2. Effect of CORM-2 and CoPP on HO-1 protein expression from sciatic nerve-injured WT and NOS2-KO mice. The protein expression in the ipsilateral site of the dorsal root ganglia (A) and the lumbar section of the spinal cord (B) from sciatic nerve-injured (CCI) WT and NOS2-KO mice treated with vehicle, CORM-2 or CoPP at 20 days after surgery is represented. The expression of HO-1 in the dorsal root ganglia and spinal cord from sham-operated WT and NOS2-KO mice treated with vehicle has been also represented as controls (sham-vehicle). In both figures and genotypes, *indicates significant differences when compared vs. their respective sham-operated vehicle treated mice ($*p<0.05$, one-way ANOVA followed by the Student Newman Keuls test), +indicates significant differences when compared vs. their respective sciatic nerve-injured vehicle treated mice ($+p<0.05$, one-way ANOVA followed by the Student Newman Keuls test), #indicates significant differences when compared vs. their respective sciatic nerve-injured CORM-2 treated mice ($\#p<0.05$, one-way ANOVA followed by the Student Newman Keuls test). Representative examples of western blots for HO-1 protein (32 kDa) in which β-actin (45 kDa) was used as a loading control are also shown. Data are expressed as mean values ± SEM; n = 5 samples per group.
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expression of HO-1 induced by CoPP in the dorsal root ganglia and spinal cord of sciatic nerve-injured WT mice is higher than those produced by CORM-2 treatment ($p<0.001$; one-way ANOVA followed by Student Newman Keuls test).

The protein levels of HO-2 in the dorsal root ganglia (A) and spinal cord (B) from sciatic nerve-injured WT or NOS2-KO mice treated with vehicle, CORM-2 or CoPP are shown in Fig. 3. The expression of HO-2 from sham-operated WT or NOS2-KO mice treated with vehicle is also shown. Sciatic nerve injury significantly increased the dorsal root ganglia and spinal cord levels of HO-2 in vehicle, CORM-2 and CoPP treated WT mice ($p<0.001$; one-way ANOVA vs. sham-operated vehicle treated WT mice). In contrast, the dorsal root ganglia and spinal cord expression of HO-2 was not altered in vehicle, CORM-2 and CoPP treated nerve-injured NOS2-KO mice.

Effect of CORM-2 and CoPP on CD11b/c Protein Expression in the Spinal Cord from WT and NOS2-KO Sciatic Nerve-injured Mice

We next investigated whether the increased spinal cord expression of CD11b/c induced by nerve injury was altered by CORM-2 and CoPP treatments. The expression of CD11b/c from sham-operated WT or NOS2-KO vehicle treated mice is also evaluated (Fig. 4). Our results showed that the repeated treatment with CORM-2 and CoPP inhibited the increased expression of CD11b/c induced by sciatic nerve injury in WT mice ($p<0.001$; one-way ANOVA vs. sham-operated WT mice). Interestingly, sciatic nerve injury did not increase the protein levels of CD11b/c in NOS2-KO mice, which expression remains unaltered after CORM-2 or CoPP treatment.

Effect of CORM-2 and CoPP on NOS1 and NOS2 Protein Expression in the Spinal Cord from WT and NOS2-KO Sciatic Nerve-injured Mice

The protein levels of NOS1 (A) and NOS2 (B) in the spinal cord from sciatic nerve-injured WT or NOS2-KO mice treated with vehicle, CORM-2 or CoPP are shown in Fig. 5. The expression of NOS1 and NOS2 from sham-operated WT or NOS2-KO mice treated with vehicle has been also shown. Sciatic nerve injury significantly increased the protein levels of NOS1 and NOS2 in the spinal cord of WT mice ($p<0.001$; one-way ANOVA vs. sham-operated vehicle treated WT mice), which levels were significantly reduced by the repeated intraperitoneal administration of CORM-2 and CoPP. In contrast, sciatic nerve injury did not alter the spinal cord expression of NOS1 as well as of NOS2 in vehicle, CORM-2 or CoPP treated NOS2-KO animals, which protein levels were similar to those obtained in sham-operated NOS2-KO mice.

A summary of the results from the protein expression studies obtained in the ipsilateral site of the dorsal root ganglia and/or the lumbar section of the spinal cord from sciatic nerve-injured WT and NOS2-KO mice treated with vehicle, CORM-2 or CoPP is shown in Table 1.

Discussion

In the present study we demonstrated, for first time, that the repeated intraperitoneal administration of CORM-2, CORM-3 or CoPP significantly reduced the mechanical allodynia, thermal hyperalgesia and thermal allodynia induced by the chronic constriction of sciatic nerve in WT mice. Our results also indicate that these effects are mainly produced by CO synthesized by HO-

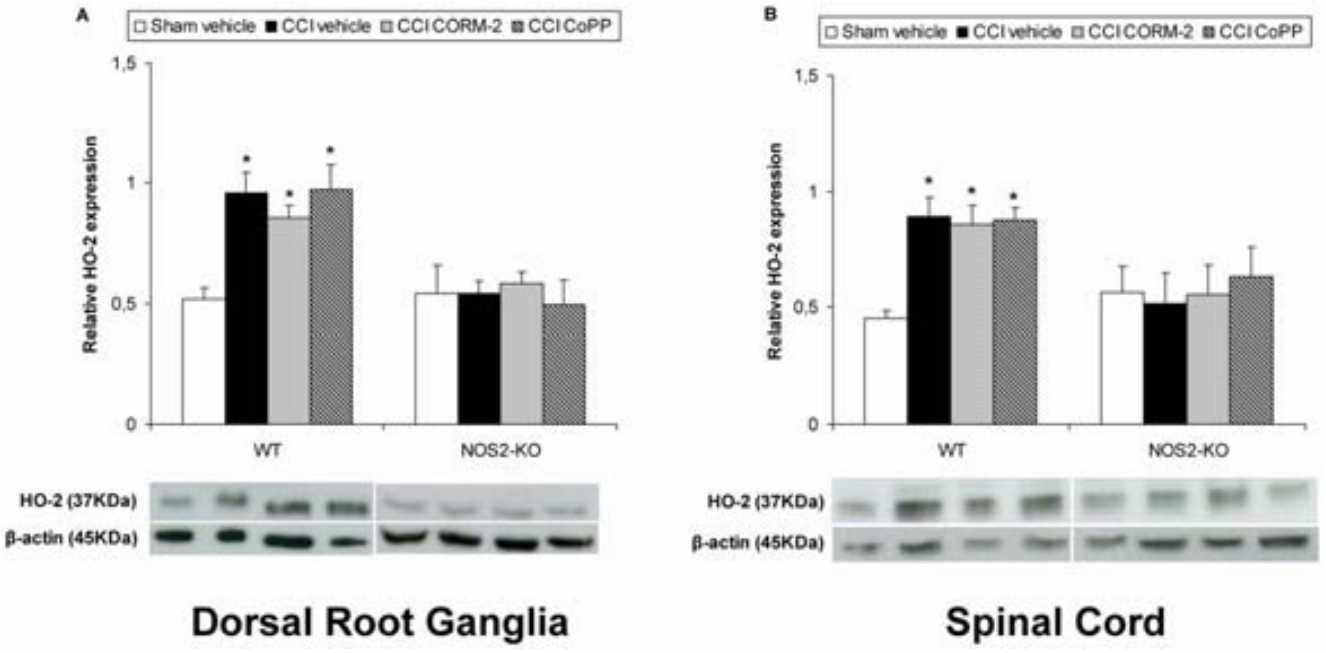

Figure 3. Effect of CORM-2 and CoPP on HO-2 protein expression from sciatic nerve-injured WT and NOS2-KO mice. The protein expression in the ipsilateral site of the dorsal root ganglia (A) and the lumbar section of the spinal cord (B) from sciatic nerve-injured (CCI) WT and NOS2-KO mice treated with vehicle, CORM-2 or CoPP at 20 days after surgery is represented. The expression of HO-2 in the dorsal root ganglia and spinal cord from sham-operated WT and NOS2-KO mice treated with vehicle has been also represented as controls (sham-vehicle). In both figures and genotypes, *indicates significant differences when compared vs. their respective sham-operated vehicle treated mice (*$p<0.05$, one-way ANOVA followed by the Student Newman Keuls test). Representative examples of western blots for HO-2 protein (37 kDa) in which β-actin (45 kDa) was used as a loading control are also shown. Data are expressed as mean values ± SEM; n = 5 samples per group.
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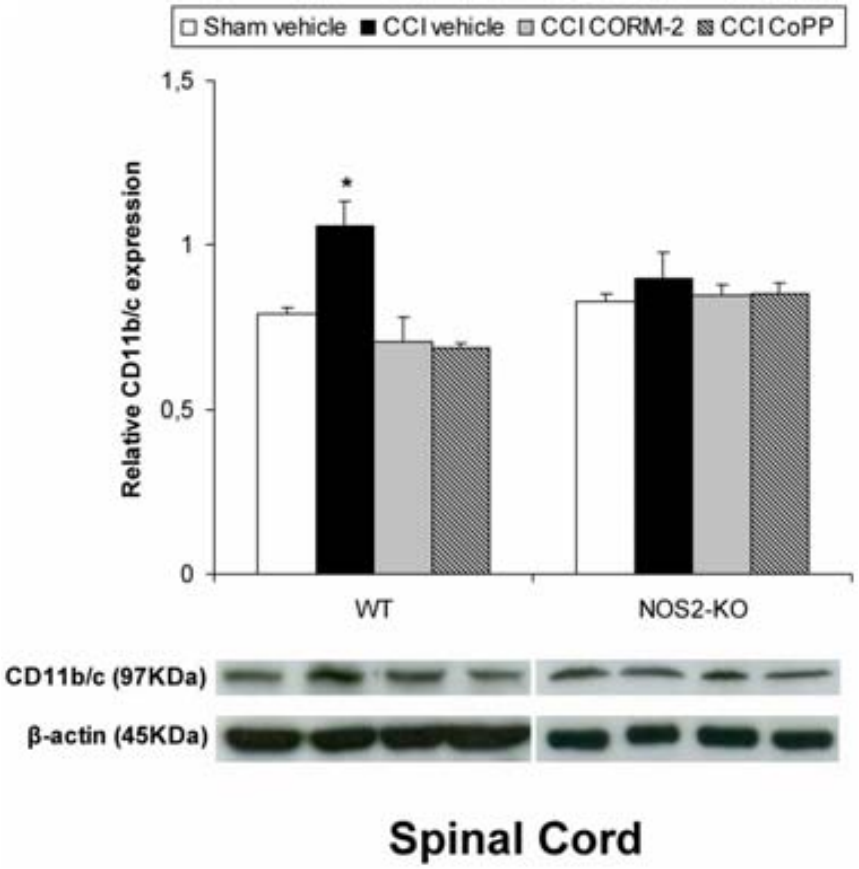

Figure 4. Effect of CORM-2 and CoPP on CD11b/c protein expression from sciatic nerve-injured WT and NOS2-KO mice. The protein expression in the ipsilateral site of the lumbar section of the spinal cord from sciatic nerve-injured (CCI) WT and NOS2-KO mice treated with vehicle, CORM-2 or CoPP at 20 days after surgery is represented. The expression of CD11b/c in the spinal cord from sham-operated WT and NOS2-KO mice treated with vehicle has been also represented as controls (sham-vehicle). In this figure *indicates significant differences when compared vs. sham-operated vehicle treated WT mice (*$p<0.05$, one-way ANOVA followed by the Student Newman Keuls test). Representative examples of western blots for CD11b/c protein (97 kDa) in which β-actin (45 kDa) was used as a loading control are also shown. Data are expressed as mean values ± SEM; n = 5 samples per group.
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1, through reduction of spinal microglial activation and NOS1/NOS2 over-expression.

It is well known that CO has potent antinociceptive and anti-inflammatory effects during inflammatory diseases [12,24,26]. In accordance, the results presented here further demonstrate that the intraperitoneal administration of CO by using CORM-2 and CORM-3 as well as the induction of endogenous CO by injection of the HO-1 inducer CoPP during 11 consecutive days significantly reduced the mechanical and thermal hypersensitivity induced by sciatic nerve injury in WT mice. Our results also revealed that, although at 11 days of treatment all compounds inhibited neuropathic pain with similar effectiveness, the anti-allodynic and antihyperalgesic effects produced by CORM-2 and CORM-3 on days 1 and 5 of treatment were significantly higher than those produced by CoPP. These results could be related to the more direct release of CO by CO-RMs, in contrast to the HO-1 over-expression required by CoPP to synthesize CO. Our data also indicate that CO-RMs are more effective on the inhibition of thermal hyperalgesia than of mechanical and thermal allodynia induced by sciatic nerve injury.

In accordance to our previous studies [6], our findings showed that the principal manifestations of neuropathic pain induced by sciatic nerve injury were completely abolished in NOS2-KO mice. In addition, the lack of thermal and mechanical hypersensitivity observed in sciatic nerve-injured NOS2-KO mice remained unchanged after CO-RMs or CoPP treatments, similarly to that occurs in sham-operated WT or NOS2-KO treated animals.

As previously shown in other inflammatory models [27,28], our results also demonstrated that the expression of HO-1 was significantly increased in the dorsal root ganglia and spinal cord of sciatic nerve-injured WT mice treated with CORM-2 or CoPP. As expected, the enhanced expression of HO-1 induced by CoPP (a HO-1 expression inducer) was higher than those produced by CORM-2 treatment. Interestingly, in sciatic nerve-injured NOS2-KO mice the expression of HO-1 was only increased after CoPP treatment. These results indicate that CORM-2 requires the presence of NOS2 to enhance the expression of HO-1, revealing that NO plays a key role in the positive feedback regulation of HO-1 over-expression during neuropathic pain. In accordance to our results other studies have been also demonstrated that the antihyperalgesic effects induced by CO in acute pain depend on the integrity of NO pathway [13].

In contrast to HO-1-derived CO, CO synthesized by HO-2 contributes to the progression of neuropathic pain. Thus, the lack of HO-2 appears to prevent the mechanical and thermal hypersensitivity to pain induced by nerve injury and the expression of this enzyme increases during neuropathic pain [14,15,29]. Our data support these findings and also demonstrate that the

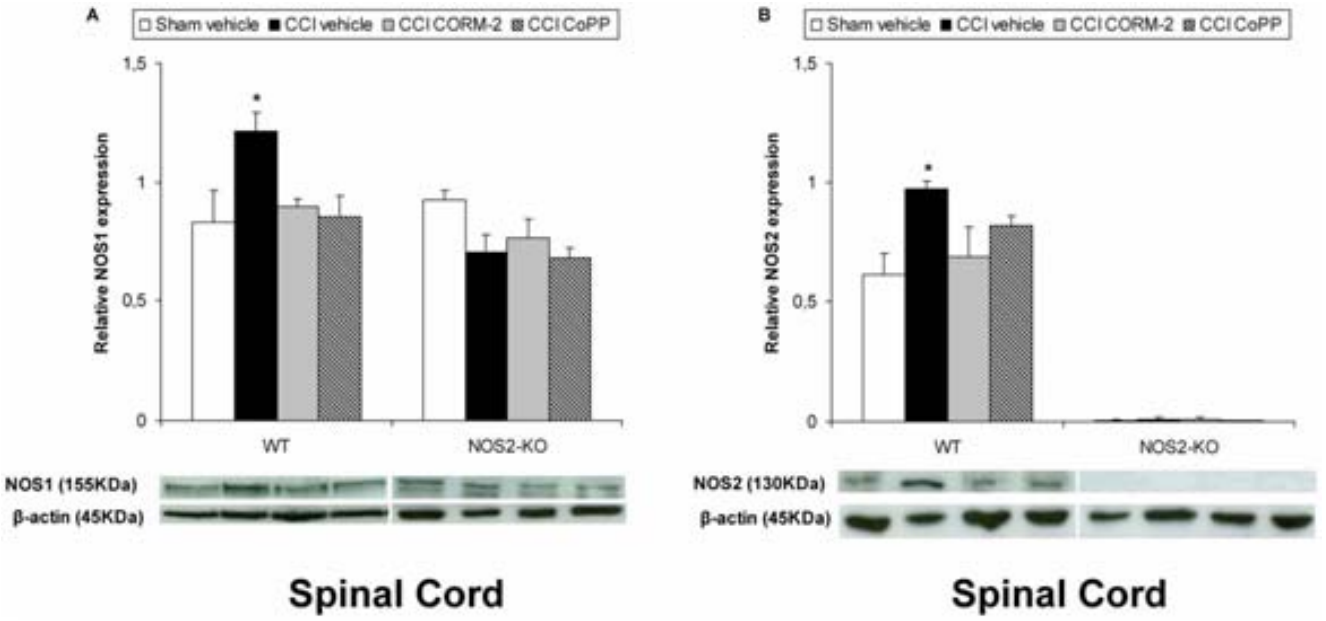

Figure 5. Effect of CORM-2 and CoPP on NOS1 and NOS2 protein expression from sciatic nerve-injured WT and NOS2-KO mice. The protein expression in the ipsilateral site of the lumbar section of the spinal cord of NOS1 (A) and NOS2 (B) from sciatic nerve-injured (CCI) WT and NOS2-KO mice treated with vehicle, CORM-2 or CoPP at 20 days after surgery is represented. The expression of NOS1 and NOS2 in the spinal cord from sham-operated WT and NOS2-KO mice treated with vehicle has been also represented as controls (sham-vehicle). In both figures and genotypes, *indicates significant differences when compared vs. their respective sham-operated vehicle treated mice (*$p<0.05$, one-way ANOVA followed by the Student Newman Keuls test). Representative examples of western blot for NOS1 (155 kDa) and NOS2 (130 kDa) proteins in which β-actin (45 kDa) was used as a loading control are also shown. Data are expressed as mean values ± SEM; n = 5 samples per group.
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increased expression of this isoenzyme in the dorsal root ganglia and spinal cord of sciatic nerve-injured WT mice is not altered by CORM-2 or CoPP treatments, indicating that the antiallodynic and antihyperalgesic effects produced by the chronic administration of both compounds are not produced by the inhibition of the enhanced peripheral or central expression of HO-2 induced by nerve injury. In addition, the fact that the expression of HO-2 did not increase in the spinal cord and dorsal root ganglia of sciatic nerve-injured NOS2-KO mice, provide evidence that the up-regulation of HO-2 induced by nerve injury required the presence of NOS2, further supporting the relevant interactions between NOS/NO and HO/CO pathways previously demonstrated in other *in vivo* and *in vitro* models [11].

Table 1. A summary of the results from protein expression studies obtained in the ipsilateral site of the dorsal root ganglia (DRG) and/or the lumbar section of the spinal cord (SC) from sciatic nerve-injured (CCI) WT and NOS2-KO mice treated with vehicle, CORM-2 or CoPP is shown.

		WT			NOS2-KO		
		CCI	CCI	CCI	CCI	CCI	CCI
Protein	**Tissue**	**Vehicle**	**CORM-2**	**CoPP**	**vehicle**	**CORM-2**	**CoPP**
HO-1	DRG	→	↑	↑↑	→	→	↑↑
	SC	→	↑	↑↑	→	→	↑↑
HO-2	DRG	↑	↑	↑	→	→	→
	SC	↑	↑	↑	→	→	→
CD11b/c	SC	↑	→	→	→	→	→
NOS1	SC	↑	→	→	→	→	→
NOS2	SC	↑	→	→	→	→	→

The arrows indicate: → unchanged, ↑ increased and ↑↑ more increased expression as compared to the expression obtained in their corresponding sham-operated animals treated with vehicle.
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The molecular mechanism implicated in the inhibitory effects produced by CO after neuropathic pain is currently unknown. It has been reported that nerve injury promotes the activation of spinal glial cells, and that this activated glia may contribute to the initiation and maintenance of neuropathic pain [30]. Indeed, the administration of inhibitors of microglial cells activation significantly reduced the behavioral symptoms of neuropathic pain [25]. Several studies also demonstrated the presence of HO-1 in glial cells [31], but the possible effect of CO liberated by CORM-2 or synthesized by HO-1 on the modulation of activated microglia induced by nerve injury is not yet well established. Thus, in order to evaluate if this gas could reduce microglial activation and to establish the role played by NO, synthesized by NOS1 and NOS2, in this process we evaluated the expression of CD11b/c (as a measure of microglial activation), as well as of NOS1 and NOS2 in the spinal cord of sciatic nerve-injured WT mice treated with CORM-2 or CoPP. It is interesting to note that CORM-2 and CoPP treatments reduced the spinal microglial activation as well as the enhanced NOS1 and NOS2 expression induced by sciatic nerve injury in WT mice. Thus, the alleviation of the behavioral manifestations of neuropathic pain in CO-RMs or CoPP-treated WT animals could be due to the inhibition of inflammatory responses that are linked to the microglia activation in the spinal cord. In contrast to WT mice, the expression of CD11b/c and NOS1 remains unaltered after nerve injury in NOS2-KO mice and neither CORM-2 nor CoPP treatment had any effect in these animals. These results support the hypothesis that the activation of NOS/NO pathway promotes the activation of microglia and

contributes to the behavioral pain responses evoked by nerve injury, as previously demonstrated by the lack or reduced mechanical and thermal hypersensitivity induced by nerve injury in NOS2-KO mice [6,8].

Recent studies indicate that CORM-2, but not CORM-3, is also an antagonist of P2X4 receptors [32] and it is well known that the up-regulation of these receptors in microglia is an important process in producing neuropathic pain [33]. However, the similar behavioral inhibitory effects produced by CORM-2 and CORM-3 in the present study indicate that P2X4 receptors are not the main molecular targets for the antinociceptive effects produced by CORM-2 under neuropathic pain conditions. The modulation of neuropathic pain by the HO-1/CO pathway after sciatic nerve injury could be explained by the inhibition of excessive NO generated by the increased NOS1 expression from activated neurons, which plays an important role in the maintenance of neuropathic pain trough microglial activation. The activated microglia promotes the consolidation and progression of neuropathic pain by the up-regulation of several inflammatory pathways including the NOS2/NO pathway, among others. Thus, the activation of the HO-1/CO pathway on microglial cells would control and limit the spreading of this neuroinflammatory process by regulating the enhanced expression of NOS2. In addition, CO located in neurons could also participates in the modulation of neuropathic pain by decreasing the production of NOS1 which would restrict the activation of microglia and attenuates the development of neuropathic pain.

This study reports for first time that an interaction between the CO and NO systems is taking place following sciatic nerve injury. Our data also reveal that exogenous delivery of CO using CO-releasing molecules or increasing the endogenous CO production with cobalt protoporphyrin IX may represent a novel stratagem in the management of neuropathic pain.

Materials and Methods

Ethics Statement

Animal procedures were conducted in accordance with the guidelines of the European Communities, Directive 86/609/EEC regulating animal research and approved by the local ethical committee of our Institution (Comissió d'Etica en l'Experimentació Animal i Humana de la Universitat Autònoma de Barcelona, #6266). All efforts were made to minimize animal suffering, and to reduce the number of animals used.

Animals

In vivo experiments were performed in male NOS2-KO mice (C57BL/6J background) purchased from Jackson Laboratories (Bar Harbor, ME, USA) and in WT mice with the same genetic background (C57BL/6J) acquired from Harlan Laboratories (Barcelona, Spain). All mice weighing 21 to 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 conducted between 9:00 AM and 5:00 PM.

Induction of Neuropathic Pain

Neuropathic pain was induced by chronic constriction of the sciatic nerve [6]. 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 [34]. 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 over-tightening 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 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 10, 14 and 20 days after sciatic nerve injury.

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 [35]. 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.

Thermal hyperalgesia was assessed as previously reported by Hargreaves et al [36]. 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 ×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.

Thermal allodynia to cold stimulus was assessed by using the hot/cold-plate analgesia meter (Ugo Basile, Italy), previously described by Bennett and Xie [34]. The number of elevations of each hind paw was recorded in the mice exposed to the cold plate (4 ± 0.5°C) for 5 minutes.

Western Blot Analysis

Sham-operated and sciatic nerve-injured mice were sacrificed at 20 days after surgery by cervical dislocation. Tissues from the ipsilateral lumbar section of spinal cord and dorsal root ganglia (L3 to L5) were removed immediately after sacrifice, frozen in liquid nitrogen and stored at −80°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 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 nM NaCl, 1% NP-40, 2 mM EDTA,

1 mM phenylmethylsulfonyl fluoride, 0.5 Triton X-100, 0.1% SDS, 1 mM Na_3VO_4, 25 mM NaF, 0.5% protease inhibitor cocktail, 1% phosphatase inhibitor cocktail). All reagents were purchased at Sigma (St. Louis, MO, USA) with the exception of NP-40 from Calbiochem. The crude homogenate was solubilised 1 hour at 4°C, sonicated for 10 seconds and centrifugated at 4°C for 15 min at 700×g. The supernatants (50 or 100 µg of total protein) were mixed with 4× laemmli loading buffer and then loaded onto 4% stacking/10% separating SDS-polyacrylamide gels. The proteins were electrophoretically transferred onto PVDF membrane for 120 minutes for HO-1, HO-2 and CD11b/c or over night for NOS1 and NOS2 detection, blocked with PBST +5% nonfat dry milk, and subsequently incubated overnight at 4°C with a polyclonal rabbit anti-HO-1 (1:300, Stressgen, Ann Arbor, MI), a polyclonal rabbit anti-HO-2 (1:1000, Stressgen, Ann Arbor, MI), a polyclonal rabbit anti-CD11b/c (1:300, Novus Biologicals) antibody against the type 3 complement receptor to detect activated microglial cells [25], a polyclonal rabbit anti-NOS1 antibody (1:100, BD Transduction Laboratories, San Diego, CA, USA) or a polyclonal rabbit anti-NOS2 antibody (1:200, Chemicon, Millipore). The proteins were detected by a horseradish peroxidase-conjugated anti-rabbit secondary antibody (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and visualized by chemiluminescence reagents provided with the ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and exposure onto hyperfilm (GE, Healthcare). The intensity of blots was quantified by densitometry. The membranes were stripped and reproved with a monoclonal rabbit anti-β-actin antibody (1:10.000, Sigma, St. Louis, MO, USA) 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 the chronic constriction of sciatic nerve previously used by us [6]. After the habituation period, baseline responses were established in the following sequence: von Frey filaments, plantar and cold plate tests. After that neuropathic pain was induced, and WT or NOS2-KO animals were again tested in each paradigm at days 10, 14 and 20 after surgery. Sham-operated mice were used as controls.

Sciatic nerve-injured or sham-operated WT or NOS2-KO animals received the intraperitoneal administration of two CO-RMs (CORM-2 or CORM-3, at 5 mg/kg of body weight twice a day) [21,27], an HO-1 inducer (CoPP, at 2.5 mg/kg of body weight twice a day) [37] or vehicle, from days 10 to 20 after surgery.

In other set of experiments, taking into account the analogous effects produced by CORM-2 and CORM-3 on the inhibition of the allodynia and hyperalgesia induced by sciatic nerve injury and in order to minimize the number of animals used, the protein levels of 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 WT and NOS2-KO mice at 20 days after surgery, were only evaluated in CORM-2 and CoPP treated animals, by using western blot assay. In these experiments sham-operated mice treated with vehicle have been used as a control.

Drugs

CORM-2 (tricarbonyldichlororuthenium(II)dimer) was purchased from Sigma-Aldrich (St. Louis, MO), CoPP from Frontier scientific (Livchem GmbH & Co, Frankfurt, Germany) and CORM-3 (tricarbonylchloro (glycinate)ruthenium (II)) was synthesized as previously described by Motterlini et al. [21]. CORM-2 and CoPP were dissolved in dimethyl sulfoxide (DMSO; 1% solution in saline) and CORM-3 in saline. All drugs were freshly prepared before use and intraperitoneally administered in a final volume of 10 ml/kg, twice a day. The control group received the same volume of vehicle.

Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM). For each genotype and test assessed, the comparison of the effects produced by CORM-2, CORM-3 or CoPP vs. the effects produced by vehicle in nerve-injured and sham-operated WT or NOS2-KO mice were evaluated by using a using a three way ANOVA (surgery, treatment and time as between factors of variation) followed by the corresponding one way ANOVA and the Student Newman Keuls test.

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

Author Contributions

Conceived and designed the experiments: OP AH. Performed the experiments: AH RN SL. Analyzed the data: AH. Contributed reagents/materials/analysis tools: RM. Wrote the paper: OP AH. Helped to write the manuscript: RM.

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5.6. *Treatments with carbon monoxide-releasing molecules and an HO-1 inducer enhance the effects and expression of μ-opioid receptors during neuropathic pain.*

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Anesthesiology. Under review

Treatment with carbon monoxide-releasing molecules and an HO-1 inducer enhances the effects and expression of μ-opioid receptors during neuropathic pain

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Number of words in Discussion: 1330

Running title: opioid/cannabinoid regulation by CO-RMs and CoPP

What We Already Know about This Topic

• The local administration of μ- and δ-opioid receptor as well as cannabinoid 2 receptor agonists attenuates neuropathic pain, a disorder where the effectiveness of μ-opioid receptor are lower than δ-opioid and cannabinoid 2 receptor agonists.

• The nitric oxide-cGMP-PKG pathway modulates the antinociceptive effects and expression of opioid and cannabinoid receptors during chronic pain but the role played by carbon monoxide has not been studied.

What This Article Tells Us That Is New

• Carbon monoxide-releasing molecules or an HO-1 inducer attenuate neuropathic pain symptoms through the increased HO-1 expression and the inhibition of nerve injury-induced microglial activation and nitric oxide synthesis.

• Carbon monoxide-releasing molecules or an HO-1 inducer increase the local effectiveness of morphine on the inhibition of neuropathic pain through enhanced the peripheral expression of μ-opioid receptors

ABSTRACT

Background: The administration of μ-(MOR) and δ-opioid receptors (DOR) as well as cannabinoid 2 receptor (CB2R) agonists attenuates neuropathic pain. We investigated if the treatment with two carbon monoxide-releasing molecules (CORM-2 and CORM-3) or an HO-1 (inducible heme oxygenase) inducer (cobalt protoporphyrin IX, CoPP) could modulate the local and systemic effects and the expression of MOR, DOR and CB2R during neuropathic pain.

Methods: In C57BL/6 mice, at 10 days after chronic constriction of sciatic nerve, we evaluated the effects of 10 mg/kg of CORM-2, CORM-3 or CoPP in the antiallodynic and antihyperalgesic actions of a MOR (morphine), DOR (DPDPE) or CB2R (JWH-015) agonist locally or systemically administered. The effects of CORM-2 and CoPP on the expression of MOR, DOR, CB2R, heme oxygenases (HO-1 and HO-2), neuronal (NOS1) and inducible (NOS2) nitric oxide synthases and microglia activation marker (CD11b/c) were also assessed.

Results: Treatments with CO-RMs and CoPP reduced the mechanical and thermal hypersensitivity induced by nerve injury ($p < 0.001$; n= 6 per group), increased the local, but not systemic, antinociceptive effects of morphine and decreased those produced by DPDPE and JWH-015 ($p < 0.05$; n= 6 per group). CORM-2 and CoPP enhanced MOR and HO-1 expression, unaltered DOR and HO-2 expression and decreased the over-expression of CB2R, CD11b/c, NOS1 and NOS2 induced by injury ($p < 0.01$; n= 5 samples per group).

Conclusions: CO-RMs and CoPP treatments increase the local antinociceptive effects of morphine through enhanced MOR peripheral expression and inhibit spinal microglial activation and NOS1/NOS2 over-expression.

Keywords: cannabinoid receptors, carbon monoxide, carbon monoxide-releasing molecules (CO-RMs), heme oxygenase1 (HO-1), microglia, neuropathic pain, opioid receptors, nitric oxide (NO), nitric oxide synthase (NOS).

Introduction

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. [1-2] In contrast, the local administration of μ-(MOR) and δ-opioid receptor (DOR) as well as cannabinoid 2 receptor (CB2R) agonists elicits antiallodynic and antihyperalgesic effects during neuropathic pain. [3-8] However, whereas the local antinociceptive effects of morphine are produced by activation of the peripheral nitric oxide (NO)-cGMP-protein kinase (PKG)-ATP-sensitive K+ (KATP) channels signaling pathway, [6] the activation of this pathway is implicated as a mechanism limiting the local antiallodynic and antihyperalgesic efficiency of DOR and CB2R agonists under neuropathic pain conditions. [5] Accordingly, while the local antiallodynic effects of morphine were significantly reduced by their local co-administration with selective neuronal (NOS1) or inducible (NOS2) nitric oxide synthase, L-guanylate cyclase or PKG inhibitors, [6] the local antinociceptive effects of DOR and CB2R agonists were significantly increased. [5] Moreover, NO is also implicated in the dorsal root ganglia down- (MOR and DOR) and up-regulation (CB2R) of these receptors after sciatic nerve-injury. [5-6]

Carbon monoxide (CO), another gaseous neurotransmitter, synthesized by inducible (HO-1) and constitutive (HO-2) heme oxygenases, also activates the cGMP-PKG pathway. [9-10] The overexpression of HO-2 isoform exerts a pronociceptive effect after nerve injury. [11-13] In contrast, the enhanced expression of HO-1 produces potent anti-inflammatory and antinociceptive effects. [14,15-17] Indeed, the administration of HO-1 inducers 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 CO, inhibits inflammation and/or acute nociception. [15,18-21] Although the exact contribution of CO 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,[22,23] but the role played by CO-RMs or CoPP in the effects and expression of MOR, DOR, CB2R as well as in the possible mechanisms implicated in these actions are still unknown. Therefore, in sciatic nerve-injury induced neuropathic pain we evaluated: 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 carbon monoxide-releasing molecules, tricarbonyldichlororuthrnium(II) dimer (CORM-2) and tricarbonylchloro (glycinate)ruthenium (II) (CORM-3) or a classical inducer of HO-1, CoPP intraperitoneally administered; 2) the antinociceptive effects of morphine, DPDPE or JWH-015 subplantarly or subcutaneously injected, alone or combined, with the HO-1 inhibitor, tin protoporphyrin IX (SnPP); 3) the reversibility of morphine, DPDPE and JWH-015 effects by their co-administration with specific antagonists and 4) the effect of CORM-2 and CoPP on the expression of MOR, DOR, CB2R, HO and NOS isoforms as well as CD11b/c, as a marker of microglial activation, in the dorsal root ganglia or spinal cord from sciatic nerve-injured mice.

Material and Methods

Animals

The experiments were performed in male C57BL/6 mice acquired from Harlan Laboratories (Barcelona, Spain). 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 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. [5] 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. [24] 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 over-tightening 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 10 days after surgery.

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. [25] 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.

Thermal hyperalgesia was assessed as previously reported by Hargreaves et al. [26] 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.

Thermal allodynia to cold stimulus was assessed by using the hot/cold-plate analgesia meter (Ugo Basile, Italy), previously described by Bennett and Xie. [24] 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.

Western Blot Analysis

Sham-operated and sciatic nerve-injured mice were sacrificed at 10 days after surgery by cervical dislocation. Tissues from the ipsilateral lumbar section of spinal cord and dorsal root ganglia (L3 to L5) were removed immediately after sacrifice, frozen in liquid nitrogen and stored at -80ºC 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 nM NaCl, 1% NP-40, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.5 Triton X-100, 0.1% SDS, 1 mM Na_3VO_4, 25 mM NaF, 0.5 % protease inhibitor cocktail, 1% phosphatase inhibitor cocktail). All reagents were purchased at Sigma (St. Louis, MO, USA) with the exception of NP-40 from Calbiochem. The crude homogenate was solubilised 1 hour at 4ºC, sonicated for 10 seconds and centrifugated at 4°C for 15 min at 700 × g. The supernatants (50 or 100 µg of total protein) were mixed with 4 x laemmli loading buffer and then loaded onto 4% stacking/10% separating SDS-polyacrylamide gels.

The proteins were electrophoretically transferred onto PVDF 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°C with polyclonal rabbit anti-MOR (1:1000, Chemicon, Millipore), anti-DOR (1:2500, Chemicon, Millipore), anti-CB2R (1:500, Abcam, Cambridge, UK), anti-HO-1 (1:300, Stressgen, Ann Arbor, MI), anti-HO-2 (1:1000, Stressgen, Ann Arbor, MI), anti-CD11b/c (1:300, Novus Biologicals)

antibody against the type 3 complement receptor to detect activated microglial cells, [27] anti-NOS1 (1:100, BD Transduction Laboratories, San Diego, CA, USA) 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, UK) and visualized by chemiluminescence reagents provided with the ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and exposure onto hyperfilm (GE, Healthcare). The intensity of blots was quantified by densitometry. The membranes were stripped and reproved with a monoclonal rabbit anti-β-actin antibody (1:10.000, Sigma, St. Louis, MO, USA) 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 the chronic constriction of sciatic nerve previously used by us. [6] 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 carbon monoxide-releasing molecules (CORM-2 and CORM-3) [17,28,29], their inactive forms (iCORM-2 and iCORM-3), an HO-1 inducer (CoPP) [30] or an HO-1 inhibitor (SnPP) [16] 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 intraperitoneal administration of a low dose of morphine (50 μg or 1 mg/kg) or high doses of DPDPE (100 μg or 5 mg/kg) or JWH-015 (30 μg or 3 mg/kg) [5,6] 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 μg or 10 mg/kg of SnPP alone or combined with the subplantar or subcutaneous administration of high doses of morphine (100 μg or 5 mg/kg) or low doses of DPDPE (25 μg or 0.5 mg/kg) or JWH-015 (5 μg or 0.15 mg/kg) [5,6] 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 [15,17,20,30-32] 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, [5,6,33] 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 5mg/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 antihyperlagesic effects after sciatic nerve injury, [5,6] by their subplantar or subcutaneous co-administration with specific (CTAP;

120 µg or 4 mg/kg, naltrindole; 50 µg or 2 mg/kg; AM630; 30 µg or 1 mg/kg) and unspecific opioid peripheral antagonist (naloxone methiodide, NX-ME 20 µg or 1 mg/kg) or cannabinoid 1 receptor (CB1R) antagonist (AM251; 150 µg or 5 mg/kg) [5,6] at 10 days after surgery were 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. [5,6,33]

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. In these experiments sham-operated mice treated with vehicle have been used as controls (n = 5 samples per group).

Drugs

CORM-2 (tricarbonyldichlororuthenium(II)dimer) was purchased from Sigma-Aldrich (St. Louis, MO), CoPP and SnPP from Frontier scientific (Livchem GmbH & Co, Frankfurt, Germany) and CORM-3 (tricarbonylchloro (glycinate)ruthenium (II)) was synthesized as previously described by Clark et al. (2003) [29]. Morphine hydrochloride was obtained from Alcaiber S.A. (Madrid, Spain), DPDPE ([D-Pen 2,5]-enkephalin), CTAP, naltrindole and naloxone methiodide were acquired from Sigma-Aldrich (St. Louis, MO). JWH-015, AM630 and AM251 were purchased from Tocris (Ellisville, MI).

CORM-2, CoPP and SnPP were dissolved in dimethyl sulfoxide (DMSO; 1 % solution in saline). JWH-015, AM630, AM251 were dissolved in DMSO (50 % solution in saline). CORM-3, morphine-HCl, DPDPE, CTAP, naloxone methiodide 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) were prepared by leaving solutions of CORM-2 or CORM-3 in DMSO or PBS, at room

temperature for two days, respectively. The iCORM-2 and iCORM-3 solutions were finally bubbled with nitrogen to remove any residual CO present in the solutions.

All drugs were freshly prepared before use. CORM-2, CORM-3 and CoPP were intraperitoneally administered, 3-4 hours 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 ± standard error of the mean (SEM). For each test and dose, the comparison of the effects produced by the intraperitoneal administration of CO-RMs, iCO-RMs, CoPP or SnPP vs. 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 dose, the comparison of the effects produced by the subcutaneous administration of morphine, DPDPE or JWH-015 vs. the effects produced by their corresponding vehicle was evaluated by using a Student's t test. For each behavioral test, the comparison of the effects produced by morphine, DPDPE and JWH-015 combined with CORM-2, CORM-3, CoPP or SnPP was evaluated by using a one way ANOVA followed by the 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 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 test, the reversal of the 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

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 1). 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). While the intraperitoneal administration of 10 or 20 mg/kg of SnPP did not alter the principal symptoms of neuropathic pain.

Tacking 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 CO-RMs in order to maintain the same dosage tested for CoPP (10 mg/kg, a dose that produce inhibitory effects) or SnPP (10 mg/kg). In addition and due to that the intraperitoneal administration of 10 mg/kg of iCORM-2 or iCORM-3 did not produce any significant inhibitory effect we did not test its 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 in the contralateral paw of sciatic nerve-injured or sham-operated animals (data not shown).

Table 1. 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) on the mechanical allodynia, thermal hyperalgesia and thermal allodynia induced by injury in the ipsilateral paw of sciatic nerve-injured animals.

		Mechanical allodynia			**Thermal hyperalgesia**			**Thermal allodynia**		
Treatments	Dose (mg/kg)	Maximal possible effect (%)			Maximal possible effect (%)			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 SNK). Results are shown as mean values ± SEM; n= 6 animals per experimental group.*

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, DPDPE or JWH-015 dose-dependently inhibited the mechanical allodynia (Fig. 1A), thermal hyperalgesia (Fig 1B) and thermal allodynia (Fig. 1C) induced by sciatic nerve injury in mice. Thus, the mechanical antiallodynic, thermal antihyperalgesic and thermal antiallodynic effects produced by different doses of morphine (1-10 mg/kg), DPDPE (0.5-10 mg/kg) or JWH-015 (0.15-3 mg/kg) in the ipsilateral paw of sciatic nerve-injured mice were significantly higher than those produced by their corresponding vehicle treated mice ($p < 0.023$; Student's t 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).

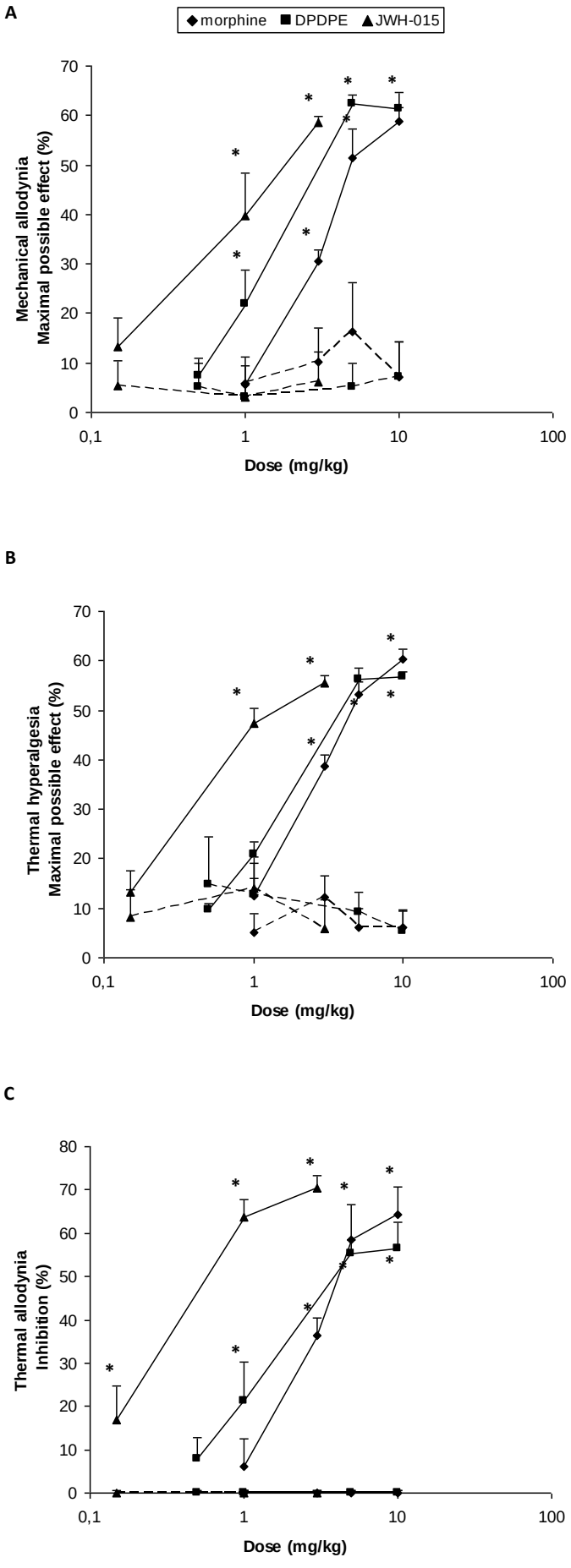


Figure 1. Effects of the subcutaneous administration of morphine, DPDPE and JWH-015 on the mechanical allodynia, thermal hyperalgesia and thermal allodynia induced by sciatic nerve injury in mice. *Effects of the subcutaneous administration of different doses (logarithmic axis) of morphine, DPDPE, JWH-015 or its respective vehicle on the mechanical allodynia (A), thermal hyperalgesia (B) and thermal allodynia (B) induced by sciatic nerve injury in the ipsilateral paw of 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 ± SEM (6 animals for dose). In all tests, for each dose, * P< 0.05 denote significant differences between drug and vehicle treated animals (Student's t test).*

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.

Our results show that the intraperitoneal administration of CORM-2, CORM-3 or CoPP alone significantly attenuated the mechanical allodynia (Fig. 2A,and 2D), thermal hyperalgesia (Fig. 2B and 2E) and thermal allodynia (Fig. 2C and 2F) induced by sciatic nerve injury ($p < 0.001$; one way ANOVA vs. control-vehicle treated mice). Our results also demonstrate that the subplantar administration of a low dose of morphine (50 μg) in CORM-2, CORM-3 or CoPP treated mice significantly increased the local mechanical antiallodynic (Fig. 2A), thermal antihyperalgesic (Fig. 2B) and thermal antiallodynic (Fig. 2C) effects produced by morphine, CORM-2, CORM-3 or CoPP administered alone in the ipsilateral paw of sciatic nerve injured mice ($p < 0.001$, one way ANOVA vs. respective vehicle or control groups). In contrast, the subcutaneous administration of a low dose of morphine (1 mg/kg) in CORM-2, CORM-3 or CoPP treated mice significantly increases the local mechanical antiallodynic (Fig. 2D), thermal antihyperalgesic (Fig. 2E) and thermal antiallodynic (Fig. 2F) effects produced by morphine alone but not to those produced by CORM-2, CORM-3 or CoPP administered alone in the ipsilateral paw of sciatic nerve injured mice ($p < 0.05$, one way ANOVA).

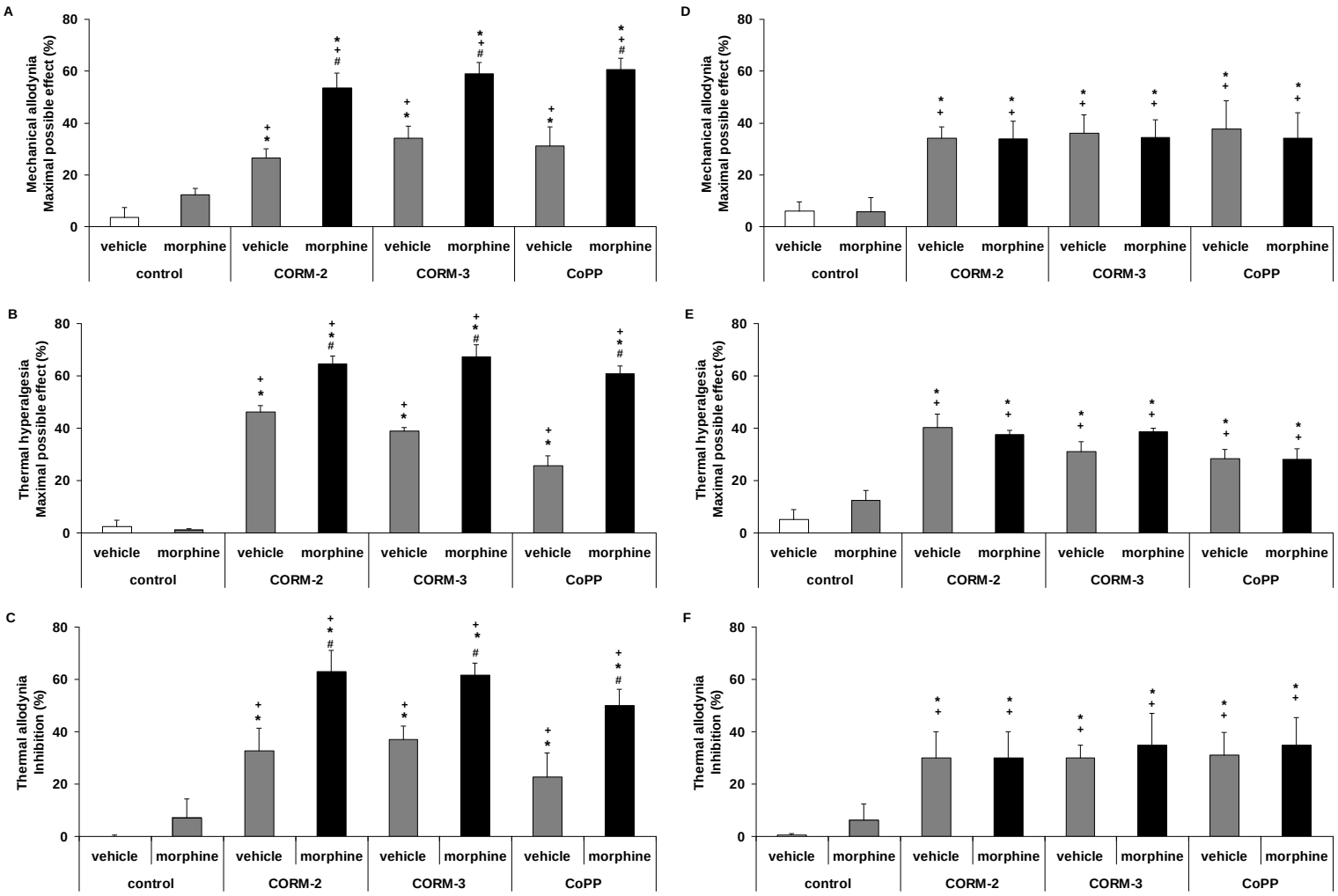


Figure 2. Effects of CORM-2, CORM-3 and CoPP on the antiallodynic and antihyperalgesic responses to morphine. *Mechanical antiallodynic (A,D), thermal antihyperalgesic (B,E) and thermal antiallodynic (C,F) effects of the subplantar (50 µg; A, B, C) or subcutaneous (1 mg/kg; D, E, F) administration of 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 ± SEM (6 animals per group). For each behavioral test, * denotes significant differences vs. control group treated with vehicle ($p < 0.05$, one way ANOVA followed by SNK), + denotes significant differences vs. control group treated with morphine ($p < 0.05$, one way ANOVA followed by the SNK) and # denotes significant differences vs. group treated with CORM-2, CORM-3 or CoPP plus vehicle ($p < 0.05$; one way ANOVA followed by the SNK).*

The mechanical antiallodynic (Fig. 3A and 3D), thermal antihyperalgesic (Fig. 3B and 3E) and thermal antiallodynic (Fig. 3C and 3F) effects produced by the subplantar or subcutaneous administration of a high dose of DPDPE (100 µg or 5 mg/kg) in the ipsilateral paw of sciatic nerve-injured mice were significantly reduced in CORM-2, CORM-3 or CoPP treated animals ($p < 0.02$, one way ANOVA vs. their respective control-DPDPE treated mice).

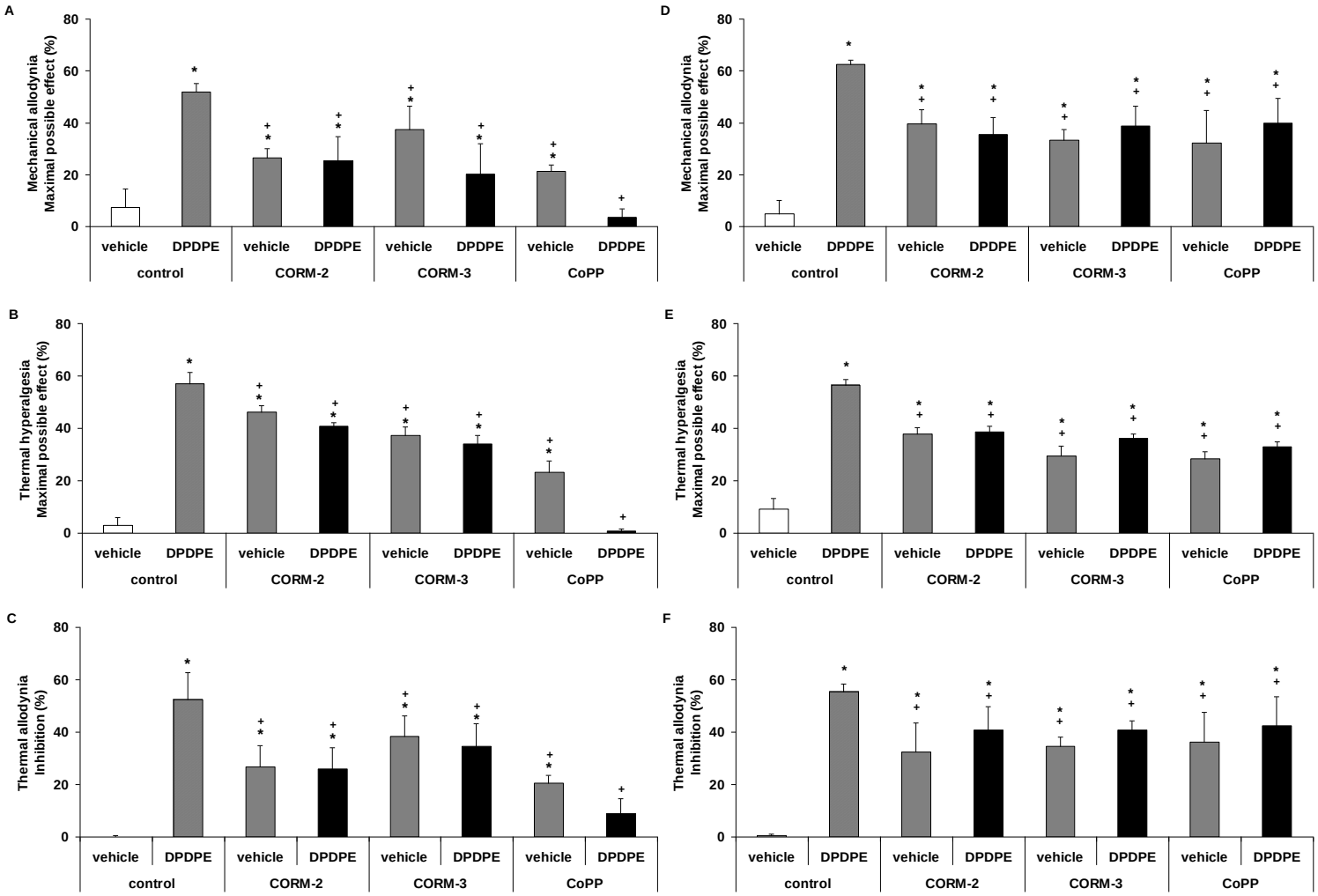


Figure 3. Effects of CORM-2, CORM-3 and CoPP on the antiallodynic and antihyperalgesic responses to DPDPE. *Mechanical antiallodynic (A,D), thermal antihyperalgesic (B,E) and thermal antiallodynic (C,F) effects of the subplantar (100 µg; A, B, C) or subcutaneous (5 mg/kg; D, E, F) administration of DPDPE 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 ± SEM (6 animals per group). For each behavioral test, * denotes significant differences vs. control group treated with vehicle ($p < 0.05$, one way ANOVA followed by SNK) and + denotes significant differences vs. control group treated with DPDPE ($p < 0.05$, one way ANOVA followed by the SNK).*

In a similar way, the subplantar or subcutaneous administration of a high dose of JWH-015 (30 µg or 3 mg/kg) in CORM-2, CORM-3 or CoPP treated animals also reduced the mechanical antiallodynic (Fig. 4A and 4D), thermal antihyperalgesic (Fig. 4B and 4E) and thermal antiallodynic (Fig. 4C and 4F) effects produced by JWH-015 alone in the ipsilateral paw of sciatic nerve-injured mice ($p < 0.001$, one way ANOVA vs. their respective control-JWH-015 treated animals).

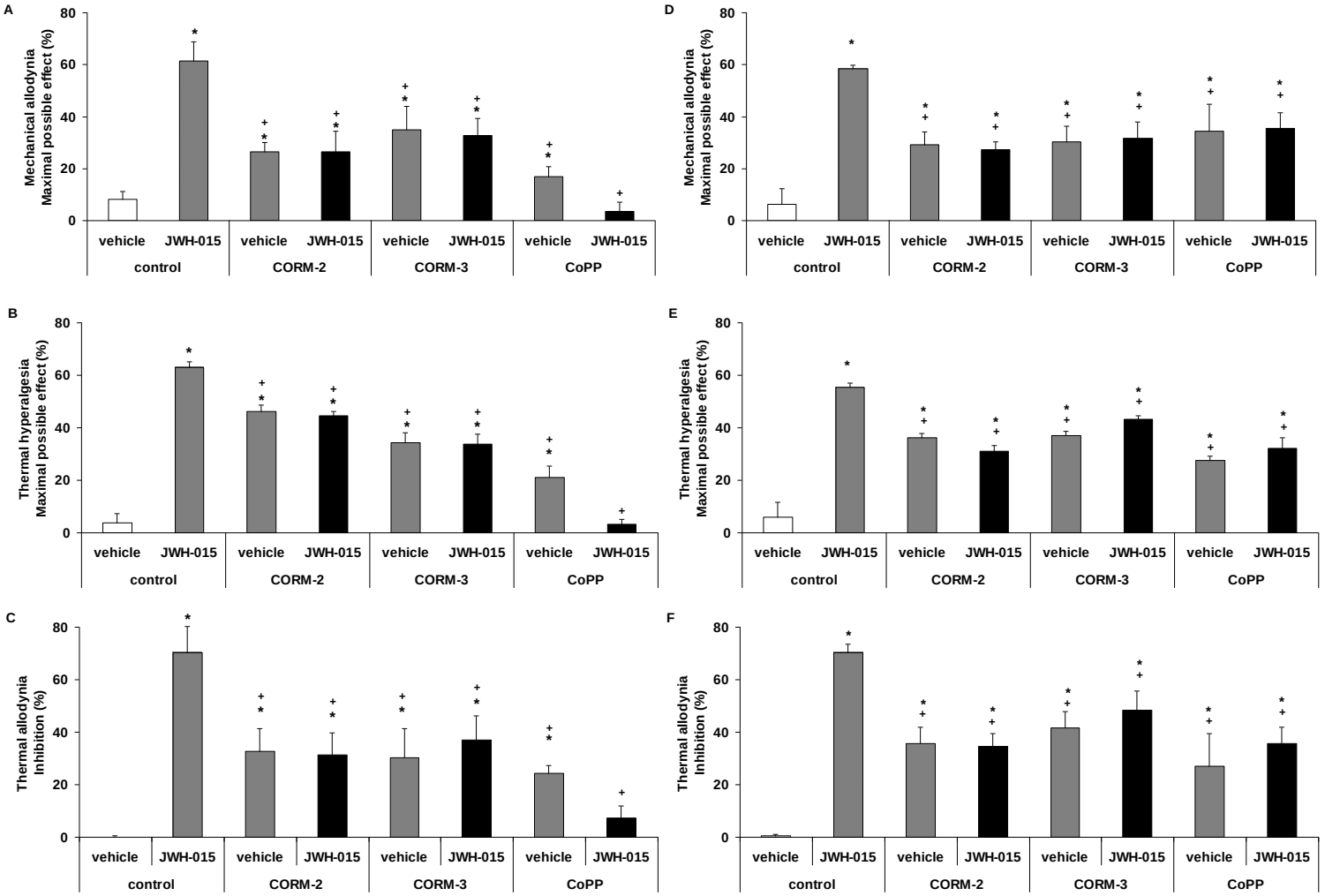


Figure 4. Effects of CORM-2, CORM-3 and CoPP on the antiallodynic and antihyperalgesic responses to JWH-015. *Mechanical antiallodynic (A,D), thermal antihyperalgesic (B,E) and thermal antiallodynic (C,F) effects of the subplantar (30 µg; A, B, C) or subcutaneous (3 mg/kg; D, E, F) administration of 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 ± SEM (6 animals per group). For each behavioral test, * denotes significant differences vs. control group treated with vehicle (p < 0.05, one way ANOVA followed by SNK) and + denotes significant differences vs. control group treated with JWH-015 (p < 0.05, one way ANOVA followed by the SNK).*

The subplantar or subcutaneous administration of morphine, DPDPE or JWH-015 alone or combined with CORM-2, CORM-3 or CoPP intraperitoneally administered did not have any significant effect neither on 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 the HO-1 inhibitor tin protoporphyrin IX on the Antinociceptive response to Morphine, DPDPE and JWH-015 in Sciatic Nerve-Injured Mice

The effects of the subplantar administration of 290 µg of tin protoporphyrin IX (SnPP) on the mechanical antiallodynic, thermal antihyperalgesic and thermal antiallodynic effects produced by the subplantar administration of morphine (100 µg), DPDPE (25 µg), JWH-015 (5 µg) or vehicle in sciatic nerve-injured mice at 10 days after surgery were assessed.

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. Our results also demonstrate that local co-administration of a high dose of morphine (100 µg) with SnPP significantly decreases the local mechanical antiallodynic (Fig. 5A), thermal hyperalgesic (Fig. 5B) and thermal antiallodynic (Fig. 5C) effects produced by morphine alone

in the ipsilateral paw of sciatic nerve injured mice ($p < 0.001$, one way ANOVA vs. vehicle group treated with morphine). In contrast to morphine, the co-administration of a low dose of DPDPE (25 μg) or JWH-015 (5 μg) with SnPP (290 μg) did not alter the antiallodynic and antihyperalgesic effects produced by these drugs administered alone in the ipsilateral paw of sciatic nerve injured mice (Fig. 5A, B and C).

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

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. Our results also demonstrate that the subcutaneous co-administration of a high dose of morphine (5 mg/kg) with SnPP did not alter the mechanical antiallodynic (Fig. 5D), thermal hyperalgesic (Fig. 5E) and thermal antiallodynic (Fig. 5F) effects produced by morphine alone in the ipsilateral paw of sciatic nerve injured mice ($p < 0.001$, one way ANOVA vs. vehicle group treated with morphine). Moreover, the co-administration of a low dose of DPDPE (0.5 mg/kg) or JWH-015 (0.15 mg/kg) with SnPP did not alter the antiallodynic and antihyperalgesic effects produced by these drugs administered alone in the ipsilateral paw of sciatic nerve injured mice (Fig. 5D, E and F).

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 in the contralateral paw of sciatic nerve-injured or sham-operated animals (data not shown).

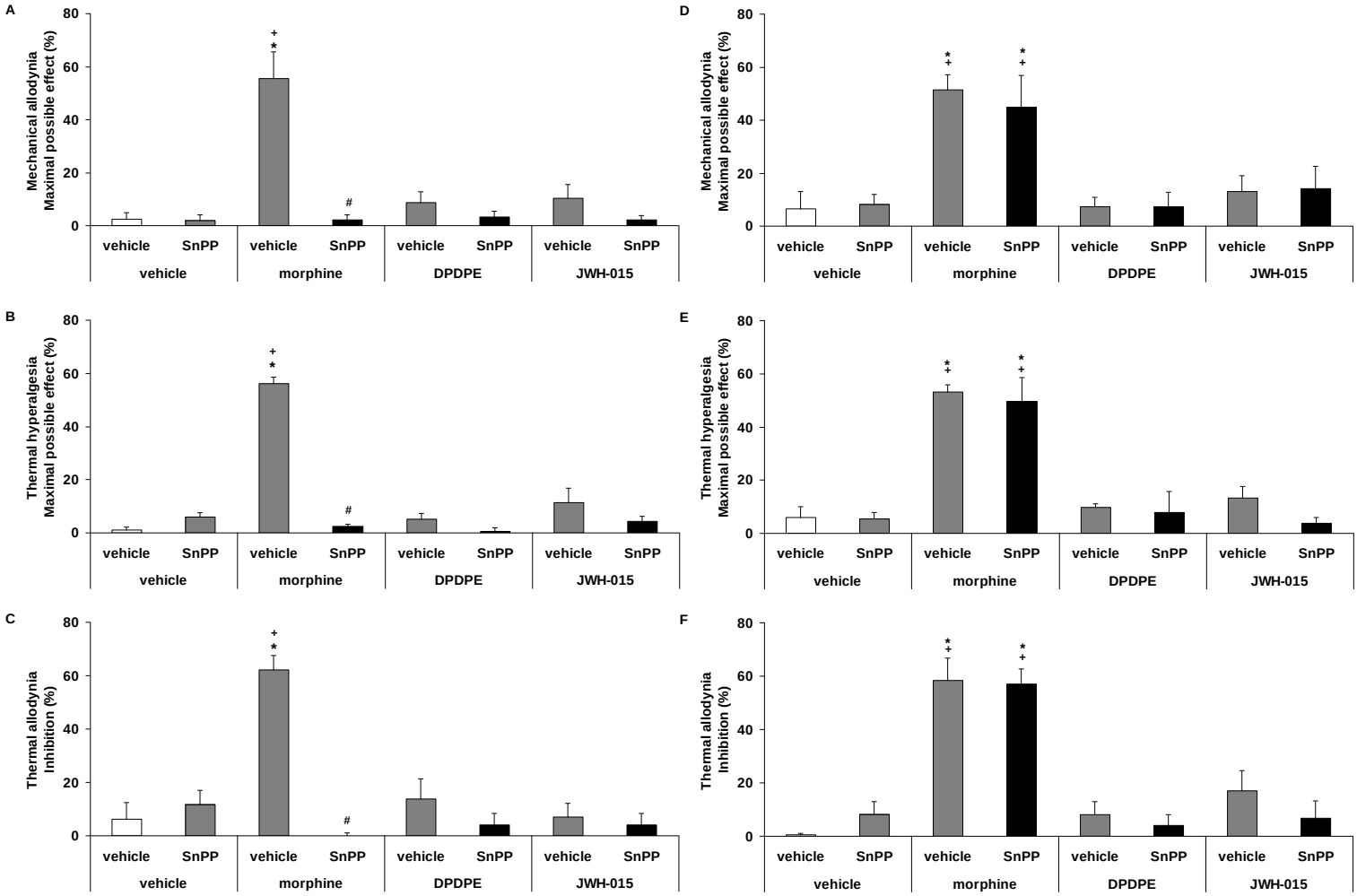


Figure 5. Effects of SnPP treatment on the antiallodynic and antihyperalgesic responses to morphine, DPDPE or JWH-015. *Mechanical antiallodynic (A,D), thermal antihyperlagesic (B,E) and thermal antiallodynic (C,F) effects of the subplantar (A,B,C) or systemic (D,E,F) administration of morphine (100 µg or 5 mg/kg), DPDPE (25 µg or 0.5 mg/kg) or JWH-015 (5 µg or 0.15 mg/kg) with SnPP (290 µg 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 ± SEM (6 animals per group). For each behavioral test, * denotes significant differences vs. group treated with vehicle ($p < 0.05$, one way ANOVA followed by SNK), + denotes significant differences vs. group treated with SnPP plus vehicle ($p < 0.05$, one way ANOVA followed by the SNK) and # denotes significant differences vs. group treated with morphine, DPDPE or JWH-015 plus vehicle ($p < 0.05$, one way ANOVA followed by the SNK).*

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 µg of morphine in the ipsilateral paw of sciatic nerve-injured mice were completely reversed by its subplantar co-administration with selective MOR (CTAP, 120 µg) or peripheral opioid receptor (NX-ME, 20 µg) antagonists ($p < 0.05$; one way ANOVA, followed by Student Newman Keuls test, Table 2). In a similar way, the mechanical and thermal antiallodynic as well as the antihyperalgesic effects produced by 100 µg of DPDPE in the ipsilateral paw of sciatic nerve-injured mice were completely reversed by its subplantar co-administration with a selective DOR (naltrindole, 50 µg) or a peripheral opioid receptor (NX-ME, 20 µg) 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 µg of JWH-015 in the ipsilateral paw of sciatic nerve-injured mice were also completely reversed by its subplantar co-administration with a selective CB2R antagonist (AM630, 30 µg; $p < 0.040$; one way ANOVA, followed by Student Newman Keuls test). The subplantar administration of AM251 (a selective CB1R antagonist; 150 µg) 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 co-administration 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 3). In contrast, the inhibitory effects produced by 5 mg/kg of DPDPE in the ipsilateral paw of sciatic nerve-injured mice were completely reversed by its co-administration 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 in the

ipsilateral paw of sciatic nerve-injured mice were completely reversed by its subcutaneous co-administration 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 did not have any significant effect on the different nociceptive responses evaluated in this study (Tables 2 and 3). In addition, the subplantar or subcutaneous administration of all tested agonists 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)

Table 2. 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 naloxone methiodide (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		Von Frey filaments strength (g)	Withdrawal latency (s)	Paw lifts (number)
vehicle	vehicle	1.05 ± 0.04	3.61 ± 0.14	5.75 ± 0.63
morphine	vehicle	2.68 ± 0.38*	6.98 ± 0.15*	2.25 ± 0.48*
	CTAP	1.24 ± 0.11	3.67 ± 0.11	5.25 ± 0.48
	NX-ME	0.97 ± 0.05	3.36 ± 0.14	4.75 ± 0.48
vehicle	CTAP	1.31 ± 0.09	3.10 ± 0.13	4.20 ± 0.20
	NX-ME	1.11 ± 0.22	3.42 ± 0.14	5.00 ± 0.79
vehicle	vehicle	1.19 ± 0.12	3.18 ± 0.28	5.00 ± 0.91
DPDPE	vehicle	2.43 ± 0.08*	8.50 ± 0.39*	2.75 ± 0.48*
	naltrindole	1.19 ± 0.13	3.53 ± 0.26	4.25 ± 0.48
	NX-ME	1.25 ± 0.09	3.36 ± 0.14	4.71 ± 0.71
vehicle	naltrindole	1.41 ± 0.22	4.13 ± 0.07	5.50 ± 0.87
	NX-ME	1.11 ± 0.22	3.42 ± 0.14	5.00 ± 0.79
vehicle	vehicle	1.17 ± 0.11	3.06 ± 0.27	5.25 ± 0.85
JWH-015	vehicle	2.60 ± 0.19*	8.69 ± 0.19*	1.75 ± 0.63*
	AM630	0.99 ± 0.12	4.58 ± 0.19	5.25 ± 0.32
	AM251	2.36 ± 0.07*	7.74 ± 0.19*	2.40 ± 0.24*
vehicle	AM630	1.42 ± 0.17	3.70 ± 0.24	5.75 ± 0.75
	AM251	1.38 ± 0.07	3.37 ± 0.11	5.00 ± 0.85

*For each test and drug tested. * $p < 0.05$ denotes significant differences vs. vehicle treated group (one way ANOVA, followed by SNK). Results are shown as mean values ± SEM; n= 6 animals per group.*

Table 3. 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 naloxone methiodide (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		Von Frey filaments strength (g)	Withdrawal latency (s)	Paw lifts (number)
vehicle	vehicle	1.62 ± 0.11	3.64 ± 0.13	4.00 ± 0.32
morphine	vehicle	2.56 ± 0.09*	8.57 ± 0.56*	1.50 ± 0.20*
	CTAP	1.56 ± 0.05	4.24 ± 0.24	4.33 ± 0.26
	NX-ME	2.50 ± 0.16*	8.41 ± 0.30*	1.60 ± 0.24*
vehicle	CTAP	1.49 ± 0.02	3.96 ± 0.17	4.40 ± 0.40
	NX-ME	1.56 ± 0.05	4.19 ± 0.26	4.40 ± 0.40
vehicle	vehicle	1.46 ± 0.04	3.48 ± 0.18	4.75 ± 0.62
DPDPE	vehicle	2.64 ± 0.16*	8.29 ± 0.18*	2.20 ± 0.20*
	Naltrindole	1.62 ± 0.11	4.48 ± 0.09	4.33 ± 0.26
	NX-ME	1.80 ± 0.13	3.81 ± 0.25	4.75 ± 0.62
vehicle	Naltrindole	1.54 ± 0.06	4.29 ± 0.24	4.20 ± 0.37
	NX-ME	1.55 ± 0.04	3.88 ± 0.16	4.60 ± 0.40
vehicle	vehicle	1.46 ± 0.04	3.85 ± 0.15	4.60 ± 0.24
JWH-015	vehicle	2.56 ± 0.09*	8.38 ± 0.11*	1.20 ± 0.20*
	AM630	1.62 ± 0.11	4.53 ± 0.14	4.00 ± 0.32
	AM251	2.40 ± 0.12*	8.25 ± 0.15*	1.80 ± 0.37*
vehicle	AM630	1.56 ± 0.07	4.43 ± 0.17	4.20 ± 0.20
	AM251	1.49 ± 0.02	4.30 ± 0.24	4.20 ± 0.37

*For each test and drug tested. * $p < 0.05$ denotes significant differences vs. vehicle treated group (one way ANOVA, followed by SNK). Results are shown as mean values ± SEM; n= 6 animals per group.*

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 Fig. 6. Our results show that the expression of MOR (A) was significantly increased by CORM-2 or CoPP treatments ($p < 0.001$; one-way ANOVA vs. sham-operated and nerve-injured vehicle treated mice). The unchanged protein levels of DOR (B) in the dorsal root ganglia from sciatic nerve-injured mice were not altered by CORM-2 or CoPP treatments while the enhanced peripheral expression of CB2R induced by nerve injury was significantly reduced by both treatments ($p < 0.001$; 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 over-expression of HO-2 induced by sciatic nerve injury (Fig. 7B) was unaltered by CORM-2 or CoPP treatments ($p < 0.001$; one-way ANOVA as 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 WT 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).

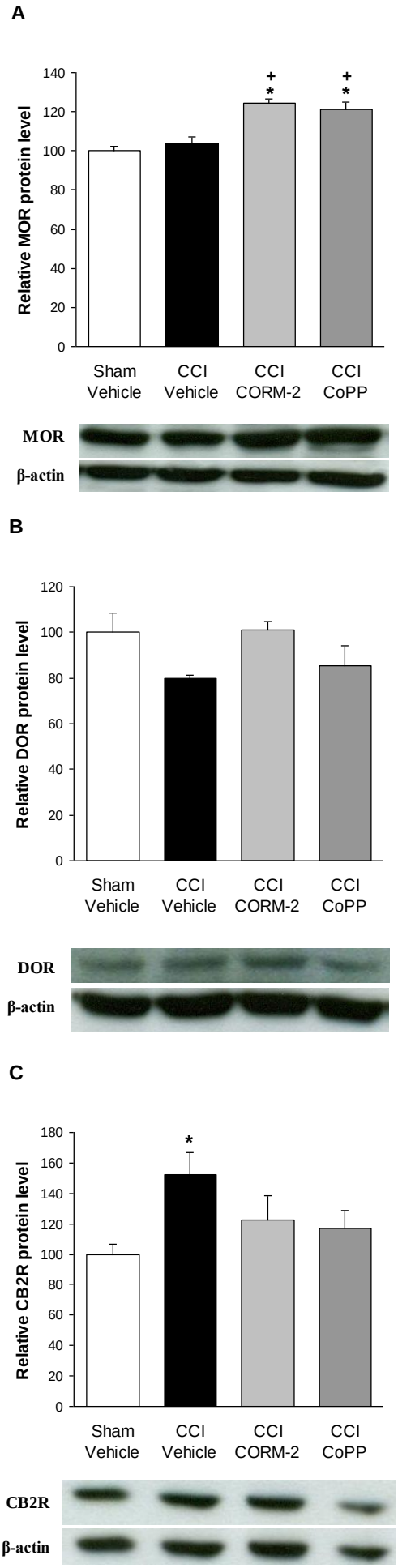
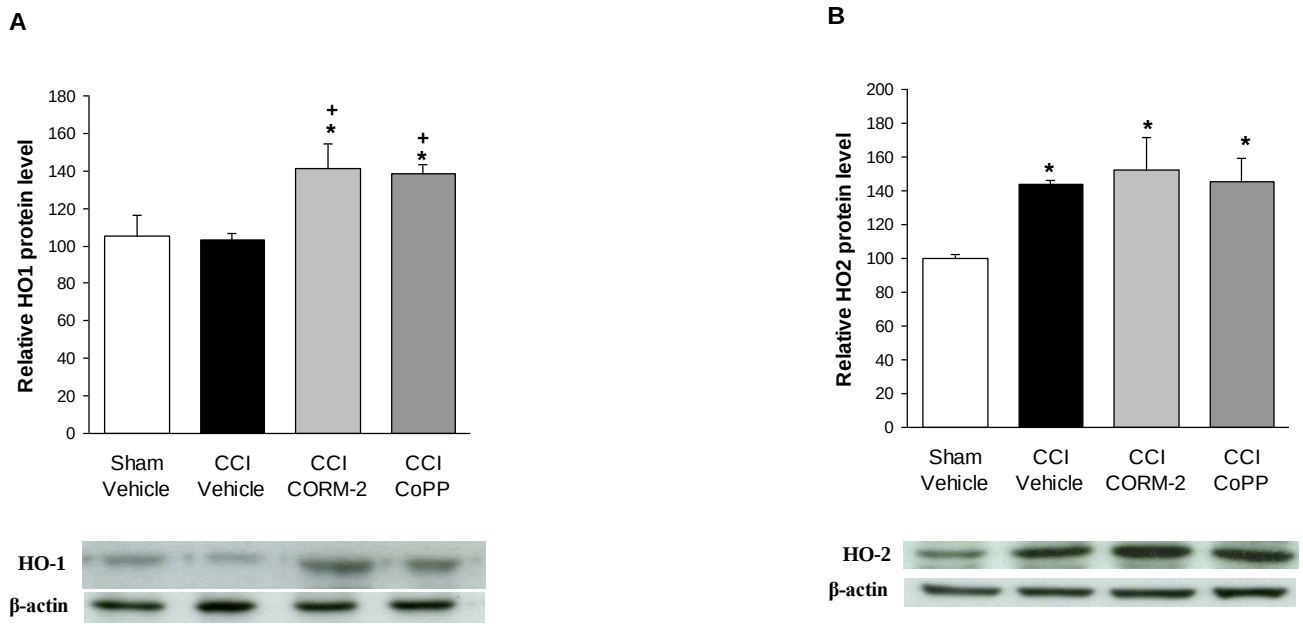


Figure 6. Effect of CORM-2 and CoPP on MOR, DOR and CB2R protein expression from sciatic nerve-injured mice*. The protein expression of MOR (A), DOR (B) and CB2R (C) in the ipsilateral site of the dorsal root ganglia from sciatic nerve-injured (CCI) mice treated with vehicle, CORM-2 or CoPP at 10 days 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 vs. sham-operated vehicle treated mice ($p < 0.05$, one-way ANOVA followed by SNK) and + indicates significant differences when compared vs. sciatic nerve-injured vehicle treated mice ($p < 0.05$, one-way ANOVA followed by SNK). Representative examples of western blots for MOR, DOR and CB2R proteins, in which β-actin was used as a loading control, are also shown. Data are expressed as mean values ± SEM; n = 5 samples per group.*



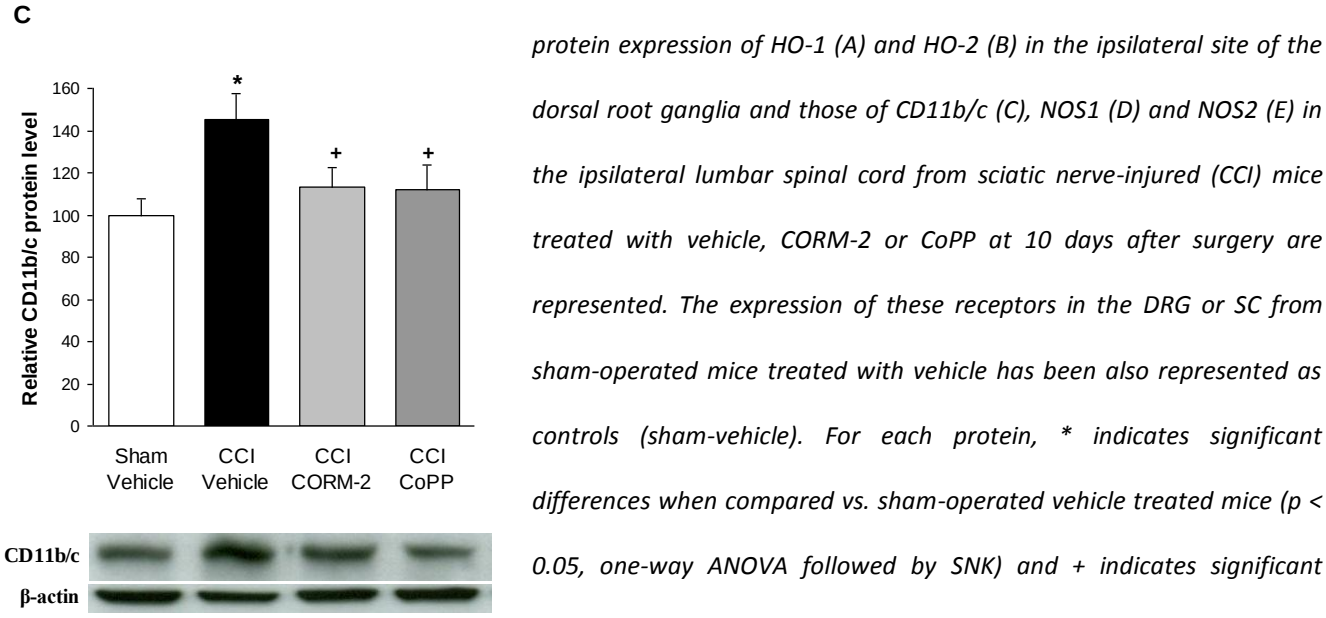


Figure 7. Effect of CORM-2 and CoPP on the HO-1, HO-2, CD11b/c, NOS1 and NOS2 protein expression from sciatic nerve-injured mice*. The protein expression of HO-1 (A) and HO-2 (B) in the ipsilateral site of the dorsal root ganglia and those of CD11b/c (C), NOS1 (D) and NOS2 (E) in the ipsilateral lumbar 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 receptors in the DRG or SC from sham-operated mice treated with vehicle has been also represented as controls (sham-vehicle). For each protein, * indicates significant differences when compared vs. sham-operated vehicle treated mice (p < 0.05, one-way ANOVA followed by SNK) and + indicates significant differences when compared vs. sciatic nerve-injured vehicle treated mice (p < 0.05, one-way ANOVA followed by SNK). Data are expressed as mean values ± SEM; n = 5 samples per group.*

Discussion

In the present 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, injection 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 increased the expression of MOR and HO-1, did not change DOR and HO-2 expression and decreased the over-expression of CB2R, CD11b/c, NOS1 and NOS2 induced by nerve injury.

The antinociceptive and anti-inflammatory effects produced by CO-RMs or CoPP during inflammatory diseases have been previously reported. [15,21] In accordance to 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 CO, [29] supports the hypothesis that the antinociceptive effect produced by CORM-2 and CORM-3 after sciatic nerve injury could probably be due to the release of CO. The present 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 co-administration 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 agonist, the administration of SnPP did not alter their antiallodynic and antihyperalgesic effects following nerve injury. In accordance to these results, a clear relationship between the local antinociceptive effects of MOR agonists, but not DOR or CB2R, and the NO-GMPc-PKG-KATP signaling peripheral pathway activation was previously demonstrated under neuropathic pain conditions. [5,6] Thus, while the local pharmacological inhibition of the NO-cGMP-PKG 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, [34,35] while the activation of CB2R reduces neuropathic pain by inhibiting the activated microglia induced by nerve injury. [36] 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. [37-39] 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 the co-administration 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 reversion of their effects by the co-administration with the non-selective 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 co-administration 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 were administered alone.

In accordance to 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. [5,7,40-42] The present 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 over-expression 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, even though a reduction of the overall inflammation produced by both treatments [20,21,43] could be also implicated in the enhanced antinociceptive effects produced by morphine after CORM-2 or CoPP treatments.

Thus, 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 to other inflammatory

models [17,43] 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 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 over-expression 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 [44] and the administration of microglial activation inhibitors significantly reduced the behavioral symptoms of neuropathic pain in animals. [27] Moreover, the activated microglia promotes the consolidation and progression of neuropathic pain state by the up-regulation of several inflammatory mediators including NO. Activated microglia after nerve injury can also modify the opioid specific signaling which diminished the antinociceptive potency of morphine after nerve injury. Accordingly, treatment with glial inhibitors enhanced the effectiveness of morphine in animals with neuropathic pain. [45]

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 over-expression. In accordance to these data, the suppression of microglial activation and/or the NO 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. [6,27] 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 NO in microglia activated with endotoxin or thrombin as well as under hypoxic conditions. [46]

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

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6. General discussion

Several studies relate the NO/CO signaling pathways in the development and expression of different types of chronic pain, as well as in the analgesic effects produced by opioids or cannabinoids in serveral pain models. The present thesis tries to clarify the role exerted by these small gaseous neurotransmitters on the development and maintenance of neuropathic pain as well as in the antinociceptive effects and expression of MOR, DOR and CB2 receptors after sciatic nerve injury-induced neuropathic pain.

Numerous studies have implicated the NO in the regulation of several neuropathic pain symptoms (Nathan and Xie, 1994; LaBuda, et al., 2006). Indeed, different authors have demonstrated that NO mediates the maintenance of neuropathic pain through the activation of spinal NO-sGC-PKG pathway, triggered by NOS1 and NOS2, in where the sGC and PKG enzymes are essentially required for the signal transduction of NO in the spinal cord after nerve injury (Guan, et al., 2007; Schmidtko, et al., 2008; Tanabe, et al., 2009).

In this thesis, we demonstrated the participation of the peripheral NOS1/NOS2-sGC-PKG signaling pathway in the development and maintenance of the mechanical and thermal allodynia as well as thermal hyperalgesia induced by the chronic constriction of sciatic nerve (CCI) in mice (Hervera, et al., 2010a; Hervera, et al., 2010b). Indeed, our results demonstrated that the mechanical and thermal hypersensitivity induced by CCI was completely abolished in both NOS1-KO and NOS2-KO mice (Hervera, et al., 2010b). Moreover, the peripheral administration of specific NOS1, NOS2, sGC or PKG inhibitors produced potent dose-dependent antiallodynic and antihyperalgesic effects after peripheral nerve injury (Hervera, et al., 2010a). These data suggest that NO, synthesized by NOS1 and NOS2, mediates the maintenance of neuropathic pain induced by a peripheral nerve injury, through the peripheral sGC-PKG pathway activation. In accordance to our results, the pharmacological inhibition or genetic depletion of NOS1 or NOS2 isoenzymes also attenuated the mechanical

hypersensitivity induced by the spinal or sciatic nerve injury in mice (De Alba, et al., 2006; Guan, et al., 2007). Additionally, other studies point out the involvement of NO, synthesized by NOS1 or NOS2, in the signaling cascades activated by other types of chronic pain, such as inflammatory (Schmidtko, et al., 2009; Leanez, et al., 2009).

However, while NOS1 and NOS2 played a different role in inflammatory pain the role played by both enzymes during neuropathic pain seems to be comparable.

There are several signaling pathways involved in the neuropathic pain, and the precise molecular mechanisms implicated remain still unclear. However, one consequence of nerve injuries is the manifestation of adaptive changes in the expression patterns of diverse receptors, channels and enzymes, in which NOS isoenzymes are not an exception. Thus, peripheral nerve injury increases the transcription and expression of NOS1 in the spinal cord of WT mice, at 14 and 21 days after nerve injury, but not after 7 days, indicating that the NOS1 nerve injury-induced upregulation seem to be related to the chronic stages of neuropathic pain. Upregulation of the protein levels of NOS2 were demonstrated at 7 or 14 days after nerve injury, suggesting that spinal NOS2 seems to be more involved in the set up stages of peripheral nerve injury-induced neuropathic pain (Martucci, et al., 2008).

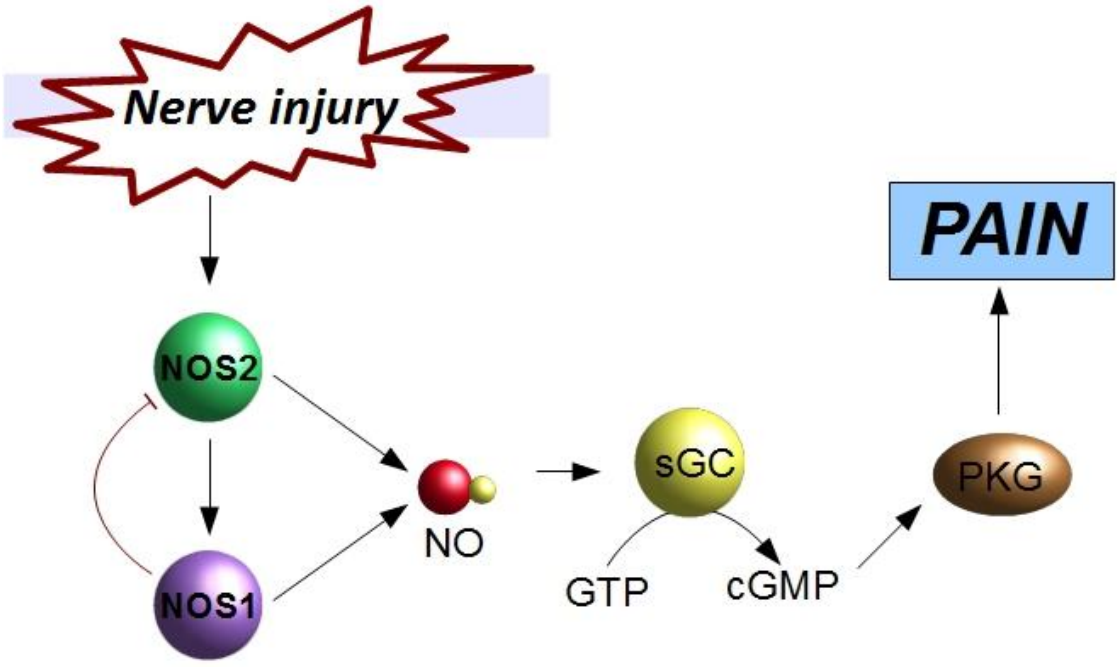

Figure 10. Schematic representation of NO signaling after nerve injury

Considering the expression patterns of the different isoenzymes, NOS2 seems to be more implicated in the development of the neuropathic pain state, whereas NOS1 seem to regulate its maintenance. Moreover, our results revealed an increased spinal cord expression of NOS1, which should be presumably regulated by NOS2, since NOS2-KO animals do not display this nerve injury-induced upregulation, indicating a cross-regulation between these two isoenzymes during neuropathic pain (Figure 10) (Hervera, et al., 2010b).

Regarding the role of CO in the development and expression of neuropathic pain, our findings demonstrated that treatments with CO donors (CORM-2 or CORM-3) or the HO1 inducer (CoPP) significantly reduced the main symptoms induced by CCI, through enhancing the expression of HO1 and reducing the nerve injury-induced spinal microglial activation and NOS1/NOS2 overexpression (Hervera, et al., 2012b). According to other studies with inflammatory models, in which the expression of HO1 was significantly increased by CORM-2 (Megias, et al., 2009; Maicas, et al., 2010), our results further demonstrated that the antinociceptive effects produced by CORMs or CoPP treatments were mainly produced by CO synthesized by HO1. In contrast to HO1, other studies reveal that CO synthesized by HO2, contributes to the progression of neuropathic pain. Indeed, the lack of HO2 appears to prevent the development of neuropathic pain, as a consequence the expression of this enzyme is upregulated after nerve injury (Li and Clark, 2000; Li and Clark, 2003). Surprisingly, our results demonstrated that the antinociceptive effects exerted by CORMs or CoPP treatments, were not due to an inhibition of the nerve injury-induced HO2 upregulation, since its increased expression remains unaltered after these treatments.

Many pathological processes within the peripheral and central nervous system are mediated by complex interactions between neurons and glial cells. In the case of painful peripheral neuropathy, spinal microglia react and undergo a series of changes, such as the release of proinflammatory substances, that directly influence the establishment of neuropathic pain states (Zhuo, et al., 2011). Thus, several studies demonstrate that attenuation of glial activation represents a novel approach in the management of neuropathic pain (Mika, 2008).

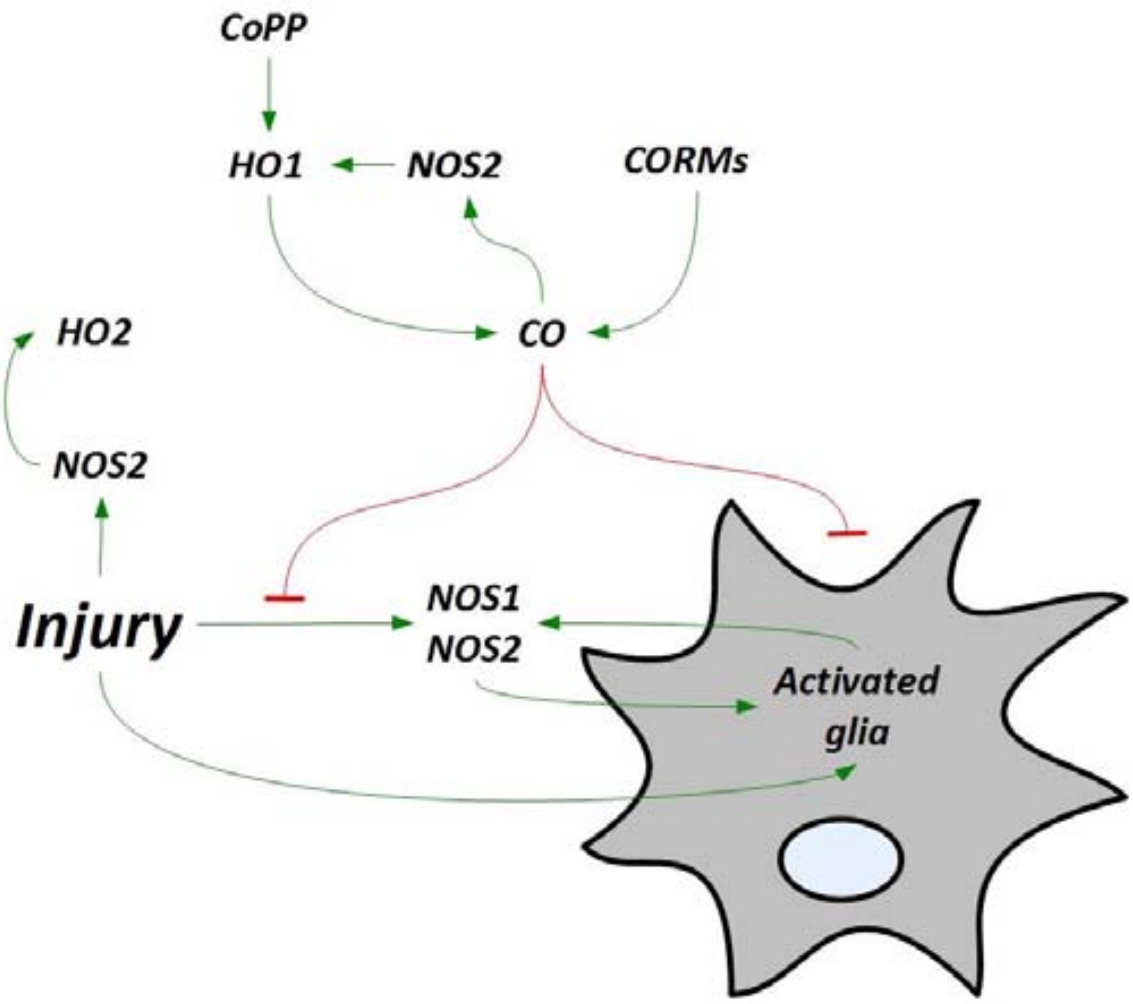

Figure 11. Hypothetic scheme of the CO signaling induced by CORMs or CoPP treatments after nerve injury

Therefore in order to evaluate the possible mechanisms implicated the inhibitory effects produced by the CORMs and CoPP after neuropathic pain, the effects of these treatments on the expression of NOS1 and NOS2 as well as on the modulation of activated microglia induced by nerve injury have been also assessed. Our study revealed, that CO liberated by CORM-2 or synthesized by HO1 reversed the nerve injury-induced NOS1 and NOS2 overexpression as well as the microglia activation in the spinal cord, suggesting these mechanisms as the possible targets for the inhibition of neuropathic pain symptoms induced by both treatments. Interestingly, sciatic nerve-injured NOS2-KO mice did not display the nerve injury induced HO2 overexpression, nor the CO-induced HO1 upregulation, indicating that both mechanisms are NOS2-

dependant, revealing that these enzyme plays a key role in the CO signaling during neuropathic pain (Figure 11). Accordingly, other studies have also verified that the effects produced by CO in acute pain depend on the integrity of the NO pathway (Steiner, et al., 2001). All of these findings support the relevant interactions between the NO and CO signaling pathways, as previously demonstrated in other studies (Fan, et al., 2011).

We were also interested in assessing the possible involvement of NO and CO as well as the peripheral sGC-PKG signaling pathway activated by both gases in the antinociceptive effects produced by MOR, DOR or CB2 receptors agonists during neuropathic pain. A clear relationship between the local antinociceptive effects of MOR agonists and the peripheral NO-sGC-PKG-K^+ATP signaling pathway activation during inflammatory pain had been already proven (Alcaraz, et al., 2000; Steiner, et al., 2001; Rosa, et al., 2008; Fan, et al., 2011). Accordingly, in our studies we demonstrated that peripheral, but not systemic, administration of MOR agonists use the NO/CO-sGC-PKG-K^+ATP pathway to produce their antiallodynic and antihyperalgesic effects, as demonstrated by the reversion of the peripheral, but not systemic, antinociceptive effects of morphine by their co-administration with specific NOS1, NOS2, HO1, sGC, PKG inhibitors or a K^+ATP channels blocker (Hervera, et al., 2011; Hervera, et al., 2012 under review). Moreover, our data also demonstrated the increased effects produced by a subanalgesic dose of morphine peripherally, but not systemically, administered in CORM or CoPP treated animals, suggesting that, an overactivation of the CO-sGC-PKG pathway could be a useful strategy for using peripheral MOR agonists in neuropathic pain management (Hervera, et al., 2012 under review). Further, HO1-CO are not only mediating the downstream signaling of peripheral MOR, but also have an essential role in the modulation of morphine-induced glial activation (Zhang, et al., 2011; Hervera, et al., 2012 under review). Furthermore, as widely described, the peripheral MOR expression decreases as a consecuence of the nerve injury (Obara, et al., 2009). Our data confirmed these results and further demonstrated that the decreased expression of MOR observed in sciatic nerve-injured WT mice was not observed in NOS1 or NOS2-KO mice, revealing these enzymes as main regulators of the nerve injury-induced down-regulation of the

peripheral MOR (Hervera, et al., 2011). Moreover, this down-regulation was prevented by CORM-2 or CoPP treatments, indicating that the CO signaling pathway plays also an important role in the modulation of the peripheral transcriptional changes of MOR during neuropathic pain. These regulatory effects of CO could be explained by the fact that both CORM-2 and CoPP treatments prevented the nerve injury-induced up-regulation of NOS1 and NOS2, main responsibles of the downregulation of MOR expression during neuropathic pain (Figure 12) (Hervera, et al., 2012 under review).

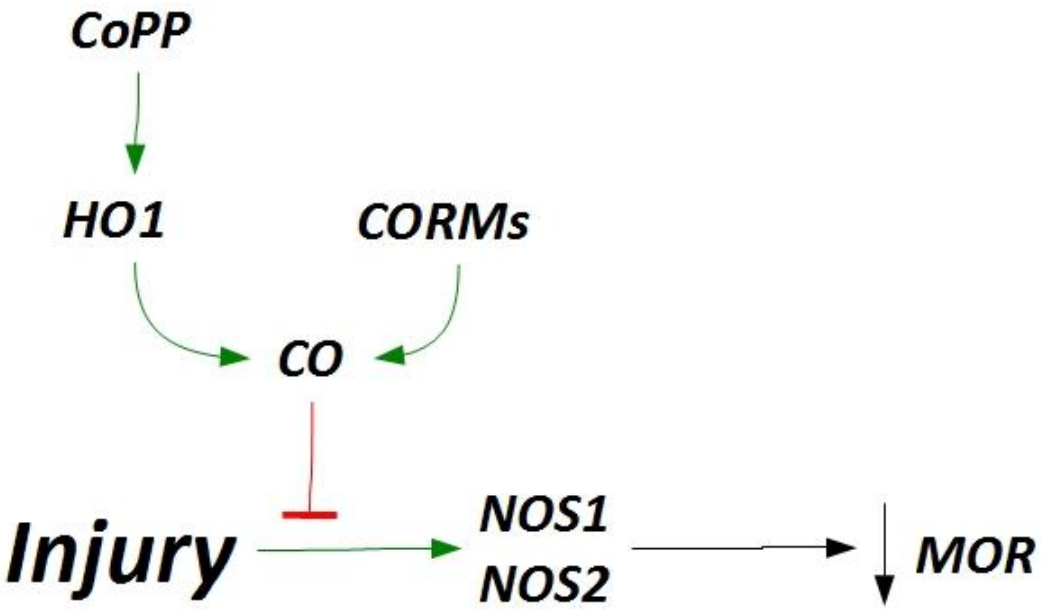

Figure 12 CO/NO regulation of peripheral MOR expression

Regarding DOR and CB2 receptor agonists, we observed that CB2 receptor agonists needed doses much lower than opioids to produce the same analgesic effects, and that DOR agonists were more efficient in the inhibition of neuropathic pain symptoms (Hervera, et al., 2010a; Hervera, et al., 2011). Further, we wanted to verify if the antinociceptive effects produced by DOR and CB2 receptor agonists, were modulated by the CO/NO-sGC-PKG-K^+ATP. In contrast to MOR agonists, the peripheral antinociceptive effects of DOR and CB2 receptor agonists were not produced by using these signaling pathway during neuropathic pain. Thus, while the local pharmacological inhibition of the NO-sGC-PKG pathway attenuated the peripheral antinociceptive effects of morphine, its blockage potentiated the peripheral

antiallodynic and antihyperalgesic effects of DOR and CB2 receptor agonists (Hervera, et al., 2010a). In addition, the administration of CORMs or CoPP blocked the peripheral and systemic inhibitory effects produced by DOR or CB2 receptor agonist under neuropathic pain conditions. 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 (Chattopadhyay, et al., 2008; Gaveriaux-Ruff, et al., 2011), while the activation of CB2 receptor reduces neuropathic pain by inhibiting the activated microglia induced by nerve injury (Racz, et al., 2008; Romero-Sandoval, et al., 2008). In accordance to other studies, our results indicated that while the peripheral expression of DOR did not change after sciatic nerve injury, the expression of CB2 receptor is increased (Zhang, et al., 2003; Obara, et al., 2009). Our results further demonstrated that NO synthesized by NOS1, but not NOS2, is an important regulator of nerve injury-induced DOR downregulation and CB2 receptor upregulation (Hervera, et al., 2010a), moreover while CORM-2 or CoPP treatments did not modify DOR expression both decreased the overexpression of CB2 receptor induced by sciatic nerve injury (Hervera, et al., 2012 under review). These results could be explain by the fact that CB2 receptor is mainly expressed in immune cells, and CORM-2 or CoPP treatments are reducing the amount of active immune cells in the lesion site.

It is well known that the repeated systemic or spinal cord administration of MOR agonists induces the development of antinociceptive tolerance under neuropathic pain conditions (Ossipov, et al., 1995; Al-Hasani and Bruchas, 2011). We also demonstrated the development of morphine tolerance after their repeated peripheral administration (Hervera, et al., 2012a). Several studies demonstrated that the activation of the NOS-sGC-PKG-JNK signaling pathway is involved in the development of opioid tolerance under basal condition and inflammatory pain states (Romero, et al., 2010; Ozdemir, et al., 2011). Our findings demonstrated that the development of peripheral morphine tolerance after sciatic nerve injury could be prevented by the co-administration of morphine with selective NOS2-sGC-PKG-JNK pathway inhibitors (Hervera, et al., 2012a). Therefore, while the single subplantar administration of morphine inhibits the mechanical and thermal allodynia induced by sciatic nerve injury through the

activation of the peripheral CO/NO-sGC-PKG-ATP-K^+ATP channels signaling pathway which concludes in the hyperpolarization of nociceptive neurons inducing analgesia (Hervera, et al., 2011; Hervera, et al., 2012 under review), the repeated peripheral administration of morphine induces tolerance by the activation of the peripheral NO-sGC-PKG-JNK signaling pathway (Hervera, et al., 2012a) which probably concludes in MOR desensitization causing morphine tolerance (Figure 13). Recent studies evidence biological changes produced by the sustained activation of MOR, thus during morphine tolerance, adaptive cellular changes take place in cerebral regulation of K^+ATP channels, which, as mentioned before, are essential in the morphine-induced antinociception (Gonzalez, et al., 2012). The precise mechanism by which JNK may induce antinociceptive opioid tolerance remains still controversial, while some studies point out that repeated morphine administration leads JNK to disrupt the G proteïn from the receptor (Melief, et al., 2010), others refer to a chronic morphine-dependent massive MAPK activation, and consequently CREB activation, that leads to an upregulation of the neuronal CGRP and substance P, sensitizing neurons (Ma, et al., 2001). Moreover, other studies indicated that this chronic morphine-dependent massive MAPK activation is leading to an upregulation of TRPV1, suggesting that this channel could be responsible for opioid tolerance and tolerance-induced hyperalgesia (Chen, et al., 2008). In contrast to MOR, the repeated peripheral administration of DOR or CB2 receptor agonists did not induce antinociceptive tolerance in sciatic nerve-injured mice (Hervera, et al., 2012a) which could be mainly explained by the different signaling pathways activated by these drugs after nerve injury.

Summarizing, these data suggest that both CO and NO systems could modulate the effects and expression of MOR, DOR and CB2 receptor during neuropathic pain, but while MOR elicits its peripheral analgesic effects through the activation of the HO1/NOS-sGC-PKG-K^+ATP signaling pathway, DOR or CB2 receptor do not use this pathway to produce their effects. Finally, although this study shows different strategies to increase the local antinociceptive effects produced by opioids and cannabinoids and avoid the development of tolerance during neuropathic pain, the investigation of new mechanisms of action of these drugs is essential to improve their therapeutic actions in neuropathic pain.

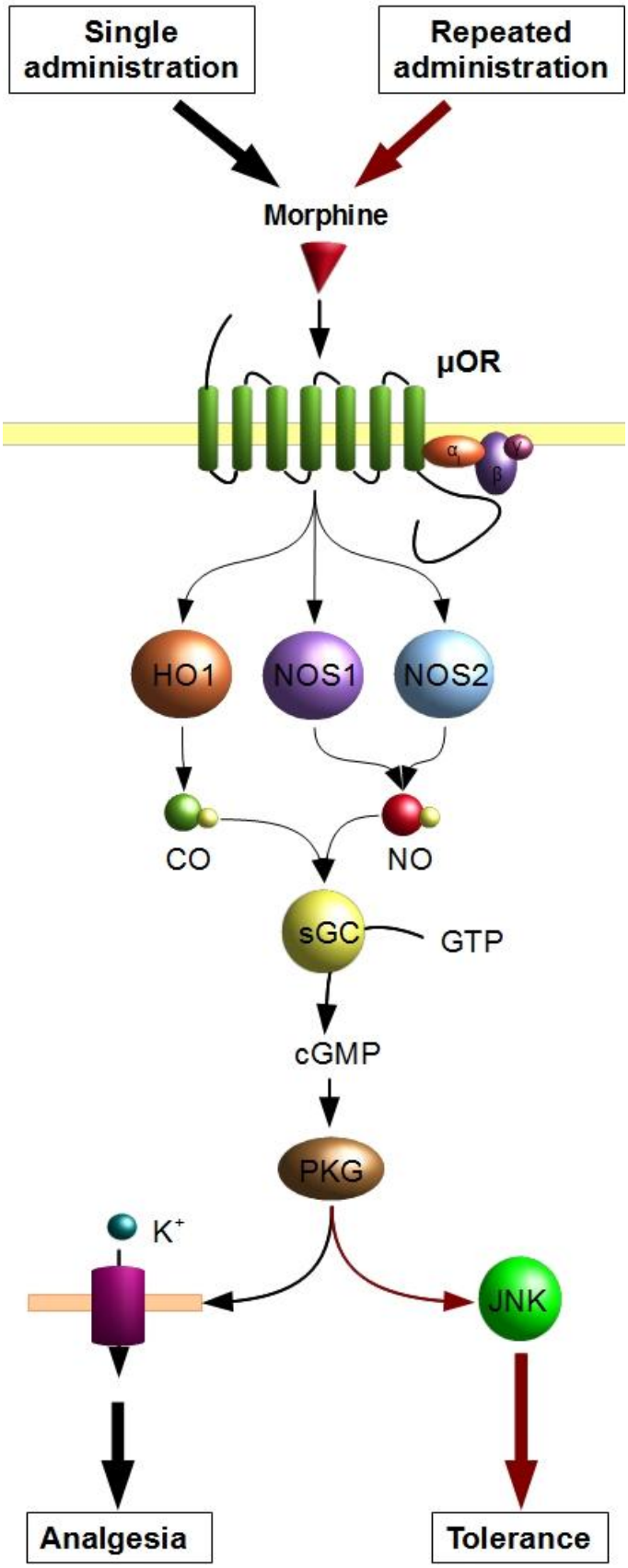

Figure 13. Schematic representation of the CO/NO-sGC-PKG signaling pathway activated by the single or repeated peripheral administration of morphine during neuropathic pain

7. Conclusions

1. The peripheral NO-sGC-PKG signaling pathway, triggered by NOS1 and NOS2, plays a key role in the development and expression of the main symptoms of neuropathic pain in mice.

2. The peripheral, but not systemic, antinociceptive effects of MOR agonists during neuropathic pain are produced through the activation of the HO1/NOS1/NOS2-sGC-PKG-K^+ATP signaling pathway. NO, synthesized by NOS1 and NOS2, is implicated in the peripheral down regulation of MOR.

3. The peripheral antinociceptive effects of DOR and CB2 receptor agonists during neuropathic pain increases by the inactivation of NOS1/NOS2-sGC-PKG signaling pathway. NO, synthesized by NOS1, is implicated in the peripheral down- and up-regulation of DOR and CB2 receptor during neuropathic pain.

4. The inhibition of the NO-sGC-PKG-JNK signaling pathway avoids the development of tolerance to the local antiallodynic effects produced by morphine during neuropathic pain.

5. CO, synthesized by HO1, inhibits neuropathic pain by the attenuation of NOS1/NOS2 overexpression and the microglial activation induced by nerve injury.

6. Treatment with CO, exogenously deliberated or endogenously synthesized by HO1, enhances the peripheral antinociceptive effects of MOR agonists by up-regulating the peripheral expression of MOR and inhibiting microglial activation.

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