UNIVERSITAT DE BARCELONA

Selective blockade of the sigma-1 receptor for the treatment of pain of different aetiology: Preclinical studies

Georgia Gris Ormo

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FACULTAD DE FARMACIA

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Georgia Gris Ormo

Barcelona, 2015

ESTEVE

UNIVERSITAT DE BARCELONA

FACULTAD DE FARMACIA

Programa de doctorado en Biotecnología

Selective blockade of the sigma-1 receptor for the treatment of pain of different aetiology: Preclinical studies

Memoria presentada por Georgia Gris Ormo para optar al título de doctor por la Universidad de Barcelona

Dr. Daniel Zamanillo Castanedo

Director

Dr. Enrique Portillo-Salido

Director

Dra. Josefa Badia Palacín

Tutora

Georgia Gris Ormo

Tesis Doctoral realizada en Esteve

(Área de descubrimiento de fármacos y desarrollo preclínico)

ESTEVE

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PRESENTATION

The present Doctoral Thesis focuses on the study of the Sigma-1 receptor (σ_1R) in the field of pain. This research has been a part of the preclinical σ_1R project focusing on drug discovery of σ_1R ligands for the treatment of pain of different aetiology at the pharmaceutical company ESTEVE.

The main goal of this Doctoral Thesis was to explore the therapeutic interest of σ_1R blockade in the pharmacological management of neuropathic, inflammatory and postoperative pain. The efficacy of the selective σ_1R antagonist S1RA (E-52862) in these different types of pain was evaluated, and its potency and efficacy was compared to other marketed analgesic drugs. To this end, two species (rat and mouse), different pain-related behavioural endpoints (hind paw withdrawal response to thermal and mechanical stimulation), and different pharmacological strategies (systemic acute and repeated E-52862 administration), were evaluated. σ_1R knockout mice were also used to study the *in vivo* specificity of E-52862 and the involvement of σ_1R in the spinal modulation of several pain-related molecular markers in order to ascertain the mechanism of action of σ_1R.

Taken together, the results of this Doctoral Thesis provide new knowledge about σ_1R and support the clinical development of selective σ_1R antagonists as a suitable therapeutic intervention to achieve analgesia in pain conditions of different aetiology.

ABBREVIATIONS

5-HT: 5-hydroxytryptamine (serotonin)

ADME: absorption, distribution, metabolism and excretion

ADMET: absorption, distribution, metabolism, excretion and toxicology

α_1, α_2, β: adrenergic receptors

ANOVA: analysis of variance

ASIC: acid-sensing ion channel

ASO: antisense oligodeoxynucleotides

ATP: adenosine triphosphate

B1/B2: bradykinin receptors

Bcl-2: B-cell lymphoma 2

BD1063: 1-[2-(3,4-dichloropheny)ethyl]-4-methylpiperazine

BDNF: brain derived neurotrophic factor

BiP: binding immunoglobulin protein

Ca^{2+}: calcium ion

CFA: complete Freund's adjuvant

CGRP: calcitonin gene related peptide

CIA: collagen type II arthritis

CMH: mechano-heat-sensitive C fibre

CNS: central nervous system

COX: cyclooxygenase

COX-1: cyclooxygenase-1

COX-2: cyclooxygenase-2

CYP: cytochrome P450 enzymes

D_2 : dopaminergic receptor

DAG: diacylglycerol

DNA: deoxyribonucleic acid

DRG: dorsal root ganglion

E-52862: see S1RA

ED_{50}: effective dose 50

EGFR: epidermal growth factor receptor

EP: Prostanoid receptor

ER: endoplasmic reticulum

ERK: extracellular signal-regulated kinase

Fig.: figure

FMO: flavin-containing monooxygenase

FRET: Fluorescence resonance energy transfer

GABA: gamma-aminobutyric acid

GDNF: glial-derived neurotrophic factor

GFAP: glial fibrillary acidic protein

GPCR: G protein-coupled receptors

H^+: protons

H1: Histamine type 1

HINT1: histidine triad nucleotide bonding protein 1

HPMC: (hydroxypropyl)methyl cellulose

5-HT: serotonin receptor (5-hydroxytryptamine)

IASP: International Association for the Study of Pain

IC_{50}: half maximal inhibitory concentration

IL: interleukin

IL-R: interleukin receptor

iNOS: inducible nitric oxide synthase

IoN: infraorbital nerve

i.d.: intradermal

i.p.: intraperitoneal

i.pl.: intraplantar

IP_3: inositol-1,4,5-triphosphate

IP_3R: inositol-1,4,5-triphosphate receptor

i.v.: intravenous

K^+: potassium ion

Ki: inhibition constant

KO: knockout

μ: mu (opioid receptor)

MAM: mitochondria-associated endoplasmic reticulum membrane

MAPK: mitogen-activated protein kinase

MIA: monosodium iodoacetate

morphine: morphine hydrochloride

mRNA: messenger ribonucleic acid

mTOR: mammalian target of rapamycin

n.a.: not applicable

n.d.: not determined

NGF: nerve growth factor

NK-1: neurokinin-1 receptor

NMDA: *N*-methyl-*D*-aspartate

NMEs: New molecular entities

nNOS: neuronal nitric oxide synthase

NO: nitric oxide

NPY: neuropeptide Y

NR1: N-methyl-D-aspartate receptor subunit

NSAID: nonsteroidal anti-inflammatory drug

OX: oxaliplatin

P2X: purinergic receptor

PAG: periaqueductal grey

PBS: phosphate buffered saline

PKA: protein kinase A

PCK: protein kinase C

PCR: polymerase chain reaction

PGE2: Prostaglandin E2

PGRMC1: progesterone receptor membrane component 1

PIP_2: phosphatidylinositol 4,5-biphosphate

PKA: protein kinase A

PLC: phospholipase C

PNS: peripheral nervous system

PRE-084: 2-(4-Morpholinethyl) 1-phenylcyclohexanecarboxylate hydrochloride

PSNL: partial sciatic nerve ligation

RIPA: radioimmunoassay precipitation assay

ROS: reactive oxygen species

RT: room temperature

RVM: rostral ventromedial medulla

S1RA: Sigma-1 receptor antagonist (E-52862; 4-[2-[[5-methyl-1-(2-naphthalenyl)-1H-pyrazol-3-yl]oxy]ethyl] morpholine)

σR: sigma receptor

σ_1R: sigma-1 receptor

σ_2R: sigma-2 receptor

SBDL: steroid binding domain like

SC: spinal cord

s.c.: subcutaneous

SKF-10,047: N-allyl-normetazocine

SP: substance P

STZ: streptozotocin

TM: transmembrane

TNC: trigeminal nucleus caudalis

TNF-α: tumor necrosis factor α

TrkA: tyrosine kinase A

TRP: transient receptor potential

TRPV1: transient receptor potential vanilloid type 1

TTXr: tetrodotoxin-resistant voltage-gated sodium channel

TTXs: tetrodotoxin-sensitive voltage-gated sodium channel

VGCCs: voltage-gated Ca^{2+}channels

WDR: wide dynamic range

WT: wild-type

INDEX

INTRODUCTION

1. PAIN

1.1. General Overview

The International Association for the Study of Pain (IASP) defines pain as:

"An unpleasant sensory or emotional experience associated with actual or potential tissue damage, or described in terms of such damage".

The concept of pain has both sensory and emotional components, and therefore its management needs to consider the multidimensional characteristics of pain by taking into account nociception, pain perception, suffering, and pain behaviour (Loeser and Melzack, 1999).

Nociception is the process by which intense noxious thermal, mechanical or chemical stimuli are detected by a subpopulation of peripheral nerve fibres called nociceptors. Nociceptive pain is described as pain occurring with a normally functioning somatosensory nervous system as opposed to the abnormal function seen in pathological pain. It is caused by a brief noxious stimulus, such as when touching something too hot, cold or sharp, and arises from actual or threatened damage to non-neural tissue, and is due to the activation of high-threshold nociceptor neurons (Fig. 1). This type of pain is maintained in the presence of noxious stimuli, so it is usually temporary and has a protective role. Loss of nociception, as in hereditary disorders associated with congenital insensitivity to pain (Indo, 2001; Cox *et al.*, 2006), leads to repeated injury and inadvertent self-mutilation, thus illustrating the highly adaptive function of nociceptive pain (Costigan *et al.*, 2009).

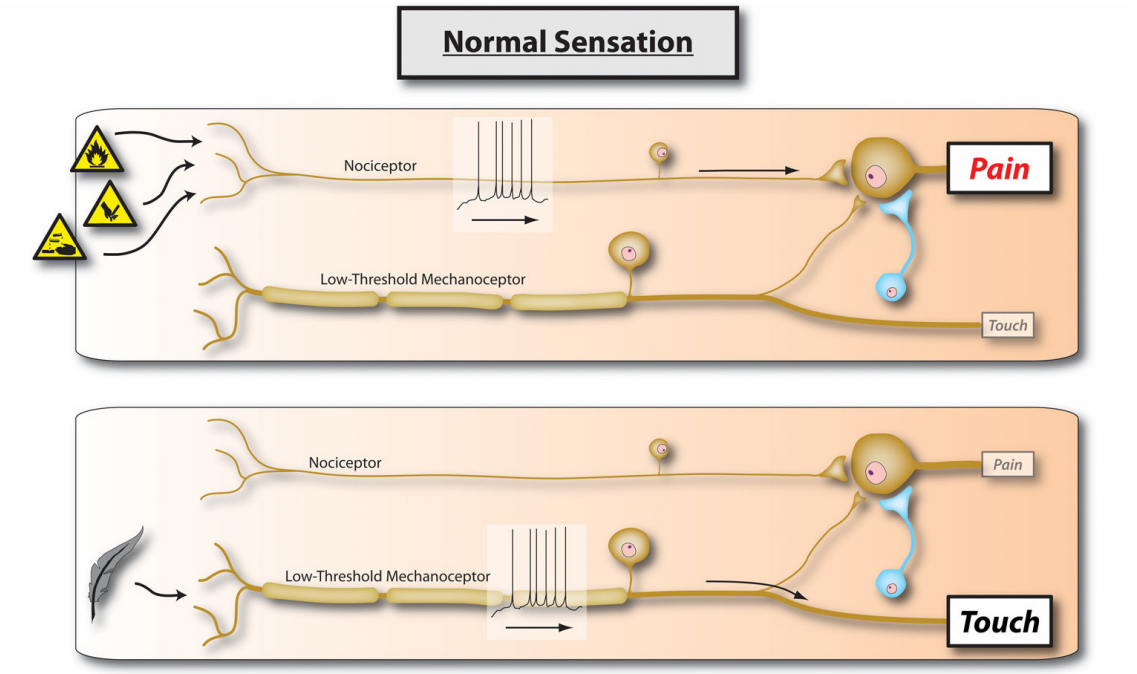


Fig. 1. The somatosensory system is organized such that the highly specialized primary sensory neurons that encode low intensity stimuli only activate the central pathways that lead to innocuous sensations, while high intensity stimuli that activate nociceptors only activate the central pathways that lead to pain and the two parallel pathways do not functionally intersect. This is mediated by the strong synaptic inputs between the particular sensory inputs and pathways and inhibitory neurons that focus activity to these dedicated circuits (From Woolf, 2011).

The sensation of pain results from an extraordinarily complex and interactive series of mechanisms integrated at all levels of the neuroaxis, from the periphery, via the dorsal horn, to higher cerebral structures (Millan, 1999). The perception of pain is controlled by both ascending and descending pathways.

1.1.1. Pain transmission

Nociceptive peripheral stimulation is transmitted through the **ascending pain pathway** as a nerve impulse from the periphery to the cerebral cortex. The ascending tract is triggered when a peripheral noxious stimulus (biological, electrical, chemical, mechanical or thermal) is received. It is converted to electrical signal —a phenomenon called **transduction**. Therefore, the action potential (nerve impulse) is conducted through the axons of primary afferent fibres (nociceptors) to the dorsal horn of the spinal cord (SC). These neurons, also called first order neurons, have their cell bodies located in the dorsal root ganglion (DRG) –in the body– and in the trigeminal ganglion –in the face. These neurons have both a peripheral and central axonal branch that innervates their target organ and the SC, respectively (Basbaum *et al.*, 2009). Thus, DRG neurons make a synapse onto dorsal horn neurons (second order neurons) in the SC, which in turn conveys projections through the contralateral side (opposite side where the insult has occurred) of the SC and ascends to supraspinal areas (brainstem nuclei) via several different ascending pathways (**transmission**). The main circuits mediating physiological pain are the lateral spinothalamic tract and the spinoparabrachial tract, differentiated by the anatomical route towards the brain (Kuner, 2010). Already in the brain, they make synapse onto third order neurons that transmit the impulse to the cerebral cortex, where the information is processed —a phenomenon called **perception** (Fig. 2).

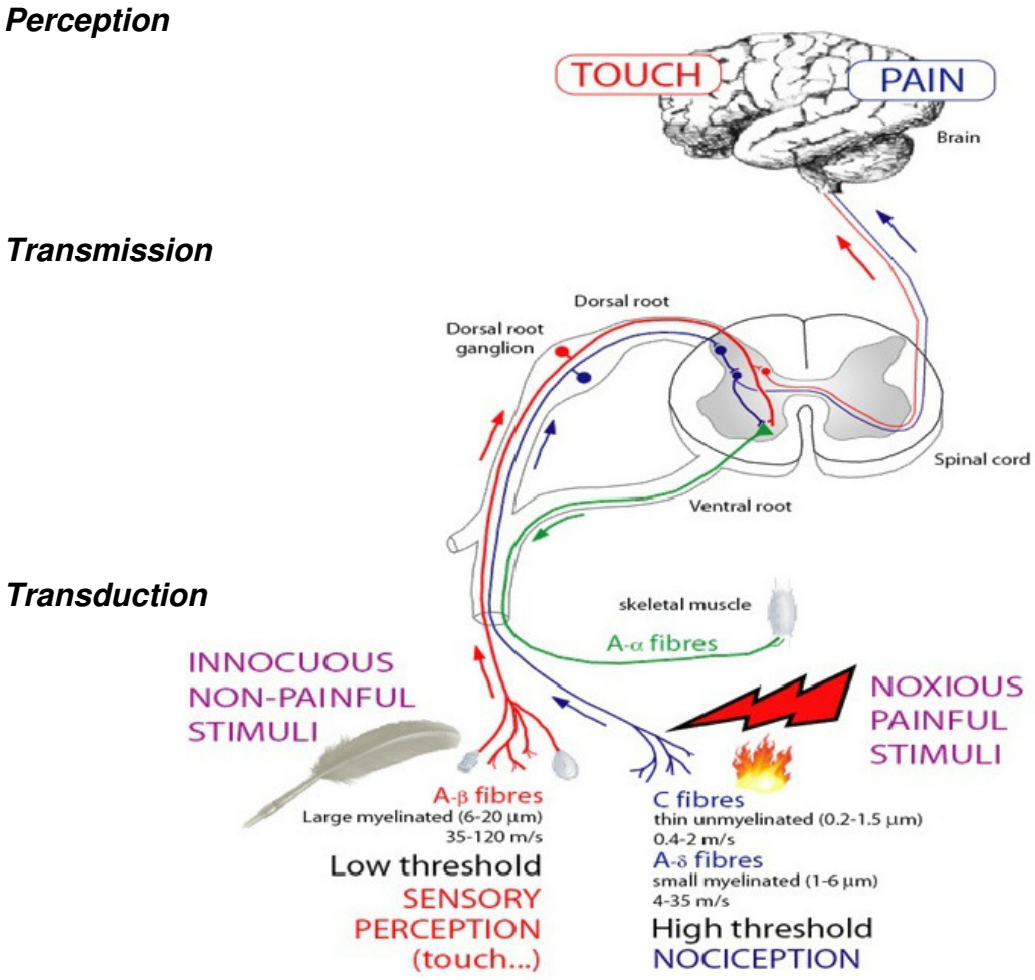


Fig. 2. Graphic representation of the pain circuitry: Transmission of the peripheral stimulation as a nerve impulse from the periphery to the cerebral cortex through the sensory afferent fibres. The ascending circuits consist mainly of three phases: transduction (a noxious stimulus is converted to an electrical impulse), transmission (synaptic conduction of the three afferent fibres towards supraspinal structures), and perception (information processing in the cerebral cortex). Aβ fibres (low-threshold neurons) transmit innocuous non-painful stimuli (touch). Nociceptive fibres include C and Aδ fibres (both are high-threshold neurons) that transmit noxious painful stimuli. Conversely, Aα fibres provide information of proprioception and also innervate the motor function to the skeletal muscles as a reaction response in order to avoid the painful stimuli.

There are three main groups of **first order neurons**, classified according to morphology and function criteria:

- **Aβ fibres:** Large myelinated (6-20 μm) and fast driving (35-120 m/s) fibres. They transmit information relating to innocuous mechanical stimuli (non-painful stimuli), such as vibration and light touch.
- **Aδ fibres:** Small myelinated (1-6 μm) and fast driving (4-35 m/s) fibres. They are mediators of acute, well-localized "first" or fast pain (Basbaum *et al.*, 2009), upon the first adaptive response to pain (withdrawal). Electrophysiological studies have further subdivided these fibres into two main categories:
  - ✓ Type I: They respond to both mechanical and chemical stimuli; they have relatively high thermal thresholds (> 50 ºC), and they mediate the acute "first" pain to mechanical stimuli.
  - ✓ Type II: Under physiological conditions, they respond to the application of thermal stimuli, but have a much lower threshold (40-45 °C). In contrast, the thresholds to mechanical stimuli are much higher. They mediate the acute "first" pain to thermal stimuli (Giordano, 2005).
- **C fibres:** Unmyelinated small diameter (0.2-1.5 μm) and slow conduction (0.4-2 m/s). They transmit poorly localized, diffuse pain, also called "second" or slow pain (Basbaum *et al.*, 2009). Unmyelinated C fibres are heterogeneous, most are polymodal nociceptors and heat- and mechanically sensitive (mechano-heat-sensitive C fibres, CMHs) (Snider and McMahon, 1998; Perl, 2007). Interestingly, there are certain nociceptors, called "silent" or "quiet" nociceptors, which respond to heat but not to mechanical stimuli, except at the site of tissue injury, where they also develop mechanical hypersensitivity (Schmidt *et al.*, 1995). These afferents are more responsive to chemical stimuli (capsaicin or histamine) as compared to CMHs, and likely come into play when the chemical medium of inflammation alters their properties (Basbaum *et al.*, 2009). C fibres

are also heterogeneous regarding the neuroactive substances (transmitters and proteins) they synthesize and release, and the receptors and ion channels they express, which confer different pain sensitivities (Tsuda *et al.*, 2005; Basbaum *et al.*, 2009):

- ✓ Peptidergic C nociceptors: they synthesize, store and release neuropeptides such as substance P (SP), neuropeptide Y (NPY) and the calcitonin gene-related peptide (CGRP); they also express the tyrosine kinase A (TrkA) neurotrophin receptor, activated by nerve growth factor (NGF) (Basbaum *et al.*, 2009).
- ✓ Non-peptidergic C nociceptors: Among other proteins, they express the c-Ret neurotrophin receptor that is targeted by glial-derived neurotrophic factor (GDNF). A large percentage of those expressing c-Ret also binds isolectin IB4 and expresses G protein-coupled receptors (GPCR) of the Mrg family (Dong *et al.*, 2001), as well as some specific purinergic receptor subtypes, particularly $P2X_3$.

In summary, under physiological conditions, Aδ and C fibres are responsible for nociceptive transmission. In contrast, Aβ fibres transmit the sensation of touch. Exceptionally, under sensitization processes (pathological pain), Aβ fibres can promote nociceptive transmission. In addition to these three main groups of fibres, **Aα fibres** are also described as responsible for transmitting the information related to proprioception (sense of the relative position).

As mentioned above, primary afferent nerve fibres project to the dorsal horn of the SC, but fall into a specific spot depending on the fibre type. The gray matter of the SC has ten layers (Rexed laminae) based on the presence of different types of neurons,

size, and the place where fibres make synapse (Rexed, 1952; Basbaum and Jessell, 2000). For example, C fibres project to laminae I and II, similarly to Aδ fibres, which project to lamina I as well as to the deeper dorsal horn (lamina V). The low threshold Aβ fibres project to deeper laminae (III, IV and V) (Basbaum *et al.*, 2009) (Fig. 3).

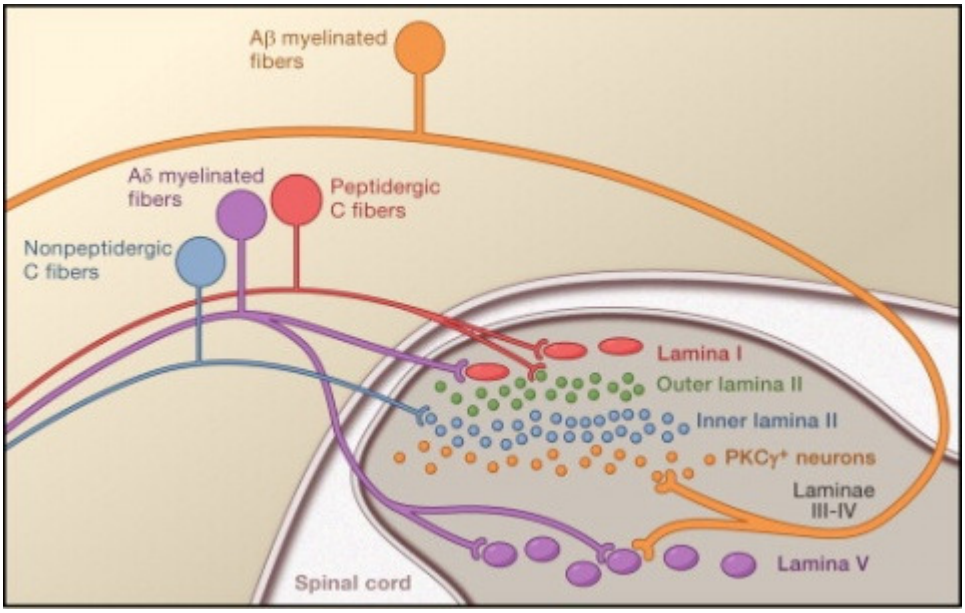


Fig. 3. Connections between primary afferent fibres and the SC. There is a very precise laminar organization in the dorsal horn of the SC; subsets of primary afferent fibres target spinal neurons within discrete laminae. The unmyelinated, peptidergic C (red) and myelinated Aδ (purple) nociceptors terminate mostly superficially, synapsing upon large projection neurons (red) located in lamina I and interneurons (green) located in outer lamina II. The unmyelinated, non-peptidergic nociceptors (blue) target interneurons (blue) in the inner part of lamina II. In contrast, innocuous inputs carried by myelinated Aβ fibres (orange) terminate on PKCγ expressing interneurons in the ventral half of the inner lamina II. A second set of projection neurons within lamina V (purple) receive convergent inputs from Aδ and Aβ fibres (From Basbaum *et al.*, 2009).

Second order neurons constitute the major output from the dorsal horn to the brain (Basbaum and Jessell, 2000). Depending on the afferent input from the first order neuron, three types of second order neurons can be distinguished (Schaible and Grubb, 1993; Calvino and Grilo, 2006):

- **Specific nociceptive neurons:** They respond exclusively to noxious stimuli, both thermal and mechanical, are mediated by high intensity Aδ and C fibres, and are mostly located in laminae I (marginal zone), II, V and VI. They are involved in the encoding pain location.
- **Wide dynamic range (WDR) neurons:** They respond to a broad range of mechanical, thermal or chemical stimulus intensities transferred by first order neurons (Aδ, C and Aβ). Given the convergence of fibres that transmit noxious and innocuous stimuli, this cell group plays a central role in the mechanisms of inhibitory pain modulation. WDR neurons are located in laminae I, II, IV, V, VI and X.
- **Non-nociceptive neurons:** They respond to low intensity innocuous stimuli (both mechanical and thermal) and also to proprioceptive stimuli. They are mainly located in laminae I, II, III and IV.

Finally, **third order neurons** are found at the supraspinal level, extending from the thalamus up to the primary somatosensory area (primary cortex), where the information is processed —a phenomenon called **perception**, resulting in the "feeling" of pain.

The **descending pathways** are integrated by neurons that project from the frontal cortex, the hypothalamus and other brain areas to the midbrain and rostral ventromedial medulla, and also down to the SC, where they are activated by ascending nociceptive transmission and other influences from the brain, such as psychological factors (e.g., fear or anxiety, which exert facilitatory influences through these modulatory systems). The descending pathways play an important role in integrating the

nociceptive input at the spinal level exerting inhibitory or excitatory/facilitatory modulation (Millan, 1999) (Fig. 4).

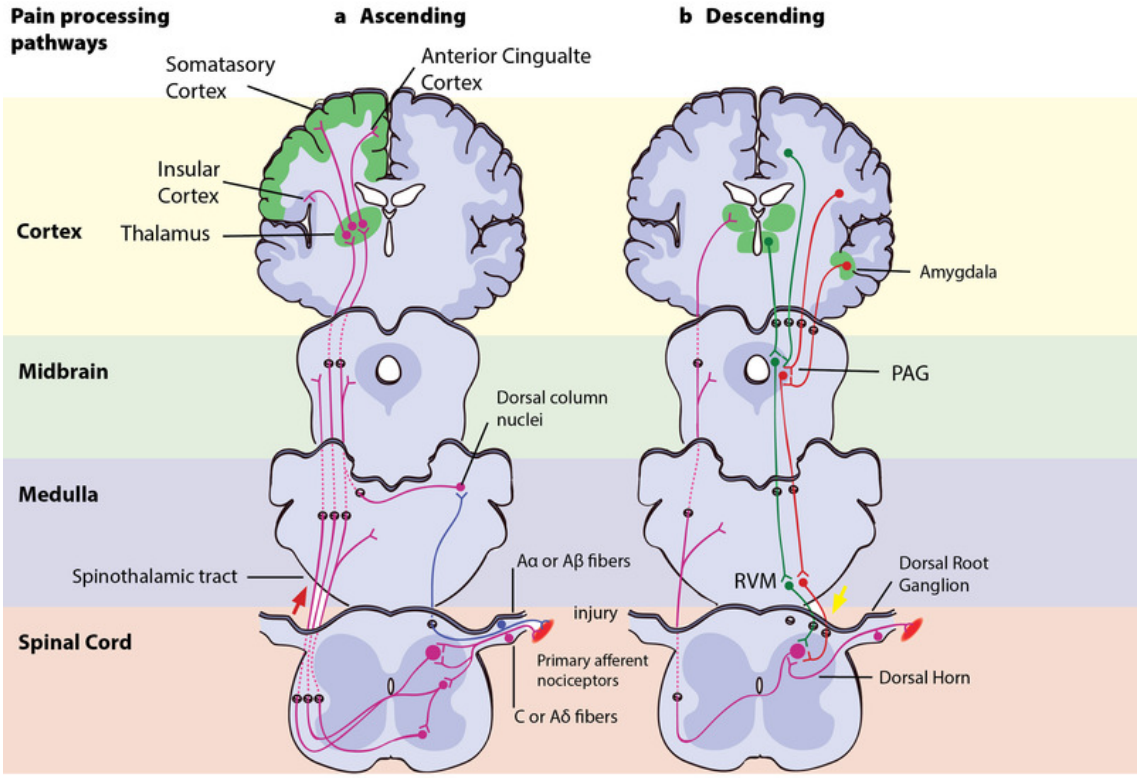


Fig. 4. Left: Ascending pain pathways: An injury is signaled simultaneously via fast-conducting Aα or Aβ fibres and slow-conducting C- or Aδ-fibres. The C- and Aδ-fibres sends pain information from nociceptors in the tissue or skin, and transmits these signals to second order neurons in the dorsal horn of the spinal cord. The second order neurons then cross over to the opposite side, where they form the ascending spinothalamic tract. This tract projects signals to nuclei in the medulla and midbrain on the way up to the thalamus. The thalamus relays the information to the somatosensory and insular cortex, as well as cortical regions mediating different aspects of the pain experience such as affective responses in the cingulate cortex. Right: Descending pain modulation pathways: Information from the environment and certain motivational states can activate this top–down pathway. Several areas in the limbic forebrain including the anterior cingulate and insular cortex, nuclei in the amygdala and the hypothalamus (H), project to the midbrain periaqueductal grey (PAG), which then modulates ascending pain transmission from the afferent pain system indirectly through the rostral ventromedial medulla (RVM) in the brainstem. This modulating system produces analgesia by the release of endogenous opioids, and uses ON- and OFF-cells to exert either inhibitory (green) or facilitatory (red) control of nociceptive signals at the spinal dorsal horn (Loseth *et al.*, 2013).

Modulation results in descending inhibition (suppression) of nociception through the release of neurotransmitters such as serotonin, norepinephrine, and endogenous opioids; or it can also potentiate descending facilitation of nociception where glia contribute to supraspinal facilitatory influences on the processing of pain messages in the SC, and consequently to pain (Basbaum *et al.*, 2009). Interneurons are also present in the SC and play a major role in the modulation of the ascending and descending sensory information, specifically in the control of excitability at the segmental level and thus determine how nociceptive information is relayed to higher structures. Regulation of inhibitory interneuron activity may therefore have critical consequences on pain perception (Labrakakis *et al.*, 2009).

1.1.2. Acute *versus* Chronic pain

Whereas acute pain is an alarm that leads into nociceptive pathways of the central nervous system (CNS) to avoid tissue damage, chronic (pathological) pain is not protective, but maladaptive, and results from abnormal functioning of the nervous system. It is not a symptom of some disorder but rather a disease of its own (Woolf, 2010; Micheletti *et al.*, 2014), given that it is low-threshold pain characterized by sensory abnormalities. This hypersensitivity state leads to both peripheral and central sensitization and temporal summation with a progressive build-up in pain response to repeated C fibre stimulation (wind-up). Both peripheral nervous system (PNS) and CNS components of the pain transmission pathways exhibit huge **plasticity both functionally and structurally**, and are manifested as changes in individual molecules, synapses, cellular function and network activity, leading to an enhancement of pain signals and producing hypersensitivity. Neuroplasticity occurs shortly after the onset of persistent acute pain and leads to the transition from acute pain to chronic pain

(Voscopoulus and Lema, 2010). The progression from acute to chronic pain, also known as pain chronification, remains not completely understood. Several factors are involved in this transition, including peripheral and central sensitization, neuroplastic changes (including protein expression), altered pain modulation, and perception.

Peripheral sensitization is the result of peripheral changes in the expression of pain-related key receptors and ion channels, some of which are only marginally expressed under physiological conditions, which determine the excitability of the nociceptor terminal.

Central sensitization is an abnormal state of the CNS characterized by the enhancement of several mechanisms:

- Function of neurons and circuits in nociceptive pathways
- Membrane excitability (hyperexcitability) and synaptic efficacy
- Plasticity of the somatosensory nervous system

All these phenomena occur in the dorsal horn of the SC and in supraspinal structures during the development of persistent pain in response to activity, inflammation, and neural injury (Latremoliere and Woolf, 2009) (Fig. 5). Central sensitization is mainly initiated and maintained by the activity of the pathologically sensitized C fibres (homosynaptic facilitation), but also by the low-threshold Aβ fibres (heterosynaptic facilitation). These normally signal innocuous sensations but, after neural lesions, begin to produce mechanical sensitivity and ectopic spontaneous pain (Khan *et al.*, 2002; Witting *et al.*, 2006; Costigan *et al.*, 2009).

Central sensitization is responsible for many of the temporal, spatial and threshold changes in pain sensitivity in acute and chronic clinical pain settings, and exemplifies the fundamental contribution of the CNS to the generation of pain hypersensitivity (Latremoliere and Woolf, 2009). In summary, the typical changes of

individual neurons following peripheral inflammation or nerve lesion are translated into several phenomena:

- Increased responses to noxious stimulation of inflamed tissue
- Lowered threshold of nociceptive specific SC neurons
- Increased responses to stimuli applied to non-inflamed tissue surrounding the inflamed tissue
- Expansion of the receptive field

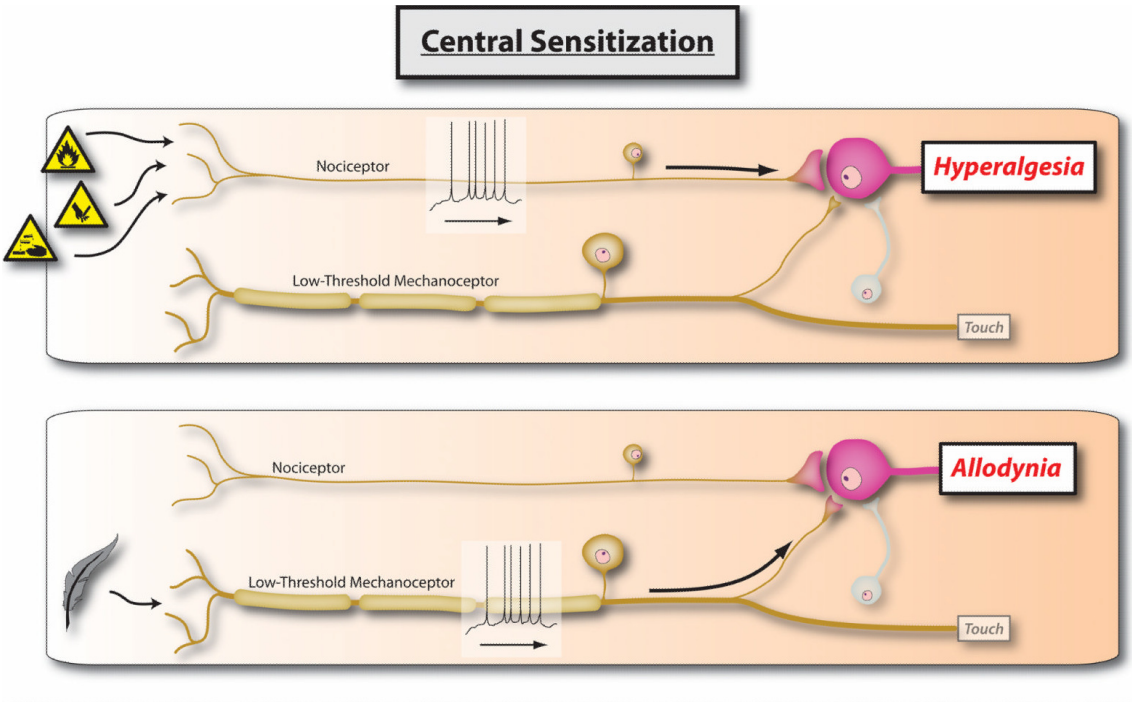


Fig. 5. Central sensitization involved in chronic pain facilitation. With the induction of central sensitization in somatosensory pathways with increases in synaptic efficacy and reductions in inhibition, a central amplification occurs enhancing the pain response to noxious stimuli in amplitude, duration and spatial extent, while the strengthening of normally ineffective synapses recruits subliminal inputs such that inputs in low threshold sensory inputs can now activate the pain circuit. The two parallel sensory pathways converge (From Woolf, 2011).

Central sensitization is translated behaviourally into **symptoms and signs** of persistent (chronic) pain, such as burning pain, spontaneous pain, and abnormal

stimulus-evoked pain (mainly hyperalgesia and allodynia, but also dysesthesia and paresthesia).

When a stimulus that usually causes mild pain is perceived by the patient as producing severe pain, this situation is called **hyperalgesia**. Depending on the nature of the stimulus, the resultant condition is known as heat, cold or mechanical hyperalgesia. In some cases, painless stimuli (such as the rubbing of clothes or water running down one's back in the shower) are felt as painful, and this situation is known as **allodynia** and can be very distressing for some patients. Otherwise, disesthesia is known as an unpleasant abnormal sensation of touch, different from pain, either spontaneous or evoked, and paresthesia is known as an abnormal sensation of tingling, tickling, prickling and pricking, different from pain, either spontaneous or evoked, not unpleasant (Baños *et al.*, 2003; Ji *et al.*, 2014).

1.1.3. Pain-related molecular markers

Several pain-related molecules are modulated in the CNS after injury, such as ion channels, receptors, cytokines, kinases, neuropeptides, and glial and neuronal cells, among others. The efforts to find a selective and predictive marker are very important because it would allow early diagnosis and the selective treatment for specific forms of pain. The SC, specifically laminae I and II of the dorsal horn, is a key area where the expression of many pain-related molecular markers are strongly modulated. Here, we summarize some of the most important molecules involved in the spinal responses to noxious stimuli.

c-Fos, an immediate early gene, and extracellular signal-regulated kinase (**ERK**), a mitogen-activated protein kinase (MAPK), are rarely expressed in the SC under physiological conditions,. Innocuous (non-noxious) stimuli, such as light touch,

warm water or Aβ fibre activation normally do not induce the expression of c-Fos and ERK (Coggeshall, 2005; Gao and Ji, 2009). Nevertheless, the activation of small-diameter cutaneous sensory afferents by noxious heat or chemical stimuli results in the rapid appearance of c-Fos immunoreactivity and the activation through phosphorylation of ERK in the superficial layers of the dorsal horn. During inflammation and neuropathy a large number of SC neurons induce c-Fos expression, thus suggesting that a large population of neurons is activated (Schaible *et al.*, 2006). In turn, the expression of pERK has been related to wind-up and central sensitization processes.

Nerve injury causes proliferation of microglia and astrocytes in the SC that could contribute to increased production of glial mediators and to the expression of hyperalgesia and allodynia (Ji *et al.*, 2014). Microglial activation occurs mainly during the early phase of peripheral injury, whereas astrocytic reaction is delayed and occurs normally a few days after injury (Choi *et al.*, 2015), thus playing both a major role in the development and maintenance of allodynia and hyperalgesia in acute, subchronic and chronic pain states. Glial fibrillary acidic protein (**GFAP**) is an intermediate filament component in astrocytes and its expression may increase with astrocytic activation after CNS injury (Stephenson and Byers, 1995; Sun *et al.*, 2005). As a consequence of a peripheral lesion that persistently generates pain impulses to the SC, inhibitory interneurons eventually die. Moreover, glial cells remodel neuronal synapses to intensify nociceptive transmission, neurons become more sensitive and new connections grow, resulting in a neuroplasticity process. Hence, after a lesion, astrocytes undergo extensive hypertrophy of their cell bodies and cytoplasmic processes. The biochemical hallmarks of astrogliosis are: (i) up-regulation of intermediate filament proteins (GFAP, vimentin and/or nestin); (ii) over-expression of the intracellular signaling molecule S100B; and (iii) increased production of a variety of

proinflammatory substances (Watkins and Maier, 2003; Raghavendra *et al.*, 2004). Microglia and astrocytes react to peripheral insults such as paw incision (Obata *et al.*, 2006; Romero-Sandoval and Eisenach, 2007; Romero-Sandoval *et al.*, 2008), carrageenan-induced paw inflammation (Hua *et al.*, 2005), CFA-induced paw inflammation and monoarthritis (Raghavendra *et al.*, 2004; Sun *et al.*, 2007) and peripheral nerve injury (Jin *et al.*, 2003; Tsuda *et al.*, 2003; Tanga *et al.*, 2005), among others. These results suggest that different types of pain may share a common pathophysiological mechanism.

Nitric oxide (NO) acts as a neuromodulator in laminae I and II of the SC and is involved in the modulation of thermal and inflammatory hyperalgesia after an insult. NO synthesis is primarily triggered by activation of *N*-methyl-*D*-aspartate (NMDA) receptors (Stanfa *et al.*, 1996; Freire *et al.*, 2009). Interestingly, the inhibition of the NO production dose-dependently reduces c-Fos expression and behavioural signs of pain following noxious mechanical, chemical and electrical stimuli (Hoskin *et al.*, 1999; Wu *et al.*, 2000; Coggeshall, 2005). Neuronal nitric oxide synthase (**nNOS**), the isoform of the enzyme for NO production in both CNS and PNS, has been shown to both genetically and pharmacologically modulate persistent pain, such as chronic inflammatory and neuropathic pain (Sardella *et al.*, 2011). Non-specific NOS inhibitors blocked CFA-evoked thermal and mechanical pain hypersensitivity at both the development (2 h) and the maintenance (24 h) phase in wild type (WT) mice, but had no effect in knockout mice for the nNOS gene (Chu *et al.*, 2005; Chen *et al.*, 2010).

The neuropeptide **SP** is a member of the tachykinin family that binds to the neurokinin-1 (NK-1) GPCR, causing its internalization (Mantyh *et al.*, 1989). Although, SP is released into the dorsal horn upon noxious or electrical stimulation of C fibres, without previous injury (Adelson *et al.*, 2009; Taylor *et al.*, 2014), it has been

extensively involved in the neurogenic inflammatory process triggered by peripheral inflammation or nerve injury (Abbadie *et al.*, 1996; Schaible *et al.*, 2005, 2006; Wang *et al.*, 2015). SP is co-localized with glutamate in primary afferent Aδ and C fibres, and both molecules interact by modulating the nociceptive transmission from the periphery to the dorsal horn of the SC, specifically concentrated in lamina I (Abbadie *et al.*, 1996; Cuesta *et al.*, 1999). One of the main experimental models in which SP expression has been widely studied is the inflammatory model induced by an intraplantar (i.pl.) injection of carrageenan (Traub, 1996; Okano *et al.*, 1998; Taylor *et al.*, 2014). Moreover, antagonists at the NK-1 receptor have shown to attenuate central sensitization (Schaible *et al.*, 2006).

Another example of biomarker widely distributed in the CNS and PNS and having an important neuromodulator role in primary sensory neurons is the neuropeptide Y (**NPY**). NPY is a 36-amino acid peptide that acts at GPCRs to modulate a variety of physiological processes. NPY is present in superficial laminae of the dorsal horn of the SC, where it is up-regulated during inflammation. However, the mechanisms underlying its effects still remain unclear. For instance, intrathecal administration of NPY has been shown to be both pro- and anti-nociceptive in inflammatory and neuropathic pain models (Intondi *et al.*, 2008; Yalamuri *et al.*, 2013). Thus, whereas one study showed that intrathecal administration of NPY was able to inhibit hyperalgesia associated with nerve injury and inflammation and consequently reduce SP release (Taiwo and Taylor, 2002; Mahinda and Taylor, 2004; Intondi *et al.*, 2008; Kuphal *et al.*, 2008; Yalamuri *et al.*, 2013), another study showed that injecting NPY directly into DRG immediately after spinal nerve ligation injury exacerbates pain-related behaviour (Sapunar *et al.*, 2011). It has been recently shown that Y1 receptors contribute to the antihyperalgesic effects of NPY by mediating the inhibition of SP release, and that Y1

receptor signaling in the dorsal horn is enhanced during inflammatory nociception. It has also shown a significant regulation in the expression of NPY after plantar incision (Spofford and Brennan, 2012).

1.2. Neuropathic pain

Neuropathic pain has been defined by the IASP as "Pain caused by a lesion or disease of the somatosensory nervous system, either peripheral or central". This type of pain is always chronic and can be extremely severe and crippling for the individual. Neuropathic pain is described by patients as a persistent, diffuse, burning-like sensation with no specific location in a given organ or tissue. In addition, they suffer from paroxysmal pain, that is, short electric shock-like sensations alternating with remission periods. Neuropathic pain is one of the most challenging types of pain as far as understanding the relationships between symptoms and mechanisms is concerned. It is not surprising, therefore, that effective and safe neuropathic pain treatment remains a largely unmet therapeutic need (Dray, 2008). The presence of symptoms or signs (e.g., touch-evoked pain) alone does not justify the use of the term neuropathic. Nevertheless, some disease entities, such as trigeminal neuralgia, are currently defined by their clinical presentation rather than by objective diagnostic testing.

Current neuropathic pain treatments show general insensitivity to non-steroidal anti-inflammatory drugs (NSAIDs) and relative resistance to opioids. Moreover, some of these drugs involve dose limitations with respect to efficacy and side effects (dizziness, sedation, and weight gain).

Pathophysiology

Neuropathic pain leads to a maladaptive plasticity caused by a lesion or disease affecting the somatosensory nervous system which, in turn, modifies the nociceptive signal processing so that pain is felt in the absence of a stimulus and responses to innocuous and noxious stimuli are enhanced (Costigan *et al.*, 2009). Depending on the scope of the lesion or disease of the nervous system, central and peripheral neuropathic pain types are distinguished.

Tissue injury can result in the activation of peripheral nociceptors producing a change in the response characteristics of the nociceptors. Thus, an insult can sensitize the peripheral nociceptors to any further stimulus promoting a reduction in threshold and, therefore, an increased responsiveness of the peripheral ends of nociceptors. Two main processes have been involved in this increased sensitivity, with changes in existing nociceptor proteins (post-translational processing) and in protein expression regulation being made by the nociceptor (altered gene expression). For instance, the modulation of the expression of transient receptor potential vanilloid type 1 (TRPV1), transient receptor potential (TRP), or voltage-gated sodium/calcium channels (Basbaum *et al.*, 2009). Another peripheral mechanism occurring during neuropathic or persistent pain and contributing to peripheral sensitization is collateral sprouting (actively growing state) of primary afferent neurons, which spread in their vicinity and eventually establish new synapses. Another level of structural plasticity is that of activity-dependent changes in connectivity, such as denervation, reinnervation, sprouting, and hypertrophy. All these alterations have been reported to occur in peripheral tissues — such as the skin, bone or visceral organs— in pathological pain states in humans and experimental animals (Kuner, 2010).

Following a peripheral nerve injury, anatomical and neurochemical changes can occur within CNS that can persist long after the injury has healed. This "CNS plasticity" may play an important role in the evolution of neuropathic pain. At the cellular level, the CNS plastic changes appear to be associated with enhanced neurotransmission via the NMDA receptor (Freire *et al.*, 2009).

Different types of neuropathic pain can be distinguished depending on their aetiology: diabetic neuropathy, post-herpetic neuropathy and trigeminal neuropathy, SC injury pain and phantom limb (post-amputation) pain, among others.

Animal models

The existence of these different pathological conditions leading to the development of neuropathic pain makes difficult the identification of a simple and reliable animal model and explains the increasing numbers of models present in the literature. Many rodent models of neuropathic pain have been used to mimic human diseases, other models have been used to explore pathophysiological mechanisms in the nervous system, and some models have been used to screen for putative analgesics. The neuropathic pain models can either have a traumatic or a non-traumatic nature (Table 1). Traumatic injury paradigms include crush, transection, ligation or any combination of these mechanical deformations to a peripheral nerve (Jaggi *et al.*, 2012). In contrast, non-traumatic injury paradigms cover a more diverse set of injury models, including the application of neurotoxic substances (such as chemotherapeutic agents), metabolic lesioning substances (such as streptozotocin), or cells or proteins associated with various neuropathic pain states (Authier *et al.*, 2009; Jaggi *et al.*, 2012). As shown in Table 1, the main experimental models have a traumatic basis as a primary mechanism.

The rat model of chronic constriction injury of the infraorbital nerve has been reported to be a good model that mimics **trigeminal neuralgia** in humans (Latrémolière *et al.*, 2008; Li *et al.*, 2014). Most neuropathic pain studies (90%) have focused exclusively on measuring hypersensitivity, i.e. mechanically (pinch/pressure) and thermally (heat/cold) evoked nocifensive behaviours (for example, vocalization and withdrawal responses) (Deseure and Hans, 2015).

Streptozotocin-induced **diabetic neuropathy** in the rat has been increasingly used as a model of painful diabetic neuropathy to assess the efficacy of potential analgesic agents. Diabetic animals were chronically ill, with reduced growth rate, polyuria, diarrhea, and had enlarged and distended bladders. Diabetic neuropathy is evidenced by a clear hypersensitivity to a noxious pressure stimulus in diabetic rats as compared to naïve (non-diabetic) rats, thus suggesting the presence of symptoms observed in diabetic humans (Gul *et al.*, 2000; Kamei *et al.*, 2001).

Chemotherapy-induced peripheral neuropathy is considered to be the most common neurological complication of cancer treatment, and is probably the most common toxic neuropathy in our setting (Velasco and Bruna, 2010). The neurotoxic effects of chemotherapy drugs not only do not reduce cancer-evoked pain but even cause additional neuropathic pain, which contributes to a major deterioration of the patient's quality of life (Farquhar-Smith, 2011). The incidence of these neuropathies depends on the antineoplastic agent, and can vary widely (10-100%) depending on the definition of neuropathy. In general, it is estimated that neuropathy affects around 30-40% of patients receiving chemotherapy. The oxaliplatin-induced neuropathic pain model in rats is widely used to mimic chemotherapy-induced neuropathy observed in cancer-treated patients (Ling *et al.*, 2007; Weickhardt *et al.*, 2011). Oxaliplatin is a third-generation platinum-based chemotherapy drug for the treatment of advanced

metastatic colorectal cancer (Screnci *et al.*, 2000; Baker, 2003), and induces painful neuropathy characterized by symptoms including cold hypersensitivity and spontaneous pain, which can lead to drug discontinuation (Ling *et al.*, 2007; Jaggi and Singh, 2012). Although oxaliplatin is less toxic than cisplatin on peripheral organs and functions, it is more toxic on sensory nerves.

Table 1. Rodent models commonly used in neuropathic pain studies.

Model primary mechanism	Human relevance/disease	Experimental pain model
Traumatic nerve injury	Nerve entrapment	Chronic constriction injury (CCI; Bennet)
		Spinal nerve ligation (SNL; Chung)
		Partial sciatic nerve ligation (PSNL; Seltzer)
		Spared nerve injury (SNI)
		Sciatic nerve crush cryoneurolysis
		Phototoxicity
		Distal nerve injury
		Complete nerve transection
	Spinal cord injury	Ischemic or traumatic contusion, compression or transection of spinal cord
	Trigeminal neuralgia	Traumatic infraorbital nerve injury
Non-traumatic nerve injury	Neuritis	Injection of inflammatory agents directly in the nerves
	Drug-induced neuropathic pain	Systemic administration of clinically used therapeutic agent (e.g. chemotherapeutics or antiretrovirals)
	Diabetic neuropathy	Injection of streptozotocin
	Post-herpetic neuralgia	Inoculation of herpes simplex virus type I
	Acute inflammatory demyelinating polyradiculo-neuropathy	Immunization with perypheral myelin P2 peptide

1.3. Inflammatory pain

This type of pain is associated with inflammatory processes and may arise in conditions such as trauma events, infections by activation of the immune system, or chronic inflammatory diseases. Inflammatory pain is adaptive and protective, and assists the healing of the injured tissue by swallowing the injured organ that avoids physical contact and movement. The nociceptive system after inflammation is sensitized and the sensory nervous system undergoes a profound change in its responsiveness, and can also be activated by low-threshold innocuous inputs. Therefore, normally innocuous stimuli produce pain, and responses to noxious stimuli are both exaggerated and prolonged, as in allodynia and hyperalgesia (Juhl *et al.*, 2008; Costigan *et al.*, 2009). In fact, inflammatory pain can become a chronic disease of its own, e.g., rheumatoid arthritis (Woolf, 2010).

As mentioned, inflammatory pain is a central characteristic of highly prevalent clinical diseases such as rheumatoid arthritis and osteoarthritis, a diverse group of conditions characterized by joint swelling, pain, deformity, and disability (Pisetsky and Ward, 2012). Although inflammatory pain should dissapear after the resolution of the initial tissue injury, the pain lasts as long as inflammation is active in chronic disorders such as rheumatoid arthritis (Michaud *et al.*, 2007). The acute inflammatory response is controlled relatively effectively with NSAIDs, paracetamol, opioids, and steroids. However, inflammatory pain associated with a chronic disease such as osteoarthritis, cancer or migraine is deleterious to health and often debilitating for the patient (Rahman and Dickenson, 2013).

Pathophysiology

Tissue injury provokes a local inflammatory reaction characterized by redness, swelling, heat, pain, and decreased nociceptive thresholds (sensitization to pain). Tissue damage leads to the release of a huge number of inflammatory pain mediators by activated nociceptors or non-neural cells that reside within or infiltrate into the injured area, including mast cells, basophils, platelets, macrophages, neutrophils, endothelial cells, keratinocytes, and fibroblasts (Fig. 6). This "inflammatory soup" of signaling molecules includes histamine, ATP, neurotransmitters (serotonin, glutamate), peptides (SP, CGRP and bradykinin), eicosanoids and related peptides (eicosanoids, prostaglandins, thromboxanes, leukotrienes and endocannabinoids), chemokines and cytokines (NGF, tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), and extracellular proteases and protons (H^+) (Basbaum *et al.*, 2009). Some of these chemicals (ATP or H^+) can directly activate or sensitize the high-threshold nociceptors by means of low intensity stimuli that signal the presence of inflamed tissue, produce pain, and result in primary hyperalgesia (Serpell, 2006). Other chemical mediators (e.g., histamine or bradykinin) are produced by activated inflammatory cells, such as neutrophils or mast cells (Julius and Basbaum, 2001). A lot of these mediators bind to membrane receptors and act via regulatory intermediates (GPCRs and second messengers) to induce changes in membrane ion channels or enzymes, thus enhancing the response to peripheral stimuli (Wang *et al.*, 2006). Peptides such as CGRP and SP produce vasodilation, vascular endothelial disruption and extravasation of plasma and blood cells, thus contributing to the recruitment of immune cells from the circulation. In parallel, these molecules interact with complex processes of events that alter the gene expression pattern of several receptors in the DRG (Serpell, 2006).

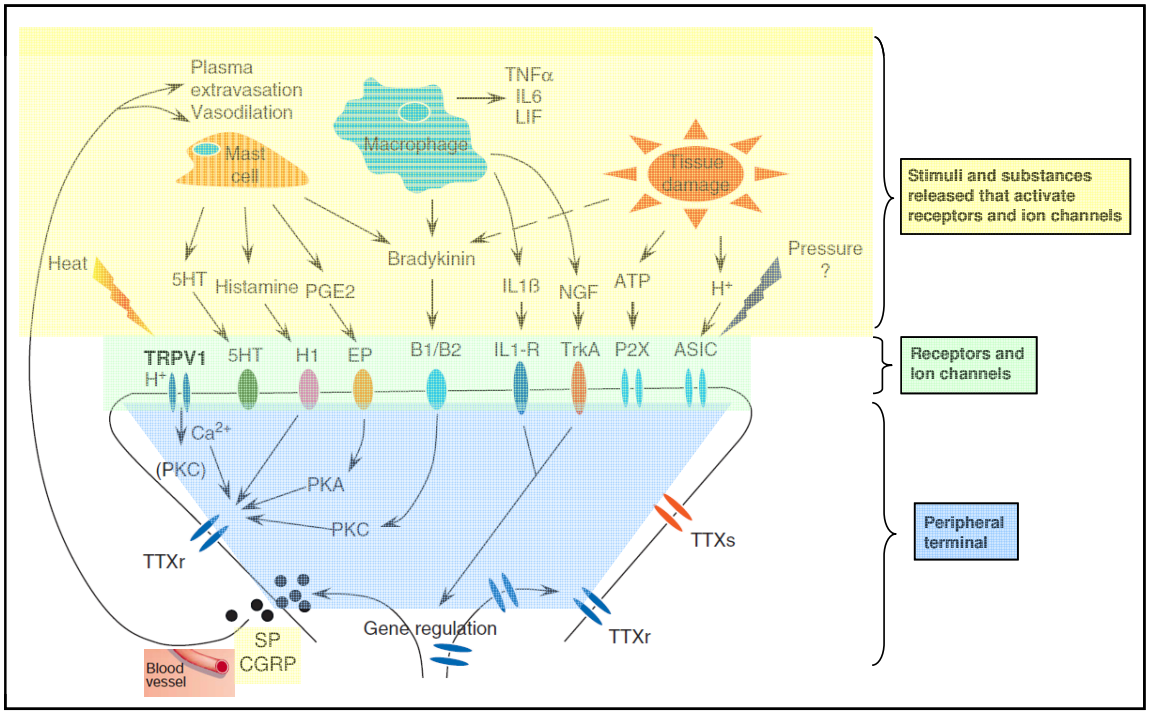


Fig. 6. Peripheral mechanisms of nerve fibre sensitization induced by tissue damage, heat or pressure. Tissue damage leads to the release of inflammatory mediators by activated nociceptors or non-neural cells that reside within or infiltrate into the injured area. Abbreviations: Protein kinases A and C (PKA, PKC), transient receptor potential vanilloid type 1 (TRPV1), 5-hydroxytryptamine (5HT) receptors, Histamine type 1 (H1), Prostaglandin E2 (PGE2), Prostanoid receptor EP subtype (EP), bradykinin (B1/B2) receptors, interleukin-1 beta (IL-1β), interleukin-1 receptor (IL1-R), nerve growth factor (NGF), tyrosine kinase A receptor (TrkA), adenosine triphosphate (ATP), purinergic receptor subtype P2X (P2X), protons (H+), calcium ion (Ca^{2+}), tetrodotoxin-resistant voltage-gated sodium channel (TTXr), tetrodotoxin-sensitive voltage-gated sodium channel (TTXs), substance P (SP), calcitonin gene related peptide (CGRP), acid-sensing channel (ASIC) (Modified from Voscopoulus and Lema, 2010).

Animal models

Several animal models have been investigated in order to mimic the physiopathological processes underlying inflammation, such as rheumatoid arthritis or osteoarthritis. Experimental inflammatory pain models can be classified according to the type of inflammation induction: spontaneous, genetic, chemical, or surgical (Table 2).

Carrageenan and CFA inflammatory pain models are chemically induced following their inoculation and are extensively used in preclinical research. The oedema induced by the injection of **carrageenan** —a polysaccharide obtained from various seaweeds— in the hind paw has been widely used as an animal model in anti-inflammatory drug screening tasks (Winter *et al.*, 1962). Intraplantar injection of carrageenan induced a transient acute inflammatory state characterized by both mechanical and thermal hypersensitivity. Cardinal signs of inflammation, such as oedema, hyperalgesia and erithema, develop immediately after intraplantar injection of carrageenan into a hind paw as a result of the action of proinflammatory agents like bradykinin, histamine, prostaglandins, thromboxanes, reactive oxygen, etc., which can be generated at the site of the insult or by infiltrating cells (Di Rosa *et al.*, 1971; Vazquez *et al.*, 2015). The responses induced after a subcutaneous injection of carrageenan are initiated by mast cells and neutrophils, and are then followed by a phagocytic response that depends on macrophage mobilization. Another feature of carrageenan administration is the wind-up of Aδ and C fibres of flexor motor neurons, which contribute to the mentioned decrease of the mechanonociceptive threshold in the injected paw (Helyes *et al.*, 2009).

Inflammation induced by Complete Freund's Adjuvant (**CFA**) has also been widely used over the years to produce an arthritic condition in an experimental model of chronic pain and inflammation in rats (Pearson and Wood, 1963; Colpaert *et al.*, 1980). CFA is a water-in-oil emulsion containing heat-killed mycobacteria or mycobacterial cell wall components. Mechanical allodynia, thermal hyperalgesia and pain upon joint movement (joint hyperalgesia) are due to the prolonged presence of antigens at the injection site. CFA was first injected intradermally (i.d.) in combination with spleen cells and adjuvants. Later, it was found that injecting CFA alone was sufficient to

induce arthritis. Antigenic substances of bacterial walls cause the activation of the immune system in the injection area, which involves neutrophil and macrophage extravasation, blood vessel dilation, and tissue damage. These inflammation events occur in the affected area.

Table 2. Rodent models commonly used in inflammatory pain studies.

Human relevance/disease	Categories	Experimental pain model
Neurogenic inflammation	Chemical	Hindpaw injection of formalin, capsaicin, mustard oil or bee venom
Osteoarthritis	Spontaneous	STR/ort mouse
	Chemical	Knee joint injection of monosodium iodoacetate (MIA), papain or collagenase
	Surgical	Partial or total menistectomy or transection of collateral or cruciate ligaments
Inflammatory monoarthritis	Chemical	Knee joint or paw injection of kaolin/carrageenan, carrageenan, zymosan or Complete Freund's adjuvant (CFA)
Rheumatoid arthritis	Spontaneous or transgenic animal	MRL/lpr mouse (spontaneous) or HLA-B27 mouse (transgenic)
	Chemical	Paw, knee joint or tail injection of Complete Freund's adjuvant (CFA) or colagen type II arthritis (CIA),
Temporomandibular joint inflammation	Chemical	Facial injection of carrageenan or CFA
Gout	Chemical	Knee joint injection of monosodium urate crystals or uric acid

1.4. Postoperative pain

Surgical injuries trigger a cascade of events designed to fight infection, limit further damage, and initiate repair. Postoperative pain involves nociception, inflammation, and nerve cell remodeling (Voscopoulus and Lema, 2010). Surgery aims either to repair a part of the body by reshaping or replacing it, or to remove dysfunctional elements. To this end, various procedures are required which may transiently but strongly activate the nociceptive network, thus inducing long-term central sensitization. The acute postoperative pain is an important clinical issue (Brennan, 2011); indeed, approximately 60% of patients undergoing surgery have moderate to severe pain despite receiving analgesic treatment (Wu and Raja, 2011). Moreover, acute postoperative pain can become chronic in a high percentage of patients (15-60%) (Lavand'homme, 2011). Whereas acute postoperative pain has been classified as nociceptive pain, the underlying mechanisms of chronic postsurgical pain with persistent pain lasting at least 3-6 months are complex and poorly understood (Vandenkerkhof *et al.*, 2013). However, the syndromes are, at least in part, neuropathic as a result of neuroplastic changes after injury (Macrae, 2008; Voscopoulos and Lema, 2010). This transition to chronicity is most obvious after surgical nerve lesions, where the extent and timing of the lesion are defined (Kehlet *et al.* 2006).

Several pharmacological approaches have been used to attenuate chronic postoperative pain. For example, pre-emptive analgesia —the analgesic treatment prior to surgery causing peripheral inflammation— has shown to prevent central and peripheral mechanisms leading to neuronal sensitization and subsequent chronic pain. Moreover, considerable controversy currently exists over whether pre-emptive treatment targeting central sensitization is better than postoperative treatment in treating either acute postoperative pain or its transition to chronic pain (Woolf and Chong, 1993;

Dirks *et al.*, 2002; Woolf, 2011). Hence, further studies of the mechanisms underlying postoperative pain are needed.

Pathophysiology

The factors contributing to postoperative pain can broadly be divided into patient factors and surgical factors. Patient factors include psychosocial status, pre-existing pain conditions, genetic predisposition to exaggerated pain response, and gender. Surgical factors include type of anaesthesia administered (general *vs.* regional technique) and surgical approach, including the ability to identify and avoid nerve injury when possible. Additional surgical factors include postoperative period, treatment of pain type and duration thereof, full assessment of pain and its consequences, and neurophysiological examination (Kehlet *et al.*, 2006; Kehlet and Rathmell, 2010; Voscopoulus and Lema, 2010). Surgery triggers a cascade of pro-inflammatory cytokines, chemokines and neurotrophins that induce both peripheral and central sensitization to limit further injury to the affected area (Voscopoulus and Lema, 2010). After surgery, patients experience ongoing pain or are sensitive to incidental, normally non-painful stimulation. This period of time varies; with uncomplicated wound healing, this type of pain should progressively abate and disappear (Voscopoulus and Lema, 2010). The transition from acute to chronic pain appears to occur in discrete pathophysiological and histopathological steps. As mentioned, surgery causes the release of inflammatory and other mediators. For many surgery-related reasons, such as prolonged inflammatory states with the insertion of mesh materials or chronic nerve stretching in bunionectomy, this process of sensitization and facilitation can cause phenotypic and pathophysiological changes in nociceptors. Once these structural

changes occur, chronic (neuropathic) postoperative pain becomes established (Voscopoulus and Lema, 2010).

Animal models

Today, few experimental models in rodents cause postoperative pain following surgical mechanical trauma: laparotomy (De Winter, 2003), ovariohisterectomy (Lascelles *et al.*, 1995; González *et al.*, 2000), postthoractomy pain (Buvanendran *et al.*, 2008), or incisional pain (Pogatzki *et al.*, 2003; Brennan, 2011). The most commonly used postoperative pain model is induced by a surgical incision in the plantar surface of mice (Brennan *et al.*, 1996) (Fig. 7). This animal model has shown to be a useful tool for studying the neurobiological mechanisms of pain after surgery.

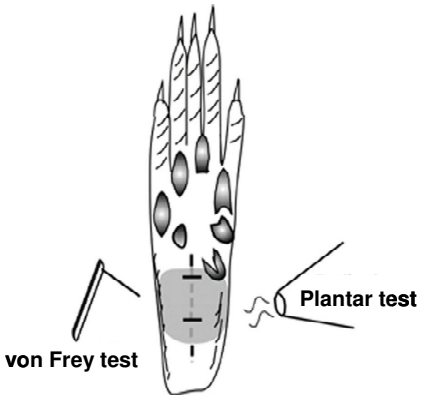


Fig. 7. Stimulation area of the hind paw in the postoperative model of rodents. The shaded area represents the area where heat and mechanical stimuli were applied. The dark horizontal lines in the shaded area represents the sutures, and the vertical line represents the surgical incision (Modified from Banik *et al.*, 2006).

1.5. Current treatment strategies

Today, pharmacological therapies for acute and chronic pain primarily rely on NSAIDs, opioids and a diverse group of drugs used as direct or adjuvant treatments (e.g., antidepressants, anticonvulsants, local anesthetics, and α_2-adrenoreceptor agonists). These types of drugs are limited in terms of clinical utility as a consequence of significant adverse effects that often limit tolerability (i.e., they possess a relatively low therapeutic index) and prevent "dosing to effect" from achieving adequate or tolerable management of some chronic pain states, such as neuropathic pain (Porreca *et al.*, 2006).

Opioid receptor agonists (e.g., morphine and tramadol) have strong efficacies for various nociceptive type of pains. The opioid agonists are the gold standard drugs for the treatment of moderate to severe chronic pain, interacting with the mu (μ), delta (δ) and kappa (κ) receptors (Martin and Eisenach, 2001).

Antipyretics like acetaminophen or **NSAID**s like indomethacin and diclofenac are the first line drugs in the treatment of arthritic pain, and these cyclooxygenase (COX) inhibitors play an adjunctive role in chronic pain. COX inhibitors are the first step in the World Health Organization scheme for the treatment of chronic pain, and the majority of patients with chronic pain receive these agents as a base throughout their treatment (Martin and Eisenach, 2001). NSAIDs inhibit COX enzymes, which in turn convert arachidonic acid to prostaglandin H_2, converted by other enzymes to several prostaglandins, mediators of pain, inflammation, and fever. Hence, the main mechanism of action of NSAID is considered to be a reduction in the production of prostaglandins and thromboxanes.

Antidepressants (e.g., amitriptyline), **antiepileptics** (e.g., carbamazepine and gabapentin) and **local anesthetics** administered intravenously (i.v.) have been

frequently used in the treatment of chronic pain in addition to their original use (Nagakura *et al.*, 2003). For instance, antidepressants are usually given at doses much lower than those required to treat depression (McQuay and Moore, 1997)(Table 3).

Table 3. Analgesic drugs developed from 1960 to 2009 and currently in use. (From Kissin, 2010).

Drugs developed for the treatment of pain					
Opioids					
Pentazocine	1967[a]	Nalbuphine	1979[a]	Alfentanil	1986[a]
Fentanyl	1968	Buprenorphine	1981[a]	Tramadol	1995[a]
Butorphanol	1978[a]	Sufentanil	1984[a]	Remifentanil	1996[a]
NSAIDs					
Indomethacin	1965[a]	Piroxicam	1982[a]	Nabumetone	1991[a]
Mefenamic acid	1967[a]	Diflunisal	1982[a]	Oxaprozin	1992[a]
Ibuprofen	1974[a]	Ketoprofen	1986[a]	Ketorolac	1992[a]
Naproxen	1976[a]	Diclofenac	1988[a]	Bromfenac	1997[a]
Tolmetin	1976[a]	Fenoprofen	1988[a]	Celecoxib	1998[a]
Sulindac	1978[a]	Flurbiprofen	1988[a]	Meloxicam	2000[a]
Meclofenamate	1980[a]	Diclofenac	1988[a]	Nepafenac	2005[a]
Other drugs					
Sumatriptan	1992[a]	Rizatriptan	1998[a]	Eletriptan	2002[a]
Pentosan	1996[a]	Almotriptan	2001[a]	Ziconotide	2004[a]
Zolmitriptan	1997[a]	Frovatriptan	2001[a]	Pregabalin	2004[a]
Naratriptan	1998[a]				

Drugs developed for indications other than pain but effective in the treatment of pain			
Anticonvulsants			
Carbamazepine	1966–1995[b] (3)[c]	Valproate	1979–2000 (FDA)[d]
Phenytoin	1964–1995 (3)	Gabapentin	1996–2002 (FDA)
Clonazepam	1975–1995 (3)	Topiramate	2001–2003 (FDA)
Antidepressants			
Amitriptyline	1964–1992 (4)	Desipramine	1984–1996 (5)[c]
Doxepin	1973–1992 (4)	Venlafaxine	1996–2005 (6)
Imipramine	1962–1996 (5)	Duloxetine	2004 (FDA)
Other drugs			
Propranolol	1968–1991 (7)	Mexiletine	1986–2005 (10)
Capsaicin (topical)	1987–1994 (8)	Ketamine	1974–2006 (21)
Cyclobenzaprine	1989–2004 (9)	Dronabinol	1975–2007 (22)
Lidocaine (systemic, topical)	1982–2005 (10,11)	Dexamethasone	1967–2008 (12)

[a] Year of Food and Drug Administration (FDA) approval as a new molecular entity.
[b] Period of publications leading to the confirmation of effectiveness in pain by meta-analysis or to FDA approval for the treatment of pain as an additional indication.
[c] Reference for meta-analysis (Cochrane review).
[d] FDA approval for the treatment of pain as an additional indication.

Pain management is most effective when the underlying disease conditions are cured. Unfortunately, the number of disease-modifying drugs is relatively low. The unmet need for better efficacy and safety has resulted in substantial interest for treating these conditions. In addition, side effects have been associated with their antinociceptive efficacy, thus hindering the effective treatment of chronic pain. For instance, the use of μ opioid agonists has been related to the development of tolerance and dependence as well as adverse effects such as sedation, dysphoria, and constipation (Martin and Eisenach, 2001). Hence, treatment of persistent/chronic pain continues to

be a troublesome clinical issue and new drugs and targets to design optimal pharmacological treatments are being pursued (Fig. 8).

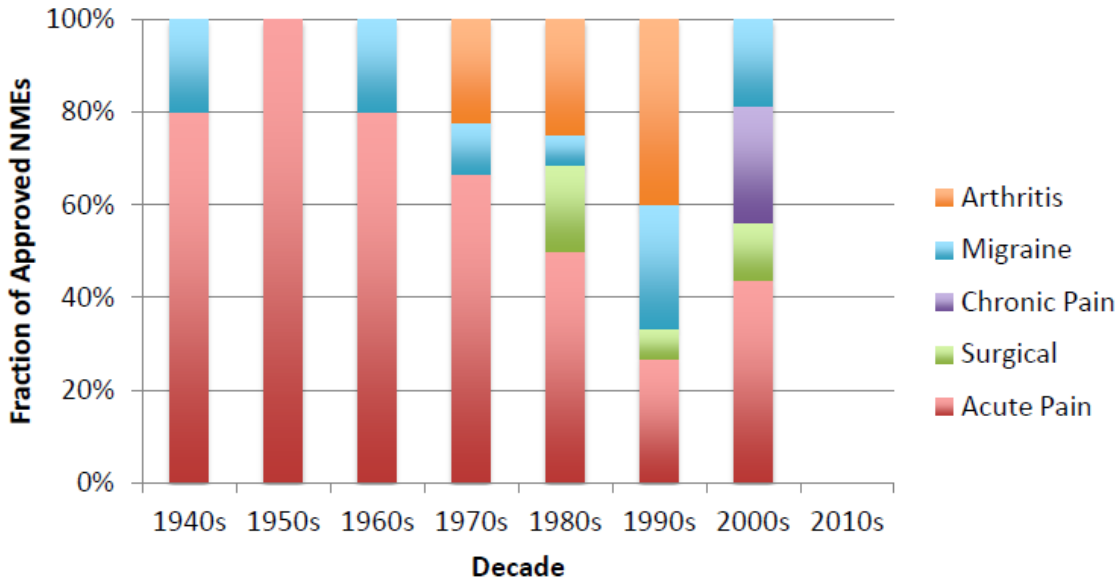


Fig. 8. New molecular entities (NMEs) targeting pain. Note that three NMEs, such as morphine, aspirin and gallamine triethiodide had been approved before 1930. Instead of acute pain, chronic pain, as well as different types of complex pain are currently filling pharmacological research worldwide (From Kinch, 2015).

2. THE SIGMA-1 RECEPTOR

2.1. General Overview

The sigma-1 receptor (σ_1R) is a ligand-regulated molecular chaperone that interacts with other proteins to modulate their activity. It has been implicated in a variety of physiological and pathological conditions such as pain, psychosis, neuroprotection and cardioprotection, cognitive processes, drug dependence, cancer and locomotor behaviours, as well as in the regulation of certain endocrine and immune functions (Walker *et al.*, 1990; Bowen, 2000; Kitaichi *et al.*, 2000; Almansa and Vela 2014).

The σ receptor (σR) was discovered almost 40 years ago. It was firstly misclassified as a subclass of the opioid receptor family to account for the psychotomimetic effects exhibited by racemic N-allyl-normetazocine ((±)-SKF-10,047) and other benzomorphans (Martin *et al.*, 1976). The differences in the enantioselectivity of ((±)-SKF-10,047) for the opioid receptors further clarified the nature of σR (Su *et al.*, 1982). σR binds with high affinity to (+)-benzomorphans such as *N*-allyl-normetazocine ((+)-SKF-10,047) or (+)-pentazocine, and exhibits lower affinity for the corresponding (-)-enantiomer (Walker *et al.*, 1990). Based on the selectivity profile of some ligands and the molecular mass, two subtypes of σR were described: σ_1R and sigma-2 receptors (σ_2R) (Hellewell and Bowel, 1990; Quirion *et al.*, 1992). They have been shown to co-localize but they are present in different ratios (Leitner *et al.*, 1994; McCann *et al.*, 1994; Bouchard and Quirion 1997; Guitart *et al.*, 2004). Recent data have found that σ_2R and the PGRMC1 (progesterone receptor membrane component 1) form a complex that shares the same binding site. In turn, this complex seems to be coupled with EGFR (epidermal growth factor receptor), mTOR (mammalian target of rapamycin), caspases, and ion channels (Xu *et al.*, 2011; Ahmed *et al.*, 2012). σ_2R has been related to cancer,

arguing that the complex σ_2R-PGRMC1 is induced in multiple types of cancer, where it regulates tumor growth and is implicated in progesterone signaling (Huang *et al.*, 2014). σ_2R has also been related to many cellular events, such as proliferation, apoptosis, dendritogenesis, synaptogenesis and neuronal plasticity, activation of cytochrome P450 and steroid signaling, among others (Cahill, 2007; Losel *et al.*, 2008). Despite these data, σ_2R remains largely elusive, since both the cloning of σ_2R and the identification of its endogenous ligand have not been successfully resolved and the lack of structural information has severely hindered the understanding of its physiological roles, its signaling pathways, and the development of more selective σ_2R ligands (Huang *et al.*, 2014).

σ_1R, in contrast, has been cloned from several tissues; it was first cloned from guinea pig liver, and later from mouse kidney, human choriocarcinoma cell line and brain, and rat and mouse brain (Hanner *et al.*, 1996; Kekuda *et al.*, 1996; Seth *et al.*, 1997; Pan *et al.*, 1998; Prasad *et al.*, 1998; Mei and Pasternak, 2001). σ_1R cloning enabled a series of expression studies and allowed studying this receptor through sequence-specific antibodies (Alonso *et al.*, 2000; Palacios *et al.*, 2003; Aydar *et al.*, 2006) and antisense oligodeoxynucleotides (Kitaichi *et al.*, 2000; Maurice *et al.*, 2001a, b; Matsumoto *et al.,* 2002; Nguyen *et al.*, 2005; Espallergues *et al.*, 2007), as well as generating a knockout (KO) mouse by homologous recombination (Langa *et al.*, 2003). The viability of σ_1R KO mice suggests that σ_1R is not determinant at the embryogenic stages, thus allowing mutant mice to grow and behave, apparently, as wild-type (WT) mice.

The σ_1R gene is positioned on chromosome 9 (9p13.3, band p13, a region known to be associated with different psychiatric disorders). It is approximately 7 kbp long and contains four exons interrupted by three introns (Prasad *et al.*, 1998). The σ_1R gene

encodes a 24-kDa protein of 223 amino acids with high sequence homology among species (above 90%). σ_1R shares no sequence homology with any known mammalian protein although exhibits 35% identity and 67% similarity to the yeast gene that codes a sterol C8-C7 isomerase, which is essential to ergosterol synthesis and cell proliferation. It has been shown that some σ_1R ligands are able to inhibit sterol biosynthesis due to the high-affinity drug binding site. However, σ_1R does not possess sterol isomerase activity because σ_1R knockdown does not alter cholesterol metabolisms in mammalian cells (Hanner *et al.*, 1996; Moebius *et al.*, 1996; Seth *et al.*, 2001).

2.1.1. Structure of σ_1R

The structure of σ_1R has been ascertained by several studies using hydrophobicity analysis, photoaffinity probes, and site-directed mutagenesis (Fig. 9). Firstly, it was proposed that σ_1R was a single transmembrane protein. Years later, however, immunohistochemistry and protease protection assays allowed Aydar and coworkers (2002) to suggest that the structure of σ_1R consists of two transmembrane (TMI and TMII) segments with the NH_2 (N-) and COOH (C-) termini on the cytoplasmic side of the membrane (Aydar *et al.*, 2002; Hayashi and Su, 2007; Ishikawa and Hashimoto, 2010) (Fig. 9). Apart from the transmembrane domains, there are two other hydrophobic regions that form the 'steroid binding domain like' (SBDL-I and SBDL-II), so named because of the sequence homology to yeast sterol isomerase, where σ_1R ligands could bind to the receptor. The extracellular loop consists of approximately 50 amino acids, whereas the intracellular C-terminal has 125 amino acids (Aydar *et al.*, 2002). The N-terminal has been described to contain a helix followed by a large dynamic region and a structured helical C-terminal region that surround a membrane-

associated domain containing four helices, three of which map the cholesterol and drug recognition sites (Almansa and Vela, 2014).

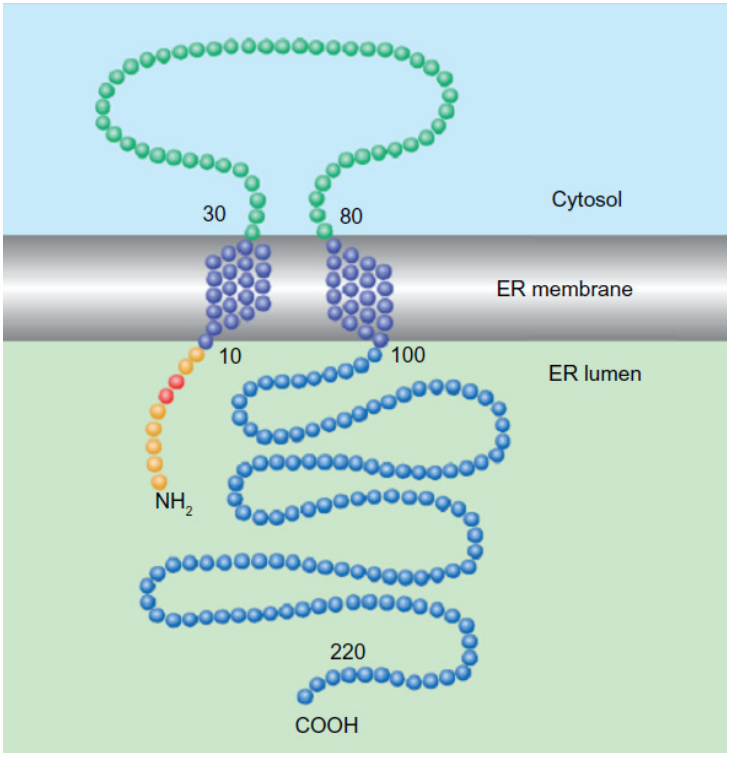


Fig. 9. The putative molecular structure model of σ_1R proposed by Aydar. σ_1R contains two hydrophobic transmembrane regions. The N- and C- terminals are shown on the intracellular side of the membrane. Circles represent amino acids (From Ishikawa and Hashimoto, 2010).

Pabba (2013) has recently described that the N- and C- terminals of σ_1R could be facing the extracellular side at the plasma membrane. This new configuration of the σ_1R placement is consistent with some recent studies on the interaction of σ_1R with other receptors and channels (Balasuriya *et al.*, 2013; Pabba, 2013; Almansa and Vela, 2014).

2.1.2. Anatomical and subcellular distribution of σ_1R

σ_1R cloning also helped document their wide distribution in the **anatomical compartments**. σ_1R is ubiquitously expressed in mammalian tissues and is distributed in peripheral organs, such as digestive tract, liver, kidney and skin, and different areas of the CNS involved in memory, emotion, and sensory and motor function (Hellewell *et al.*, 1994; Kekuda *et al.*, 1996; Zamanillo *et al.*, 2000; Bangaru *et al.*, 2013; Sánchez-Fernández *et al.*, 2014).

The localization of σ_1R in the **subcellular compartment** is dynamic in nature, σ_1R is found in several membranes —such as microsomal, endoplasmic reticulum (ER), nuclear and plasmatic membranes— as highly clustered globular structures enriched in cholesterol and in lipid-containing microdomains. At the ER, it is located at the interface with mitochondria at the mitochondria-associated ER membrane (MAM) (Vance, 1990; Hayashi and Su 2007; Cobos *et al.*, 2008; Su *et al.*, 2010) (Fig 10). It has been shown to translocate from the MAM to other areas of the cell where they can interact with several membrane targets to modulate the function or production of various intracellular secondary messengers (Hayashi and Su, 2001; Aydar *et al.*, 2002; Hayashi and Su, 2003; Su *et al.*, 2010; Kourrich *et al.*, 2012).

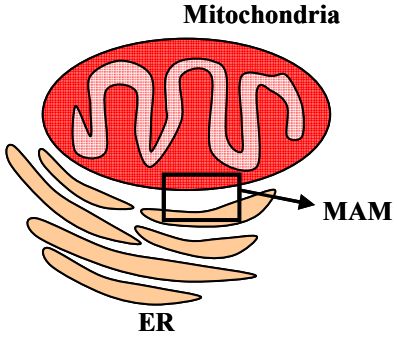


Fig. 10. Subcellular localization of σ_1R at the MAM (mitochondria-associated endoplasmic reticulum membrane). ER: Endoplasmic reticulum.

2.1.3. Mechanism of action of σ_1R

Chaperones are proteins that assist the correct non-covalent folding and operation of other proteins, either when they are being synthesized or at their functional localities. σ_1R is an integral membrane protein classified as the first unique ligand-regulated molecular chaperone whose activity focuses on modulating the inter-organelle signaling incurred on activation of the protein (receptor, enzyme or ion channel) that σ_1R is interacting with, and also through the binding of σ_1R ligands (agonists or antagonists) (Su and Hayashi, 2003; Hayashi and Su, 2007; Tsai *et al.*, 2009a; Hayashi and Su, 2010; Su *et al.*, 2010; Hayashi *et al.*, 2011; Zamanillo *et al.*, 2013).

σ_1R is associated with functionally and structurally diverse proteins, including inositol-1,4,5-triphosphate receptor (IP_3R) and binding immunoglobulin protein (BiP) (Aydar *et al.*, 2002; Hayashi and Su, 2007). Under resting conditions, σ_1R modulates Ca^{2+} signaling through functional IP_3R, particularly subtype IP_3R3, at the MAM, to ensure proper Ca^{2+} signaling from the ER into mitochondria (Mendes *et al.*, 2005; Tsai *et al.*, 2009a). Under pathological conditions where cells experience stress or when σ_1R binds to agonists, the ER loses its global Ca^{2+} homeostasis and there is a transcriptional up-regulation of chaperone proteins to counteract the ER stress response (Schroder and Kaufman, 2005; Hayashi and Su, 2007). Thus, in the presence of high concentrations of cytosolic IP_3, the Ca^{2+} concentration at the ER falls dramatically, σ_1R dissociates from BiP and begins to chaperone conformationally unstable proteins to enhance Ca^{2+} signaling and increase the production of ATP in the cell (Su *et al.*, 2010; Almansa and Vela, 2014). Then, σ_1R binds to unstable IP_3R to prevent their rapid ubiquitination and degradation by proteosomes and to ensure a proper Ca^{2+} influx into the mitochondria, a key organelle that plays a central role in energy production (Alzayady and Wojcikiewicz, 2005; Bhanumathy *et al.*, 2006; Hayashi and Su, 2007;

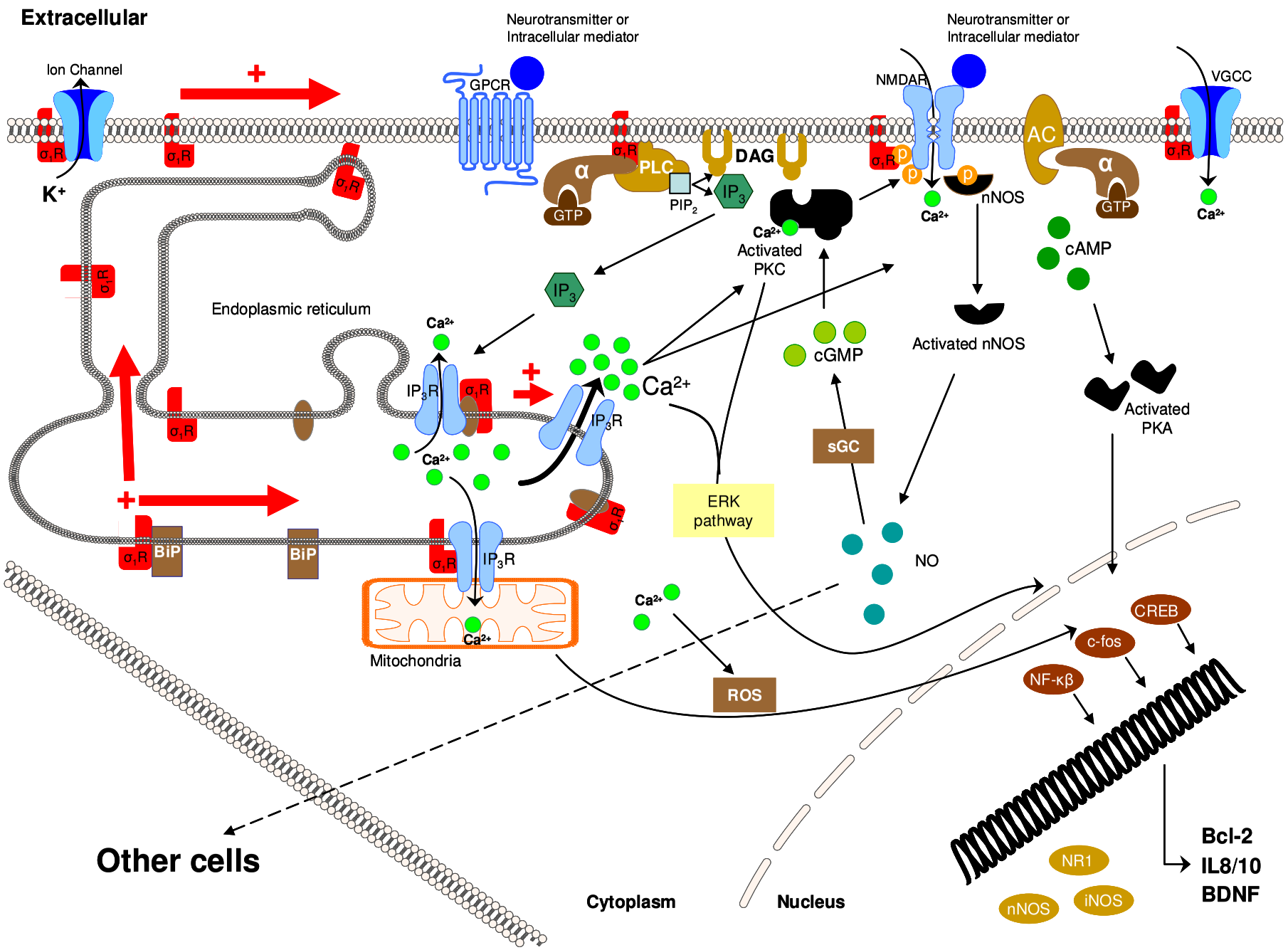
Tsai *et al.*, 2009a). σ_1R translocates from MAM to other subcellular locations (cytoplasm, plasma membranes, and nuclear envelope) and counteracts the arising apoptosis (Fig. 11).

Once σ_1R is found at the **plasma membrane**, it can interact with other receptors to form heteromers (e.g., with dopamine receptors or simultaneously with μ-opioid and NMDA receptors functioning as a unique machinery) and even with itself to generate homomers (Navarro *et al.*, 2010, 2013; Chu *et al.*, 2013; Rodríguez-Muñoz *et al.*, 2015). When σ_1R becomes activated, stimulates phospholipase C (PLC) to hydrolyze phosphorylated phosphatidylinositol 4,5-biphosphate (PIP_2) to produce diacylglycerol (DAG) and IP_3 (Morin-Surun *et al.*, 1999). IP_3 will bind to IP_3R at the ER to promote the Ca^{2+} efflux to the cytoplasm. In the same direction, the activation of σ_1R creates an amplification of the intracellular signal transduction that increases intracellular Ca^{2+} concentrations via NMDA receptors and voltage-gated Ca^{2+}channels (VGCCs) (Su and Hayashi, 2003; Martina *et al.*, 2007; De la Puente *et al.*, 2009). In parallel, the activation of σ_1R in the membrane has been reported to enhance the phosphorylation of the NMDA receptor NR1 subunit (at both the protein kinase C (PKC)-dependent Ser^{896} and the protein kinase A (PKA)-dependent Ser^{897}) (Kim *et al.*, 2008; Roh *et al.*, 2008a, b). Moreover, the activation of σ_1R reduces the recruitment of nNOS in the membrane and thereby its association with NMDA receptor subunit NR2 (Yang *et al.*, 2010; Zamanillo *et al.*, 2013). σ_1R inhibition have recently been reported to release μ opioid receptors from the negative influence of NMDA receptors (Rodríguez-Muñoz *et al.*, 2015).

At the **cytoplasm**, σ_1R has not been found in its free form. However, its activation either by σ_1R agonist binding to the membrane or by a stressful trigger, produces an increase of intracellular Ca^{2+} that in turn reduces nNOS phosphorylation.

Thus, the activity of this enzyme increases notably and forms NO that can be diffused to other cells. Then, the increment of NO promotes the activation of the NR1 subunit and ERK through PKC-dependent phosphorylation (Roh *et al.*, 2011; Zamanillo *et al.*, 2013) (Fig. 11).

At the **nucleus**, σ_1R activation transcriptionally modulates the gene expression of several proteins related to inflammation, nociception, neuronal survival, synaptogenesis and neurogenesis, such as nNOS, inducible nitric oxide synthase (iNOS), NR1, B-cell lymphoma 2 (Bcl-2), brain derived neurotrophic factor (BDNF), and interleukins 8 (IL-8) and 10 (IL-10). Some of these proteins are expressed through the regulation of their corresponding transcription factors, such as c-Fos (Yang *et al.*, 2007; Meunier and Hayashi, 2010; Hayashi *et al.*, 2011)(Fig. 11).


Extracellular
Ion Channel
K+
Neurotransmitter or
Intracellular mediator
GPCR
Neurotransmitter or
Intracellular mediator
NMDAR
VGCC
σ1R
α
GTP
PLC
PIP2
IP3
DAG
Ca2+
Activated
PKC
nNOS
AC
cAMP
Endoplasmic reticulum
IP3R
Ca2+
cGMP
Activated nNOS
Activated
PKA
sGC
ERK
pathway
NO
BiP
Mitochondria
ROS
CREB
c-fos
NF-κβ
NR1
nNOS
iNOS
Bcl-2
IL8/10
BDNF
Other cells
Cytoplasm
Nucleus

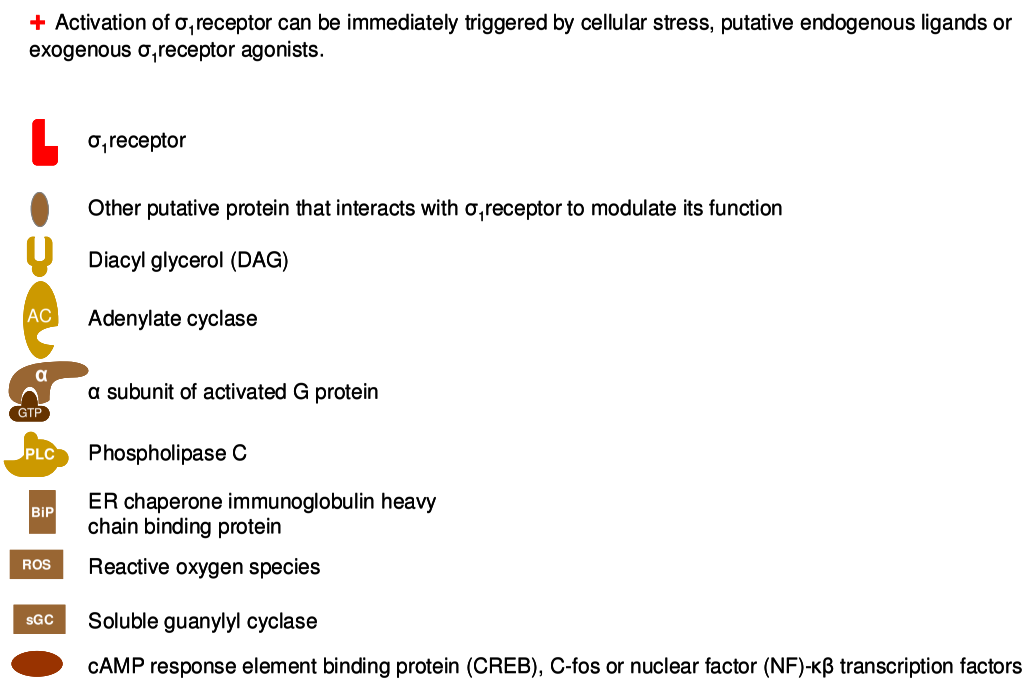


Fig. 11. Involvement of σ_1R in signal transduction pathways. σ_1R modulates downstream signaling pathways activated upon the stimulation of other targets. Under stressful conditions or when σ_1R binds to agonists, there is a transcriptional up-regulation of chaperones and an increase of the cytosolic IP_3, thus σ_1R dissociates from the chaperone BiP and binds to IP_3R at the ER to enhance Ca^{2+} signaling from the ER into mitochondria to increase ATP. In addition, its activation also promotes redistribution of σ_1R from MAM to other subcellular locations to bind to ion channels, receptors or protein kinases. At the plasma membrane, σ_1R becoming activated stimulates phospholipase C (PLC) to produce diacylglycerol (DAG) and IP_3. The active σ_1R modulates the activity of neurotransmitter receptors and ion channels, including voltage-gated Ca^{2+} channels (VGCCs), and μ-opioid and the ionotropic NMDA receptors, leading to an increased cytosolic Ca^{2+}. In parallel, σ_1R regulates protein kinases (PKC and PKA) and the subsequent phosphorylation of the NMDA receptor. At the cytoplasm, increased cytosolic Ca^{2+} reduces nNOS phosphorylation (i.e., resulting in an increase in nNOS activity). The NO generated from nNOS stimulates cGMP production via sGC, which in turn leads to an increased PKC activity (activating NR1 subunit and ERK). At the nucleus, σ_1R activation transcriptionally modulates the gene expression of several proteins and the corresponding transcription factors, such as nNOS, interleukins, and c-Fos (Modified from Zamanillo *et al.*, 2013).

2.2. Modulation of σ_1R by ligands

σ_1R is a unique target in the sense that it displays chaperone activity to modulate inter-organelle signaling while simultaneously regulated by ligands in a clear agonist/antagonist fashion.

2.2.1. σ_1R as a drug target

The discovery of selective σ_1R ligands has been intricate due to the promiscuity of σ_1R —many different molecules with diverse structures have affinity for this receptor. The overlap of the σ_1R pharmacophore with that of a number of other receptor systems and proteins responsible for off-target effects makes it intricate to find selective ligands and explains why different drug classes bind to σ_1R (Zamanillo *et al.*, 2012).

Historically, the distinction between σ_1R agonists and σ_1R antagonists has lied in the pharmacological response in specific systems, which has added to the difficulty in determining the mechanism of action of σ_1R (Schrock *et al.*, 2013). Today, the discovery of new selective ligands as well as the assessment of their functional behaviour is still complex for this target class, among others reasons due to some issues (Cobos *et al.*, 2005; Su *et al.*, 2010; Zamanillo *et al.*, 2012; Almansa and Vela, 2014):

- σ_1R is mainly localized intracellularly and exerts a modulatory action on several receptors, ion channels and enzymes, rather than an easily estimated direct effect. Ligand accessibility to σ_1R is expected to be more difficult, and the signaling outcome less predictable, than for other membrane targets as GPCRs. Thus, there is a discrepancy between the binding affinity and efficacy of σ_1R ligands. Therefore, the

hydrophobicity of ligands is a major determinant to predict the potency of σ_1R ligands *in vivo*.

- σ_1R activity can vary depending on the conformational state of the target proteins, given that σ_1R is a chaperone protein and only exerts its activity when proteins are misfolded. Therefore, the nature of the disease provides the selectivity of σ_1R ligands.
- Some σ_1R ligands do not show the classical linear dose-response curve in biochemical, behavioural and electrophysiological studies (sometimes a biphasic bell-shaped curve is obtained).
- Many of the widely accepted σ_1R antagonists (BD1063, BD1047 or NE-100) bind at nanomolar affinities to the σ_2R. The effect of these nonselective ligands could thus result in apparent discrepancies due to the activation of one subtype of receptor at low doses and the activation of other subtypes at higher doses.
- The assay conditions and readouts used notably affect the outcome (e.g., agonists can behave as antagonists depending on the readout used).

All these obstacles have been recently overcome by using several approaches to discern if a ligand is an agonist or an antagonist:

- *In vivo* tests. The antinociceptive activity in animal models such as formalin or capsaicin test has been often used for establishing the antagonist nature of ligands (Entrena *et al.*, 2009a; Cendán *et al.*, 2005b).

- Phenytoin assay. It is a low-potency allosteric modulator of σ_1R that discriminates affinities of σ_1R ligands depending on their agonistic or antagonistic nature. Phenytoin discriminates between σ_1R receptor agonists and antagonists as it is able to shift known agonists to significant higher affinities (Ki ratios without phenytoin *vs.* with phenytoin >1) while antagonists show no shift or a very little shift to lower affinity values (Ki ratios without phenytoin *vs.* with phenytoin <1) (Cobos *et al.*, 2005, 2006).
- Fluorescence resonance energy transfer (FRET) studies. They detect conformational changes due to the binding of the ligand to σ_1R, depending on the functional nature of the molecule (Gómez-Soler *et al.*, 2013).

At present, many drugs of very different structural classes that bind with high to moderate/weak affinity and with low selectivity for σ_1R have shown a wide range of therapeutic applications. Compounds launched onto the market include, among others, antipsychotics (e.g., haloperidol, chlorpromazine), antidepressants (e.g., fluvoxamine, sertraline, clorgyline), antitussives (carbetapentane, dextromethorphan, dimemorfan), drugs for the treatment of neurodegenerative disorders such as Parkinson's disease (amantadine) or Alzheimer's disease (memantine, donepezil), and drugs of abuse (cocaine, methamphetamine) (Zamanillo *et al.*, 2012; Tsai *et al.*, 2014; Vela *et al.*, 2015). Some σ_1R ligands have reached Phase II and III clinical trials for the treatment of neuropsychiatric disorders (Cutamesine and Anavex 2-73), but others have been discontinued (Table 4).

While different endogenous σ_1R ligands have been proposed, such as neurosteroids, NPY, sphingolipids and dimethyltryptamines, their exact physiological roles in the context of σ_1R modulation are still unclear.

Table 4. Summary of σ_1R ligands that are used as pharmacological tools or that are currently in clinical trials, have been discontinued, or are already marketed.

Status	Compounds	Function on σ_1R	Other pharmacological actions
Pharmacological tools	(+)-SKF-10,047	Agonist	NMDA receptor ligand (PCP site)
	DTG	?	σ_2R agonist
	PRE-084	Agonist	
	BD-1063	Antagonist	σ_2R
	BD-1047	Antagonist	σ_2R, α adrenoceptor
Marketed drugs	(+)-Pentazocine	Agonist	Highly selective σ_1R agonist
	Carbetapentane	Agonist	Muscarinic antagonist, σ_2R agonist
	Donepezil	Agonist	Cholinesterase inhibitor
	Dextromethorphan	Agonist	NMDA receptor allosteric antagonist
	Imipramine	Agonist	Monoamine reuptake Inhibitor (Tricyclic Antidepressant)
	Fluvoxamine	Agonist	Selective 5-HT reuptake inhibitor
	Sertraline	Agonist	Selective 5-HT reuptake inhibitor
	Haloperidol	Antagonist	Dopamine D_2 and D_3 antagonist, σ_2R agonist
	Opipramol	Agonist	Dopamine D_2, 5-HT_2 and histamine H1
	Verapamil	?	Ca^{2+} channels blocker
	Clorgyline	Agonist?	Irreversible monoamine oxigenase A inhibitor
	Citalopram	?	Selective 5-HT reuptake inhibitor
	(±)Fluoxetine	Agonist	Selective 5-HT reuptake inhibitor
	Tamoxifen	?	Estrogen receptor
Drugs in clinical trials discontinued	Siramesine	Antagonist	σ_2R agonist, α_1 adrenoceptor ligand
	NE-100	Antagonist	σ_2R
	Panamesine	Antagonist	DAT inhibitor
	Rimcazole	Antagonist	DAT inhibitor
	BMY-14802	Antagonist	5-HT_{1A} agonist
	Eliprodil	Antagonist	NMDA antagonist, α_1 adrenoceptor ligand
Drugs in clinical trials	**E-52862**	**Antagonist**	
	Cutamesine	Agonist	Acetylcholine release enhancer
	Anavex 2-73	Agonist	Muscarinic agonist, NMDA, Na^+ channels

2.2.2. The selective σ_1R antagonist E-52862

Esteve developed a medical chemistry program aimed at the discovery of novel and selective σ_1R ligands taking into account the pharmacophoric features of σ_1R (Díaz *et al.*, 2009). As a result of this chemical campaign, the structure-activity relationship studies of a series of 1-arylpyrazoles led to the discovery of the new chemical entity 4-[2-[[5-methyl-1-(2-naphthalenyl)-1H-pyrazol-3-yl]oxy]ethyl] morpholine (E-52862, S1RA) (Fig. 12) (Díaz *et al.*, 2012).

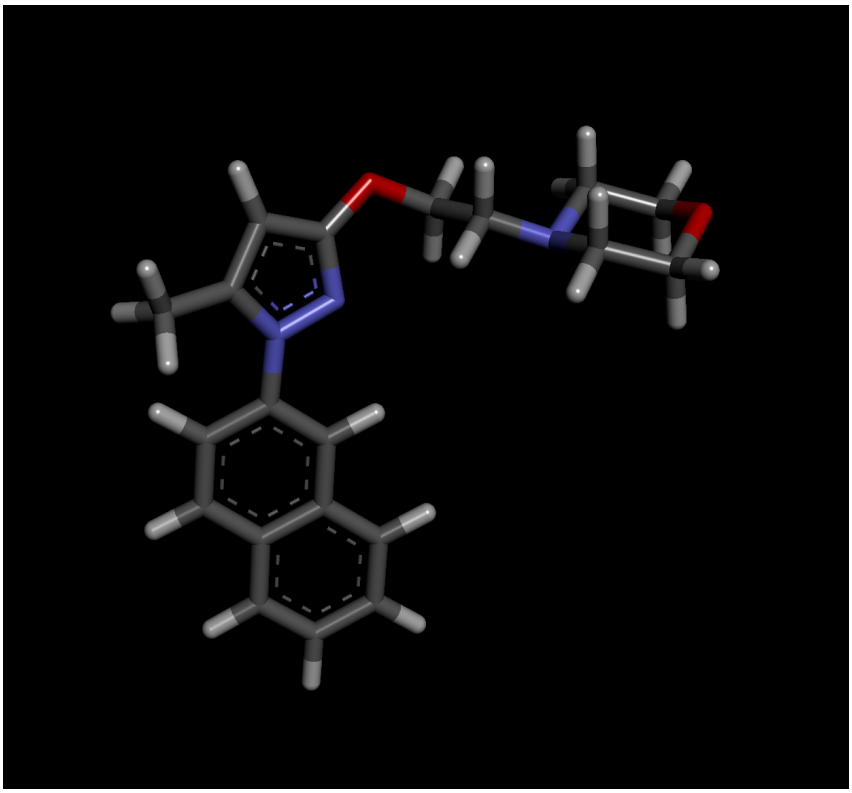


Fig. 12. Molecular structure of the selective σ_1R antagonist E-52862 in 3D layout. Nitrogen atoms are represented in blue and oxygen atoms are represented in red.

E-52862 has shown high σ_1R affinity in humans (K_i = 17 nM) and guinea pigs (K_i = 23.5 nM), whereas σ_2R affinity is not significant (K_i > 1000 nM) in guinea pigs and rats). Thus, E-52862 exhibited an excellent σ_1/σ_2 selectivity ratio (> 550).

So far, many σ_1R ligands have shown high selectivity against σ_2R, 5-HT_{1A}, 5-HT_6, 5-HT_7, alpha$_{1A}$, alpha$_2$, and NMDA receptors (Oberdorf *et al.*, 2008). However, E-52862 has not been significantly active on another 170 receptors, transporters, ion channels and enzymes (Díaz *et al.*, 2012; Romero *et al.*, 2012; Zamanillo *et al.*, 2013). In the phenytoin assay —a low-potency allosteric modulator of σ_1R that discriminates affinities of σ_1R ligands depending on their agonistic or antagonistic nature (Cobos *et al.*, 2005, 2006)— E-52862 produced a small shift to lower-affinity values when incubated in the presence of phenytoin, thus suggesting antagonistic properties of σ_1R.

E-52862 interactions with other drugs based on the inhibition of cytochrome P450 were evaluated in recombinant human cytochrome P450 (CYP) isoforms, and no alerts regarding CYP subtype inhibition or induction were found. E-52862 is metabolized *in vitro* by multiple human CYPs (CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2D6, CYP2E1 and CYP3A4) and by flavin-containing monooxygenase (FMO; FMO1 and FMO3). It is thus unlikely that E-52862 clearance *in vivo* would be significantly susceptible to interactions with drugs that induce or inhibit specific enzyme isoforms, or to inter-individual variations due to genetic polymorphisms in specific isoforms.

Regarding safety pharmacology, no significant effects of E-52862 were observed at doses associated with preclinical analgesic activity. No teratogenic, genotoxic, phototoxic or skin irritation effects were found in the toxicological studies conducted to preclude further development of E-52862.

E-52862 (cLogP=3.9) has shown to penetrate the blood-brain barrier and to bind to σ_1R in the CNS, thus showing a significant correlation between the extent of CNS σ_1R occupancy and the antinociceptive effect. At a mechanistic level and consistent with the results obtained in σ_1R KO mice, E-52862 at 30 μM produced an attenuation of

the wind-up responses in SC sensitized by repetitive nociceptive stimulation (De la Puente *et al.*, 2009; Romero *et al.*, 2012). Altogether, this makes E-52862 suitable to selectively antagonize σ_1R and to study the role of these receptors in nociception.

2.3. σ_1R and pain

σ_1R is expressed in areas related to pain control such as DRG, the superficial layers of the dorsal horn of the SC, where the first synapse with the second order neurons occurs, and also in areas related to the descending pain pathways, such as the PAG, the locus coeruleus, and the RVM (Zamanillo *et al.*, 2013; Almansa and Vela *et al.*, 2014; Sánchez-Fernández *et al.*, 2014).

2.3.1. Modulation of opioid-induced antinociception

The fact that σ_1R was related to antinociception was first suggested by studies showing a relationship between σR systems and opioid analgesia, where σ_1R had been proposed as an antiopioid system in which σ_1R exerted a tonic inhibitory control on the opioid receptor-mediated signaling pathways (Chien and Pasternak, 1994, 1995a). In the acute thermal nociception (tail-flick test), σ_1R antagonists or σ_1R antisense oligodeoxynucleotides have no effects by themselves when given alone, but significantly increase the analgesic response to mu (μ) and kappa (κ) opioid agonists (King *et al.*, 1997; Mei and Pasternak, 2002). Otherwise, the σ_1R agonist (+)-pentazocine blocks this potentiation effect (Chien and Pasternak, 1995b).

It is important to note that this increase in opioid potency appears to be limited to analgesia and not side effects. E-52862 has been used to characterize the pharmacological effect of antagonizing σ_1R on opioid-induced antinociception and adverse effects in mice. When co-administered, E-52862 enhanced the antinociceptive

effect of opioids in the tail-flick test, an effect that was attributed to the σ_1R based on studies in σ_1R KO mice (Table 5). In contrast, morphine induced antinociceptive tolerance and rewarding were attenuated whereas physical dependence, inhibition of gastrointestinal transit, or mydriasis were not modified (Vidal-Torres *et al.*, 2013).

Regarding the site of action, the modulation of opioid-induced antinociception has been observed peripherally, and centrally (spinal and supraspinal) suggesting that σ_1R-mediated pain modulation occurs at different sites (Sánchez-Fernández *et al.*, 2013, 2014; Vidal-Torres *et al.*, 2013). The σ_1R receptor agonist (+)-pentazocine supraspinally administered (microinjected in periaqueductal gray, locus coeruleus, or rostal ventromedial medulla) diminished systemic opioid analgesia in mice, and the σ_1R receptor antagonist haloperidol (and also antisense oligonucleotides) microinjected into the rostal ventromedial medulla markedly enhanced the analgesic actions of coadministered morphine In contrast, σ_1R agonists spinally administered did not alter opioid analgesia (Mei and Pasternak, 2002). Finally, BD1063 or E-52862, among other σ_1R antagonists, locally co-administered in the paw markedly potentiated the opioid antinociception and its effects were reversed by the selective σ_1R agonist PRE-084. (Sánchez-Fernández *et al.*, 2013, 2014), In addition, σ_1R KO mice exhibited an enhanced mechanical antinociception in response to morphine (local or systemic) (Sánchez-Fernández *et al.*, 2013). All these data support the use of σ_1R antagonists as systemic or local opioid adjuvants.

Table 5. Summary of the involvement of σ_1R on opioid antinociception: effects of E-52862 and σ_1R KO mice. Abbreviations: i.p.: intraperitoneal; i.pl.: intraplantar; s.c.: subcutaneous.

Nociceptive model	Anatomical scope (route)	E-52862 effect on opioid antinociception	Effect of σ_1R knockout mice on opioid antinociception	References
Tail-flick test	Systemic (i.p.)	Enhancement	No effect	Vidal-Torres *et al.* , 2013
Paw pressure test	Systemic (s.c.) and local (i.pl.)	Enhancement	Enhancement	Sánchez-Fernández *et al.* , 2013

σ_1R is physically associated with μ opioid receptors, and σ_1R antagonists (but not agonists) potentiate opioid transduction without influencing opioid receptor binding (Kim *et al.*, 2010). A crosstalk between σ_1R, NMDA and μ opioid receptors and regulated by the μ opioid receptor-associated histidine triad nucleotide binding protein 1 (HINT1) has been recently discovered. σ_1R antagonists enhance opioid analgesia in naïve mice by releasing μ opioid receptors from the negative influence of NMDA receptor, and they also reset antinociception in morphine-tolerant animals (Rodríguez-Muñoz *et al.*, 2015).

Opioids are the gold standard painkillers used for the treatment of moderate to severe pain. Although they are used worldwide, they present with side effects such as constipation, dizziness and nausea, among others, which usually lead to treatment discontinuation (Kurz and Sessler, 2003). Other side effects, such as tolerance and dependence, appear in long-term treatments with opioids, and treatment effectiveness is dramatically reduced. σ_1R antagonists have been shown to potentiate opioid analgesia but not opioid-related adverse effects (or even reducing), which suggest an application for σ_1R antagonists as opioid adjuvant therapy, even at a peripheral level.

2.3.2. Modulation of pain in sensitizing and chronic pain conditions

Unlike opioid drugs, σ_1R ligands fail to modify pain by themselves in classical models of thermal and mechanical acute nociception, as seen in the tail-flick, the hot plate and the paw pressure test in rodents (Marrazzo *et al.*, 2006; De la Puente *et al.*, 2009; Sánchez-Fernández *et al.*, 2013). However, σ_1R ligands play a key role in modulating pain behaviour, particularly in sensitizing and chronic pain conditions. These aspects have been studied using σ_1R receptor knockout mice and selective σ_1R receptor antagonists (Table 6).

σ_1R KO mice are a useful genetic tool to study the involvement of σ_1R in several pain types, given that KO mice perceive and respond normally to stimuli of different nature (mechanical, chemical and thermal). Thus, the absence of σ_1R in KO mice has been shown not to interfere with the perception of several stimuli applied to the hind paw or with the motor response required for paw withdrawal (Cendán *et al.*, 2005a; De la Puente *et al.*, 2009; Nieto *et al.*, 2012; Romero *et al.*, 2012; González-Cano *et al.*, 2013). In σ_1R KO mice, both phases of formalin-induced paw licking were clearly reduced (Cendán *et al.*, 2005b) and capsaicin injected intraplantarly did not induce mechanical allodynia (Entrena *et al.*, 2009a). Regarding the neuropathic pain models, cold and mechanical hypersensitivity were strongly attenuated in σ_1R KO mice treated with paclitaxel (Nieto *et al.*, 2012) or exposed to partial sciatic nerve ligation (PSNL) (De la Puente *et al.*, 2009). In a visceral pain model induced by intracolonic injection of capsaicin, σ_1R KO mice have shown a reduction in the number of pain behaviours as compared to WT mice (González-Cano *et al.*, 2013). From a mechanistic point of view, σ_1R KO do not completely develop the windup response after stimulation of C fibres using isolated spinal cord which is indicative of the role of σ_1R amplification in mechanisms underlying central sensitization and synaptic plasticity.

Table 6. Effects of genetic (σ_1R KO mice) and pharmacological blockade of σ_1R in different experimental pain models. Abbreviations: DRG: dorsal root ganglia; PSNL: partial sciatic nerve ligation; TNC: trigeminal nucleus caudalis. For other abbreviations see abbreviations list.

Pain type	Experimental pain model	Genetic σ_1R antagonism effect (using σ_1R KO mice)	Pharmacological σ_1R antagonism effect	References
Acute nociception	Tail-flick test	Similar to WT	No effect by itself, but reversed the facilitatory effects of agonists (increasing the tail-flick latency)	Chien and Pasternak 1995a, b; Ronsisvalle *et al.* , 2001; Cendán *et al.* , 2005a; Marrazzo *et al.* , 2006; Roh *et al.* , 2008b; De la Puente *et al.* , 2009; Tseng *et al.* , 2011
	Hot plate test	Similar to WT		De la Puente *et al.* , 2009
	von Frey test	Similar to WT	No effect by itself, but reversed the facilitatory effects of agonists (reducing the frequency of paw withdrawal)	Roh *et al.* , 2008b; De la Puente *et al.* , 2009
	Paw pressure test	Similar to WT	No effect	Sánchez-Fernández *et al.* , 2013
	Paw-pinch test		No effect by itself, but reversed the fos up-regulation induced by agonists	Roh *et al.* , 2008b
Acute and sensitization-related pain induced by chemicals	Formalin test	Attenuation	Antinociception (phase I and II) Decreased fos and pNR1 expression in the spinal cord	Cendán *et al.* , 2005a, b; Kim *et al.* , 2006; Díaz *et al.* , 2009; Romero *et al.* , 2012
	NMDA-induced pain		Reversion of the effects of agonists (both expression of pNR1 and spontaneous pain behaviour)	Kim *et al.* , 2008
	Orofacial-Formalin model		Reduced nociceptive responses Decreased fos and pp38 expression in the TNC	Roh and Yoon, 2014

	Capsaicin-induced mechanical allodynia	No development of mechanical allodynia	Antiallodynic (mechanical) effect	Entrena *et al.* , 2009a, b; Wiese *et al.* , 2009; Romero *et al.* , 2012
	Capsaicin-induced headache (migraine model)		Reduced face grooming Decreased fos and pNR1 expression in the TNC	Kwon *et al.* , 2009
Acute inflammatory (arthritic) pain	Carrageenan-induced acute inflammatory pain	Similar to WT (heat hyperalgesia) Attenuation (mechanical hyperalgesia)	Antihypersensitivity (mechanical and heat) effects	Parenti *et al.* , 2014a, b; Tejada *et al.* , 2014
Acute visceral pain	Intracolonic capsaicin-induced visceral pain	Reduced number of behaviours Similar to WT (referred hyperalgesia)	Reduced pain-related behaviours (number of behaviours and referred antihyperalgesia)	González-Cano *et al.* , 2013
Neuropathic pain	Chronic constriction injury-induced neuropathic pain (Bennett model)		Antiallodynic (mechanical) but not antihyperalgesic (heat) effects Decreased GFAP, pNR1 and pp38 expression in the spinal cord	Roh *et al.* , 2008a; Choi *et al.* , 2013; Moon *et al.* , 2013, 2014
	Chronic constriction of the dorsal root ganglion-induced neuropathic pain		Antiallodynic (mechanical and cold) effect Decreased pERK expression in the spinal cord	Son and Kwon, 2010
	PSNL-induced neuropathic pain	No development of mechanical and cold allodynia Similar to WT (heat hyperalgesia) No increase of pERK	Antihypersensitivity effects (both mechanical /cold allodynia and thermal hyperalgesia) Inhibition of anhedonic state	De la Puente *et al.* , 2009; Romero *et al.* , 2012; Bura *et al.* , 2013; Kotagale *et al.* , 2013
	Paclitaxel-induced neuropathic pain	No development of mechanical and cold allodynia No increase of pERK	Antiallodynic (mechanical and cold) effect	Nieto *et al.* , 2012, 2014; Riganas *et al.* , 2012

The **pharmacological antagonism on σ_1R** produced similar results. The antagonist haloperidol, its metabolites I and II and E-52862 inhibited formalin-induced pain (Cendán *et al.*, 2005a, Romero *et al.*, 2012) and somatic capsaicin-induced sensitization in mice (Entrena *et al.*, 2009b; Romero *et al.*, 2012). Several σ_1R antagonists, including E-52862, also inhibited visceral capsaicin-induced sensitization in mice (González-Cano *et al.*, 2013). In neuropathic pain, some pain-related behaviours have also been reversed using σ_1R antagonists in animal models, such as the chronic compression of the DRG (Son and Kwon, 2010), the migraine model induced by intracisternal infusion of capsaicin (Kwon *et al.*, 2009), PSNL (Romero *et al.*, 2012) and paclitaxel-induced neuropathic pain (Nieto *et al.*, 2012), among others. All these results are summarized in Table 6.

From a mechanistic point of view, several studies have shown the involvement of σ_1R in neuropathic pain models, with significant changes being observed in the expression of this receptor in some phases of the development of experimental neuropathic models. All these results are summarized in Table 7.

Table 7. Changes in σ_1R expression in the nervous system induced in several models of neuropathic pain. Abbreviations: DRG: dorsal root ganglia; STZ: streptozotocin.

Pain type	Experimental pain model	σ_1R molecular changes induced by pain	References
Neuropathic pain	Streptozotocin-induced diabetic neuropathy	Decreased σ_1R expression in brain homogenates 10 weeks post-stz. No changes 5 weeks post-stz	Mardon *et al.*, 1999
	Chronic constriction injury-induced neuropathic pain	Increased σ_1R expression in the spinal cord during the induction (1-3 days), but not the maintenance phase of neuropathic pain	Roh *et al.*, 2008a; Moon *et al.*, 2014
	Chronic constriction of the dorsal root ganglion-induced neuropathic pain	Increased σ_1R expression in the spinal cord 3-14 days post-surgery	Son and Kwon, 2010
	Spinal nerve ligation-induced neuropathic pain	Decreased σ_1R expression in axotomized DRG neurons 21 days post-injury	Bangaru *et al.*, 2013

2.3.3. E-52862 as analgesic drug

E-52862 has so far been pharmacologically characterized *in vivo* as an effective analgesic in several pain types (results summarized in Table 8).

- **Acute nociception:** E-52862 potentiates morphine analgesia (tail-flick and paw pressure tests) without affecting some undesirable side effects of morphine such as gastrointestinal transit (section 2.3.2, Vidal-Torres *et al.*, 2013).
- **Chemical-induced sensitization:** E-52862 was able to systemically reverse capsaicin-induced allodynia and formalin-induced paw licking in mice (Romero *et al.*, 2012; Vidal-Torres *et al.*, 2014).
- **Visceral pain:** E-52862 has shown to exert a σ_1R selective effect on visceral pain induced by intracolonic injection of capsaicin, given that its effect on referred hyperalgesia is completely abolished in σ_1R KO mice (González-Cano *et al.*, 2013).
- **Neuropathic pain:** E-52862 has been widely studied in neuropathic pain models like PSNL and paclitaxel-induced neuropathic pain. These antihypersensitivity effects have been observed using several treatment paradigms (acute and chronic) and studying different endpoints (Nieto *et al.*, 2012; Romero *et al.*, 2012). Interestingly, the effects of E-52862 have been shown on both pain sensitivity and motivation, thus addressing both the neurophysiological and the affective aspects of neuropathic pain. E-52862 has been associated with an improvement of the emotional negative state without reinforcing effects, as observed by a sucrose preference for E-52862 as well as an operant response to obtain this drug in animals subjected to nerve ligation (Bura *et al.*, 2013).

In parallel to our findings, Tejada and coworkers recently described for the first time that σ_1R is involved in **inflammatory pain** where E-52862 showed analgesic activity both systemically and locally. These findings are summarized in the article 3 of this Doctoral Thesis.

From a mechanistic point of view, E-52862 did not modify the Aβ fibre-mediated non nociceptive signaling and the response to single stimuli in C fibres in isolated neonatal mice SC, which is consistent with the behavioural observation that σ_1R antagonists do not alter the normal response to sensory and nociceptive inputs in non-sensitizing conditions (Romero *et al.*, 2012; Mazo *et al.*, 2014, Vela *et al.*, 2015). However, E-52862 reduced spike number and wind-up index elicited by repetitive stimulation of nociceptive C fibres in isolated neonatal mice spinal cords, which is also in accordance with the observation that the wind-up response was attenuated in the σ_1R KO mice. This is also consistent with the behavioural observation that under sensitizing conditions, the effect of blocking σ_1R is antiallodynic and antihyperalgesic (de la Puente *et al.*, 2009; Romero *et al.*, 2012; Mazo *et al.*, 2014). Finally, a factor contributing to its antinociceptive effects has been suggested to be the E-52862-induced potentiation of descending noradrenaline pain inhibitory control, evidenced in the formalin-induced pain model. Local administration of E-52862 (intracerebroventricular, intrathecal and intraplantar) evidenced not only a central, but also a peripheral site of action for E-52862's antinociceptive effects (Vidal-Torres *et al.*, 2014).

At present, E-52862 has successfully completed single- and multiple-dose phase I studies showing good safety, tolerability, and pharmacokinetic and pharmacodynamic profiles in healthy subjects after single (5-800 mg) and multiple (50-400 mg x eight days) oral doses (Abadias *et al.*, 2013). E-52862 is currently undergoing phase II

clinical trials for the treatment of pain and is the first-in-class compound for this indication (Laurini 2013).

Table 8. Effects of pharmacological blockade by E-52862 in different experimental pain models. Abbreviations: b.i.d: twice a day; i.c.v.: intracerebroventricular; i.p.: intraperitoneal; i.pl.:intraplantar; i.t.: intrathecal; i.v.: intravenous; p.o.: oral; PSNL: Partial sciatic nerve ligation; s.c.: subcutaneous (Adapted from Zamanillo *et al.*, 2013).

Experimental pain model	Specie	Dose/Route	E-52862 effect	References
Tail-flick test	Mouse	40 mg/kg, i.p.	No effect	Vela *et al.* , 2010; Vidal-Torres *et al.* , 2013
Paw pressure test		32 mg/kg, s.c.; 100 µg/paw, i.pl.	No effect	Sánchez-Fernández *et al.* , 2013
Formalin test		20-80 mg/kg, i.p.	Antinociception (phase I and II)	Romero *et al.* , 2012
	Rat	40-80 mg/kg, i.p.; 160-320 µg/area, i.t., i.c.v.; 320 µg/paw, i.pl.	Antinociception (phase I and II)	Vidal-Torres *et al.* , 2014
Capsaicin-induced mechanical allodynia	Mouse	16-64 mg/kg, i.p.; 32-128 mg/kg, p.o.	Antiallodynic (mechanical) effect	Romero *et al.* , 2012
Carrageenan-induced acute inflammatory pain		2-32 mg/kg, s.c.; 75-150 µg/paw, i.pl.	Antihyperalgesic (mechanical and thermal) effects, not due to an antioedematous effect. Selective thermal antihyperalgesic effect of E-52862 in σ_1R (no effect in σ_1R KO mice)	Tejada *et al.* , 2014
Intracolonic capsaicin-induced visceral pain		32-128 mg/kg, s.c.	Reduction of the number of behavioural responses and referred antihyperalgesic (mechanical) effect. Selective effect of S1RA in σ_1R (no effect in σ_1R KO mice)	González-Cano *et al.* , 2013
PSNL-induced neuropathic pain		16-64 mg/kg, i.p.	Antiallodynic (mechanical) and antihyperalgesic (thermal) effects	Romero *et al.* , 2012
		25 mg/kg, i.p., b.i.d. x 21 days (chronic treatment)	Inhibition of the development of mechanical and thermal hypersensitivity	Romero *et al.* , 2012
		6 mg/kg/infusion, i.v. x 10 days (operant self-administration)	Antiallodynic (mechanical and thermal) and antihyperalgesic (thermal) effects	Bura *et al.* , 2013
		25 mg/kg, i.p.	Inhibition of anhedonic state (inhibition of the decreased consumption of 2% sucrose solution)	Bura *et al.* , 2013
Paclitaxel-induced neuropathic pain		32-128 mg/kg, s.c.	Antiallodynic (mechanical and cold) effect	Nieto *et al.* , 2012
		32-64 mg/kg, s.c. (cotreatment with paclitaxel)	Antiallodynic (mechanical and cold) effect in a preventive paradigm	Nieto *et al.* , 2012

HYPOTHESIS

Since σR was discovered over 35 years ago, a large number of therapeutic applications related to central nervous system disorders have been proposed for σR ligands. Lately, selective σ_1R antagonists have shown promising results in the field of pain. The first preclinical evidences of the σ_1R in the field of analgesia were related to acute nociception, where pharmacological antagonism effectively potentiated the antinociceptive effects of opioid agonists (Chien and Pasternak, 1995a, b). Later on, it has also been related to pain sensitization processes induced by chemicals such as formalin and capsaicin (Cendán *et al.*, 2005a, b; Entrena *et al.*, 2009a, b; Kwon *et al.*, 2009) or induced by peripheral nerve injury (De la Puente *et al.*, 2009, Romero *et al.*, 2012). Since then, the potential interest of σ_1R as a therapeutic target for neuropathic pain has moved the selective σ_1R antagonist E-52862 forward into clinical development.

However, there are still some gaps of information in the existing preclinical pain studies. Firstly, there is no information about other rat neuropathic pain models different from those triggered by a traumatic extra-cephalic nerve injury. Secondly, the efficacy of repeated administration of σ_1R antagonists has been poorly explored. Thirdly, no information is available on the pain relief effect of σ_1R antagonists or on the spinal expression of any molecular pain markers in σ_1R KO in other types of pain such as inflammatory or postoperative pain.

Finally, very little information is available regarding the σ_1R antagonistic efficacy in comparison to other marketed analgesic drugs. These studies are important because σ_1R is considered to be a unique ligand-regulated molecular chaperone, very different from many other target proteins for pain where the concept of antagonist/inhibitor is more clearly defined. In addition, some of the data found in the literature regarding the antinociceptive effects of σ_1R may be challenged because of the use of non-selective tools. In general, most pharmacology studies have been performed

with antagonists that showed a low selectivity for σ_1R, as they also bind to σ_2R at the nanomolar range, among other receptors (Zamanillo *et al.*, 2013, Almansa and Vela, 2014). Thus, further studies using highly selective σ_1R antagonists such as E-52862 are required to validate previous observations as well as to investigate other unexplored pain models of different aetiology.

Thus, studies focusing on the clarification of these knowledge gaps and using adequate pharmacological tools will be important to validate σ_1R antagonism as a new strategy for pain management. Based on previous findings reported in the literature and in the context of ESTEVE's Sigma-1 receptor project seeking new approaches to pain management, **we hypothesise that σ_1R triggers general mechanisms leading to a sensitization process in pathological pain conditions that could be counteracted when it is blocked. Therefore:**

1) **The use of selective σ_1R antagonists could attenuate, not only neuropathic pain, but also inflammatory and postoperative pain.**
2) **The genetic ablation of σ_1R may induce changes in both behavioural and molecular endpoints after peripheral injuries from inflammatory and/or postsurgery origin that will determine the involvement of σ_1R in the development of inflammatory and postoperative pain.**

OBJECTIVES

The **global objective** of this Doctoral Thesis was to explore the therapeutic interest of selective σ_1R blockade for the treatment of pain of different aetiology**: neuropathic, inflammatory and postoperative.** The following specific objectives were established to meet this global objective:

1. To use three different **neuropathic pain models** in rats representative of clinical conditions with unmet needs regarding pharmacologic treatment: the chronic constriction injury of the infraorbital nerve as a translational model of trigeminal neuropathic pain; the streptozotocin-induced diabetes as a diabetic neuropathic pain model; and the oxaliplatin-induced neuropathy as an antitumoral-induced neuropathic pain model.

2. To evaluate the acute analgesic effects of the selective σ_1R antagonism E-52862 as compared to other marketed drugs measuring several endpoints (mechanical allodynia, mechanical hyperalgesia and thermal/cold allodynia) in these neuropathic pain models.

3. To compare the analgesic efficacy of σ_1R antagonism in acute and repeated treatment schedules in these models.

4. To set up the carrageenan-induced or complete Freund's adjuvant (CFA)-induced hypersensitivity in mice as acute and chronic **inflammatory pain models**, respectively.

5. To study the involvement of σ_1R in the development of pain-related behaviours as well as in the spinal expression of several molecular pain markers in these models of inflammatory pain . σ_1R KO mice were used to meet this objective.

6. To evaluate the acute analgesic efficacy of σ_1R antagonism as compared to other marketed drugs (morphine, ibuprofen and celecoxib) measuring several endpoints (mechanical allodynia and thermal/heat hyperalgesia) in these inflammatory pain models. The σ_1R antagonist E-52862 was used to meet this objective.

7. To set up the plantar incision-induced hypersensitivity in mice as a model of **postoperative pain**.

8. To study the involvement of σ_1R in the development of pain-related behaviours as well as in the spinal expression of several molecular pain markers in this model of postoperative pain. σ_1R KO mice were used to meet this objective.

9. To evaluate the acute analgesic efficacy of σ_1R antagonism in comparison to other marketed drugs (morphine, ibuprofen and celecoxib) measuring several endpoints (mechanical allodynia and thermal/heat hyperalgesia) in this model. The σ_1R antagonist E-52862 was used to meet this objective.

METHODS

Materials and methods used in the present Doctoral Thesis are widely described in its respective published article or manuscript. Here, we present a summary to give a quick picture of the different experimental approaches (Table 1 and 2).

Table 1. Summary of methods for the behavioural studies

Experimental model	Animals	Pain-related behaviours	Method
Chronic constriction injury of the infraorbital nerve	Sprague-Dawley rats	Mechanical allodynia	von Frey test
Oxaliplatin-induced neuropathy		Cold allodynia	Acetone test
Streptozotocin-induced neuropathy	Wistar rats	Mechanical hyperalgesia	Randall-Selitto test
Carrageenan-induced acute inflammatory pain	CD-1 (WT and σ_1R KO) mice	Mechanical allodynia and thermal hyperalgesia	von Frey test and Plantar test
CFA-induced chronic inflammatory pain			
Plantar incision-induced postoperative pain			

Table 2. Summary of methods for the molecular studies

Experimental model	Animals	Pain-related molecular markers	Method
Carrageenan-induced acute inflammatory pain	CD-1 (WT and σ_1R KO) mice	pERK, c-Fos, GFAP, nNOS, SP and NPY	Western Blot and Immunohistochemistry
CFA-induced chronic inflammatory pain			
Plantar incision-induced postoperative pain			

1. Subjects

- **Adult male Sprague-Dawley** (chronic constriction of the infraorbital nerve and oxaliplatin experiments) and **Wistar rats** (streptozotocin experiments) were used for neuropathic pain studies.
- **Adult male CD-1 WT mice** were used throughout the inflammatory and postoperative pain studies.
- **Adult male CD-1 σ_1R knockout mice** were used throughout the inflammatory and postoperative pain studies. The homozygous KO mice (Langa *et al.*, 2003) were backcrossed for 10 generations onto the CD-1 background to reduce to less than 1% the genetic material remaining from the original background.

2. Drugs

- **Selective σ_1R antagonist:** E-52862 or S1RA (4-[2-[[5-methyl-1-(2-naphthalenyl)-1H-pyrazol-3-yl]oxy]ethyl] morpholine).
- **Opioid:** morphine hydrochloride (μ-opioid receptor agonist).
- **NSAIDs:** ibuprofen (non-selective COX-1 and COX-2 inhibitor) and celecoxib (selective COX-2 inhibitor).
- **Gabapentinoids:** gabapentin and pregabalin (ligands of the $\alpha_2\delta$ subunit of calcium channels).

3. Animal models of pain

- **Chronic constriction injury of the infraorbital nerve (IoN)-induced trigeminal neuropathic pain:** Rats were anesthetized and the IoN was exposed using a surgical procedure similar to that described previously (Gregg, 1973; Jacquin and Zeigler, 1983). The edge of the orbit was dissected free and two

chronic catgut ligatures (5-0, Ethicon; Johnson and Johnson, Brussels, Belgium) were loosely tied around the IoN (2 mm apart). The neuropathic pain was evidenced by mechanical allodynia (more details in Article 1).

- **Streptozotocin (STZ)-induced diabetic neuropathic pain:** Diabetes was induced in rats through chemical pancreatectomy by a single intraperitoneal (i.p.) injection of STZ (75 mg/kg body weight) dissolved in saline, whereas control rats received saline alone (Aubel *et al.*, 2004). The neuropathic pain was evidenced by mechanical hyperalgesia (more details in Article 1).

- **Chemotherapy oxaliplatin (OX)-induced neuropathic pain:** Peripheral neuropathy was induced by repeated i.p. injections of OX (3 mg/kg, i.p.) 3 times a week during 2 weeks (7 injections; cumulative dose = 21 mg/kg, i.p.) (Polomano *et al.*, 2001). The neuropathic pain was evidenced by cold allodynia (more details in Article 1).

- **Carrageenan-induced acute inflammatory pain:** Peripheral inflammation was induced by intraplantar (i.pl.) injection of 50 µl of 2.5% (w/v) carrageenan (λ-carrageenan, 1% in 0.2 M of KCl solution; Sigma-Aldrich) into the mid-plantar surface of the right hind (ipsilateral) paw of nonanaesthetized mice (adapting from the method firstly described by Winter *et al.*, 1962). Acute inflammatory pain was evidenced by mechanical allodynia and thermal hyperalgesia 3 hours after carrageenan injection (more details in Article 2).

- **Complete Freund's adjuvant (CFA)-induced chronic inflammatory pain:** Mice were intraplantarly injected with a volume of 20 µl of CFA solution (1 mg/ml of heat-killed *Mycobacterium tuberculosis* in 85% paraffin oil and 15% mannide monoleate; Sigma-Aldrich) into the mid-plantar surface of the ipsilateral hind paw. Chronic inflammatory pain was evidenced by mechanical allodynia and thermal hyperalgesia 4 days after CFA injection (more details in Article 2).

- **Plantar incision-induced postoperative pain:** Postoperative pain model was adapted to the mouse according to the previously described plantar incision method in rat by Brennan *et al.* (1996). A longitudinal incision of 0.7 cm was made with a number 11 blade through the skin, fascia, and muscle of the right hind paw, starting 0.3 cm from the proximal edge of the heel and extending towards the toes. The underlying plantaris muscle was exposed and incised longitudinally, keeping the muscle origin and insertion intact. After hemostasis with slight pressure, the skin was closed with two simple sutures of braided silk (ref. 6/0, B. Braun). Postoperative pain was evidenced by mechanical allodynia and thermal hyperalgesia 4 hours after surgery (more details in Article 4).

In this Doctoral Thesis the behavioural experiments related to the chronic constriction injury of the infraorbital nerve (IoN)-induced trigeminal neuropathic pain and the oxaliplatin-induced chemotherapy neuropathic pain have been performed under the guidance of Esteve in the following Laboratories: Laboratory of Pain Research (University of Antwerp, Belgium) and Laboratoire de Neurobiologie de la Cognition (Centre National de la Recherche Scientifique, France), respectively.

4. Evaluation of pain-related behaviours

- **Evaluation of mechanical allodynia in the IoN-induced trigeminal neuropathic pain in rats (von Frey test):** Responsiveness to mechanical stimulation of the territory of the IoN was measured using a series of five von Frey hairs (0.015 to 2.15 g). A mean score for the five von Frey hairs was determined. The scoring system described by Vos *et al.* (1994) was used to evaluate the reaction of the rats to the stimulation. The response of an animal was analysed according to different response categories: 0: no response, 1: detection, 2: withdrawal reaction, 3: escape/attack and 4: asymmetric face grooming. Lower scores indicate a weak responsiveness to mechanical stimulation, whilst higher scores indicate a strong responsiveness (more details in Article 1).
- **Evaluation of mechanical allodynia in inflammatory and postoperative pain models in mice (von Frey test):** The animals were placed into compartment enclosures in a test chamber with a framed metal mesh floor through which von Frey monofilaments (bending force range from 0.04 to 2 g) were applied onto the plantar surface, and thresholds were measured using the up-down method paradigm (more details in Articles 2 and 4) (Fig. 1).

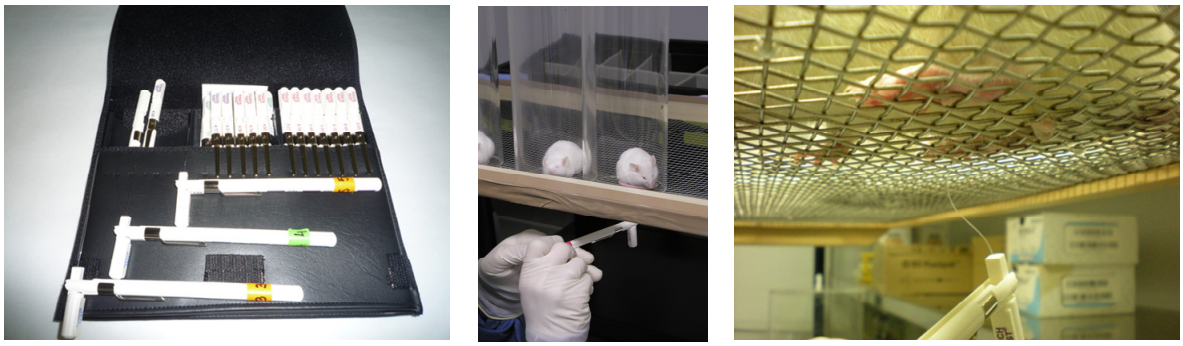


Fig. 1. von Frey test (North Coast Medical, Inc.) to evaluate hind paw withdrawal response in mice.

- **Evaluation of thermal/cold allodynia (Acetone test):** Cold allodynia was assessed using the acetone test. In this test, the intensity of the response was scored within 20 sec (cut-off) after acetone application: 0 (no response), 1 (quick withdrawal, flick of the paw), 2 (prolonged withdrawal or marked flicking of the paw), 3 (repeated flicking of the paw with licking or biting). The cumulative cold score is defined as the sum of the 6 scores for each rat together (more details in Article 1).
- **Evaluation of mechanical hyperalgesia (Randall-Selitto test):** Mechanical hyperalgesia was quantified using the Randall-Selitto test (Randall and Selitto, 1957) by means of a commercially available apparatus (Ugo Basile) (Fig. 2). This test allows determine pain threshold in response to increasing pressure applied on the dorsum of the animal's hind paw. The nociceptive threshold was defined as the pressure, in grams, at which the rat withdraws its paw. The cut-off was fixed at 270 g (more details in Article 1).

Fig. 2. Randall-Selitto test (Ugo Basile) to evaluate paw withdrawal in rats.

- **Evaluation of thermal/heat hyperalgesia (Plantar test):** Thermal (heat) hyperalgesia was assessed using the plantar test analgesia meter by determination of the hind paw withdrawal latency in response to a thermal stimulus (radiant heat) (Fig. 3). The plantar test was performed according to the Hargreaves method (Hargreaves *et al.*, 1988). The heat source, a mobile infrared photobeam, was positioned under the plantar surface of a hind paw, the nocifensive withdrawal reflex interrupts the light reflected from the photocell onto the paw and automatically turns off the light and the timer. The latency of the withdrawal response (as an indirect measure of the heat-pain threshold) was thus automatically recorded (more details in Articles 2 and 4).

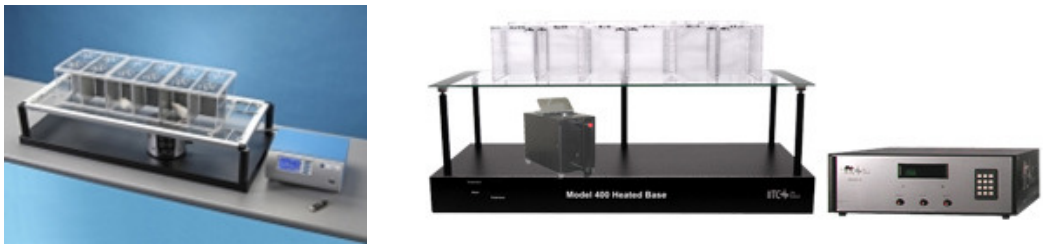


Fig. 3. Plantar test (Ugo Basile or IITC Life Science Inc).

5. Molecular studies

- **Western blotting:**

The lumbar enlargement (L4-S1) of spinal cord was removed from WT and σ_1R KO mice and ipsilateral hemicord segments were dissected, homogenized by sonication in radioimmunoprecipitation assay (RIPA) buffer and the supernatant was used. Equal amounts of protein (30 µg) were fractionated by 10% (w/v) SDS–PAGE and transferred onto a polyvinylidene difluoride membrane, blocked with 5% non-fat

dry milk in Tris–Tween 20-buffered Saline (T–TBS) for 1 h. Membranes were then incubated in 1% non-fat dry milk in T–TBS overnight at 4 ºC with rabbit primary polyclonal antibodies recognizing the mitogen-activated protein kinase (MAPK, total ERK 1/2) or mouse monoclonal antibodies recognizing the activated MAPK (diphosphorylated MAPK, pERK 1/2) at a 1:40,000 and a 1:1000 dilution, respectively. Mouse monoclonal anti-GAPDH antibody (1:80,000) or rabbit policlonal anti-GAPDH antibody (1:10,000) was used as a loading control. After washing with T–TBS, the blots were then incubated for 1 h with horseradish peroxidase–conjugated goat anti-rabbit IgG (1:4000) or goat anti-mouse IgG (1:2000). The immunoreactive bands were detected by a peroxidase reaction using an enhanced chemiluminescence method (WesternSure® PREMIUM Chemiluminescent Substrate, Li-cor) and C-DiGit® Blot Scanner (Li-cor).

- **Immunohistochemistry:**

The expression of proteins was estimated as c-Fos-, nNOS- and NPY-immunopositive neurons or immunoreactive area of GFAP and SP in the dorsal horn (laminae I + II) of the spinal cord. After perfusion, the lumbar region of the spinal cord was dissected out, postfixed for 4 h in 4% paraformaldehyde and cryopreserved in 30% sucrose solution at 4 °C for 48 h. The section from L4 to S1 of the spinal cord was selected and then embedded in frozen section medium and sliced in 40 μm sections on a cryostat, and collected in phosphate-buffered saline (PBS) to be processed immunohistochemically as free-floating sections. Sections were pre-incubated with 0.3% H_2O_2 in PBS for 30 min to block endogenous peroxidase activity and, after washing with PBS, were incubated with normal goat pre-immune serum diluted 1.5:100 in PBS for 1 h at room temperature (RT) to prevent unspecific staining. Sections were

then incubated for 48 h at 4 ºC with the primary antibody: rabbit policonal anti-c-Fos (diluted 1:6000), rabbit policlonal anti-GFAP (diluted 1:4000), rabbit policlonal anti-nNOS (diluted 1:3000), rat monoclonal anti-SP (diluted 1:750) or rabbit policlonal anti-NPY (diluted 1:10000). After washing, sections were incubated with the appropiate secondary biotinylated antibodies diluted 1:200 for 1 h at RT. After washing, an avidin–biotin–peroxidase complex was applied diluted 1:100 for 1 h at RT. The sections were washed again in PBS and placed in a chromogen solution containing 0.05% 3,3'-diaminobenzidine and 0.01% H_2O_2 in PBS for 5 min. The immunostained sections were placed on slides and coverslipped with glycergel mounting medium for microscopic observation and photography.

- **Polymerase chain reaction (PCR) assays in WT and σ1R KO mice:**

In order to ensure the proper selection of the genetic background (WT or σ_1R KO), genotype was periodically studied at the end of the behavioural testing (Entrena *et al.*, 2009).

Genomic deoxyribonucleic acid (DNA) was obtained from tail tips using the DNeasy Blood & Tissue kit (QIAGEN) according to the manufacturer's instructions. Amplifications for PCR were performed with HotStarTaq Plus Master Mix Kit (QIAGEN, Madrid, Spain) and with 0.5 μM of each primer (Invitrogen Ltd, Paisley, UK). The PCR was done with a thermal controller using the following program. Initial template denaturation at 94 ºC, followed by 35 cycles: 30 s at 94 ºC, 45 s at 55 ºC and 2 min at 70 ºC; and, as a final extension step, 10 min at 70 ºC. The oligonucleotide primer (5'– 3') sequences specific for the genes examined were as follows: 5'-AAT TTT GCT CCC CTC CTC-3' and 5'-CGT TCA CAA ATA CCC ACT G-3' for the wild-type allele; 5'-GGA CAC CAA GAT TGA ACC CAA CAG GGT GGC-3' and 5'-CGC

CAT TCA GGC TGC GCA ACT GTT GGG-3' for the mutant allele (Langa *et al.*, 2003). Amplified products were analysed by electrophoresis on 2% agarose gel containing ethidium bromide. The gels were then photographed with a UV transilluminator to visualize the ethidium bromide-stained bands (Fig. 4).

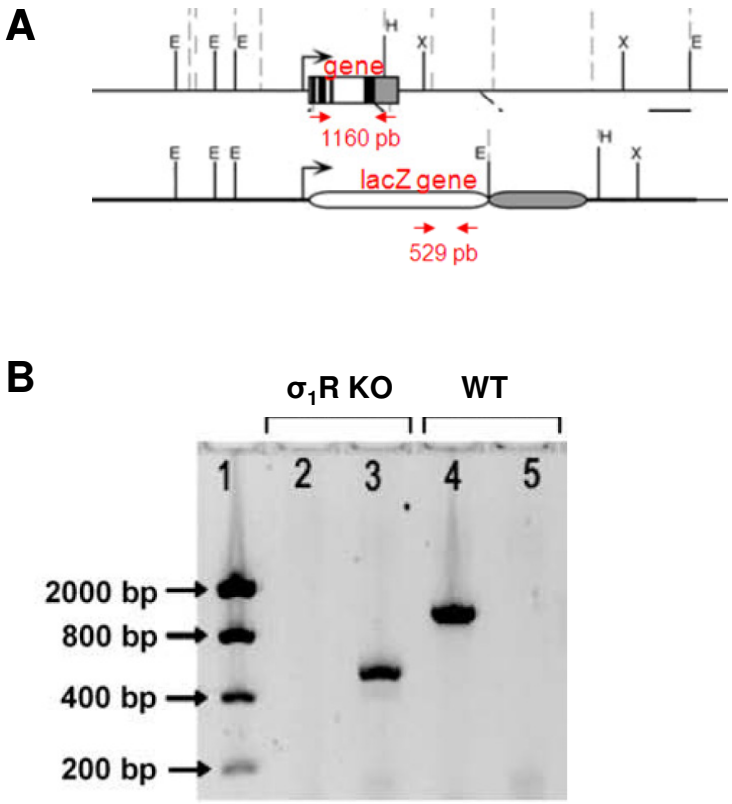


Fig. 4. Mice genotyping. (A) Map of the location of the primers inside the gene. Restriction enzyme sites for HindIII (H), EcoRI (E) and XhoI (X) are shown (for more details see Langa *et al.*, 2003). (B) Example of an electrophoresis of the PCR products.The lane 1 shows the molecular weight marker; lane 2, shows the absence of product amplification with primers for the WT allele; lane 3 shows a 529-bp product amplification with primers for the mutant allele; lane 4 shows a 1160-bp product amplification with primers for the WT allele; lane 5 shows the absence of product amplification with primers for the mutant allele (From Entrena *et al.*, 2009a).

RESULTS

LIST OF RESULTS

1. EFFECT OF σ_1R BLOCKADE ON NEUROPATHIC PAIN

1.1. Article 1: *"The selective sigma-1 receptor antagonist E-52862 attenuates neuropathic pain of different aetiology in rats"*. Submitted to *Scientific Reports*.

2. EFFECT OF σ_1R BLOCKADE ON INFLAMMATORY PAIN

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1. EFFECT OF σ_1R BLOCKADE ON NEUROPATHIC PAIN

1.1. Article 1: *"The selective sigma-1 receptor antagonist E-52862 attenuates neuropathic pain of different aetiology in rats".* (Submitted to *Scientific Reports*)

Georgia Gris[a], Enrique Portillo-Salido[a], Bertrand Aubel[a], Yassine Darbaky[b], Kristoff Deseure[c], José Miguel Vela[a], Manuel Merlos[a], Daniel Zamanillo[a].

[a]Department of Pharmacology, Drug Discovery & Preclinical Development, ESTEVE Barcelona, Spain.
[b]ANS Biotech. Facultés de Medicine et Pharmacies.
[c]Laboratory of Anesthesiology, University of Antwerp, Universiteltsplein 1, B-2610 Antwerp, Belgium.

Summary of the Article 1:

"The selective sigma-1 receptor antagonist E-52862 attenuates neuropathic pain of different aetiology in rats".

Background

The sigma-1 receptor (σ_1R) is a unique target class with chaperoning functions over different molecular targets involved in the transmission and amplification of nociceptive messages (Almansa and Vela, 2014); it is located in areas of the central and peripheral nervous system that are important for pain control, such as the spinal cord dorsal horn, periaqueductal gray matter, and dorsal root ganglia. Although previous reports have shown that σ_1R ligands did not affect acute pain perception, the role of σ_1R in modulating pain behaviour under sensitizing conditions has been widely demonstrated. The pharmacological and genetic blockade of σ_1R has shown to prevent the development of pain-related behaviours in some preclinical models of neuropathic pain. A repeated (preventive) treatment with σ_1R antagonists (E-52862 —S1RA— and BD-1063) on the development of sciatic nerve-injury or chemotherapy (paclitaxel)-induced neuropathic pain in mice supressed mechanical and thermal (heat or cold) hypersensitivity (Romero *et al.*, 2012; Nieto *et al.*, 2012). Moreover, cold and mechanical allodynia failed to develop or were strongly attenuated in σ_1R KO mice in these experimental pain models (De la Puente *et al.*, 2009; Nieto *et al.*, 2012). Acute administration of σ_1R antagonists also reversed pain-related behaviours after these had fully developed in different pain models, such as the capsaicin-induced headache model (Kwon *et al.*, 2009) or the orofacial-formalin model (Roh and Yoon, 2014).

Because neuropathic pain represents a critical unmet medical need that requires long-term pharmacological treatments in clinical practice, it is necessary to use neuropathic pain models with better predictability in human pain diseases.

Objectives

The objective of this study was to investigate the effect of single and repeat administration of E-52862 on the expression (treatment effect once pain has developed) of different pain-related behaviours of neuropathic models in rats with an elevated translational validity for different clinical conditions: trigeminal neuropathic pain following chronic constriction injury (CCI) of the infraorbital nerve (IoN), streptozotocin (STZ)-induced diabetic neuropathy, and cold allodynia in oxaliplatin (OX)-induced painful neuropathy. Finally, given the potential benefit of co-administering patients with antineoplastic drugs and drugs counteracting antineoplastic undesired effects, the possible preventive effect of E-52862 on the development of OX-induced neuropathic pain was also investigated.

Results

Firstly, the development of mechanical allodynia following CCI of the IoN from day 15 to 25 post-surgery was observed. E-52862 induced a dose-dependent attenuation of mechanical allodynia when it was acutely administered by intraperitoneal route on day 25. Moreover, a higher antiallodynic effect was obtained with repeated administration of E-52862.

Secondly, streptozotocin (STZ)-induced diabetic neuropathy was investigated 21 days after intraperitoneal injection of STZ. Diabetic rats exhibited statistically significantly reduced paw withdrawal thresholds in response to a noxious pressure

stimulus (mechanical hyperalgesia) that lasted at least until day 28. As in the trigeminal model, E-52862 was able to attenuate mechanical hyperalgesia when it was given either acutely (at day 21) or after repeated administration over 7 days (until day 28). Interestingly, repeat E-52862 administrations have a cumulative analgesic effect in diabetic rats.

Finally, in the chemotherapy OX-induced neuropathy model, repeated OX administration induced robust cold allodynia, as evidenced by paw withdrawal after acetone application on days 8, 15 and 16. As expected, E-52862 reversed nociceptive hypersensitivity in a dose-response manner in both acute and repeat administration. Interestingly, E-52862 is able to maintain its analgesic effect 24h after the completion of repeated administration. Moreover, preventive treatment with E-52862 (40mg/kg) inhibits the development of cold hypersensitivity.

Conclusions

Selective pharmacological σ_1R blockade with E-52862 was active in reducing mechanical and thermal hypersensitivity when administered both acutely and repeatedly in trigeminal neuralgia, chemotherapy-induced neuropathic pain, and diabetic painful polyneuropathy. Repeated daily administration of E-52862 caused an increased antinociceptive effect in baseline pain behaviours, thus supporting a sustained inhibitory effect on underlying pain-generating mechanisms. Hence, E-52862 could represent an alternative treatment for alleviating neuropathic pain of different aetiology.

Article 1: ***"The selective sigma-1 receptor antagonist E-52862 attenuates neuropathic pain of different aetiology in rats".***

ABSTRACT

E-52862 is a selective σ_1R antagonist currently undergoing phase II clinical trials for neuropathic pain and represents a potential first-in-class analgesic. Here, we investigated the effect of single and repeated administration of E-52862 on different pain-related behaviours in several neuropathic pain models in rats: mechanical allodynia in cephalic (trigeminal) neuropathic pain following chronic constriction injury of the infraorbital nerve (IoN), mechanical hyperalgesia in streptozotocin (STZ)-induced diabetic polyneuropathy, and cold allodynia in oxaliplatin (OX)-induced polyneuropathy. Mechanical hypersensitivity induced after IoN surgery or STZ administration was reduced by acute treatment with E-52862 and morphine, but not by pregabalin. In the OX model, single administration of E-52862 reversed the hypersensitivity to cold stimuli similarly to 100 mg/kg of gabapentin. Interestingly, repeated E-52862 administration twice daily over 7 days did not induce pharmacodynamic tolerance but an increased antinociceptive effect in all three models. Additionally, as shown in the STZ and OX models, repeated daily treatment with E-52862 attenuated baseline pain behaviours, which supports a sustained modifying effect on underlying pain-generating mechanisms. These preclinical findings support a role for σ_1R in neuropathic pain and extend the potential for the use of selective σ_1R antagonists (e.g., E-52862) to the chronic treatment of cephalic and extra-cephalic neuropathic pain.

Keywords

Allodynia, E-52862, hyperalgesia, hypersensitivity, neuropathic pain, sigma-1 receptor.

1. Introduction

Neuropathic pain is characterized by spontaneous ongoing or shooting pain and evoked amplified pain responses after noxious or non-noxious stimuli[1]. The current therapy for neuropathic pain is not satisfactory and thus new drugs acting on new molecular targets are being investigated[2-4]. Several therapeutic approaches targeting different modulatory proteins have emerged. Among them, the sigma-1 receptor (σ_1R) has been described to play a role in pain control[5]. σ_1R is an intracellular chaperone protein that interacts with other proteins, including plasma membrane and endoplasmic reticulum receptors and ion channels. In the context of pain, σ_1R modulates central sensitisation phenomena[6,7], which are responsible for many of the temporal, spatial, and threshold changes in pain sensitivity in acute and chronic pain[8]. Accordingly, pharmacological treatment with σ_1R antagonists in wild-type (WT) mice exerted antinociceptive effects and σ_1R knockout (KO) mice showed a pain-reduced phenotype in different experimental pain models[7,9-17].

The *in vitro* and *in vivo* pharmacological profile of the σ_1R antagonist E-52862 (S1RA) has been described[7]. E-52862 shows high σ_1R affinity and selectivity. It binds to σ_1R in the CNS when administered systemically, as shown by autoradiographic *ex vivo* binding assays in mice, and its efficacy correlates with the occupancy of σ_1Rs. It shows a good preclinical safety and efficacy profile in mice[7]. Specifically, formalin-induced nociception[7], capsaicin-induced mechanical allodynia[7], paclitaxel-induced cold and mechanical allodynia[17], nerve injury-induced mechanical and thermal hypersensitivity[7] and inflammation-induced mechanical and thermal hypersensitivity[15,16] were dose-dependently inhibited by acute systemic administration of E-52862.

E-52862 has completed single- and multiple-dose phase I clinical studies demonstrating good safety, tolerability and pharmacokinetic profiles in humans[18], and is currently in phase II clinical trials for the treatment of neuropathic pain. In the present study, we tested the efficacy of E-52862 in three rat models of neuropathic pain of different aetiologies: trigeminal neuropathic pain following chronic constriction injury to the infraorbital nerve (IoN)[19], streptozotocin (STZ)-induced diabetic neuropathy[20], and oxaliplatin (OX)-induced painful neuropathy[21]. These neuropathic pain models simulate clinical pain conditions with diverse aetiologies[22], such as trigeminal neuralgia[23], diabetic painful polyneuropathy[24], and chemotherapy-induced neuropathic pain[25]. As neuropathic pain is a persistent (chronic) type of pain which, in clinical practice, frequently requires long-term pharmacological treatments, E-52862 was repeatedly administered to neuropathic rats for several days, and its analgesic effects were compared with acute effects. In addition, the effects of some marketed analgesic drugs in these three models of neuropathic pain were investigated.

2. Methods

2.1. Animals

Adult male Sprague-Dawley rats (IoN and OX experiments) and Wistar rats (STZ experiments) weighing between 150-250 g at the beginning of the experimental phase were used. Animals were provided with food and water *ad libitum* and kept in controlled laboratory conditions with the temperature maintained at 21 ± 1 ºC and 12-hour light cycles (reversed dark/light cycle in IoN experiments, lights on at 20 h). Experiments were carried out in a soundproof and air-regulated experimental room. All experimental procedures and animal husbandry were conducted according to the ethical principles of the I.A.S.P. for the evaluation of pain in conscious animals[26] and the European Parliament and the Council Directive of 22 September 2010 (2010/63/ EU), and were approved by the local Ethics Committee.

2.2. Drugs

Oxaliplatin (OX) was provided by Shan Dong Boyuan Chemical Co, dissolved in distilled water and administered by intraperitoneal (i.p.) route. Streptozotocin (STZ) and acetone were provided by Sigma Aldrich. STZ was dissolved in 0.9% saline solution and administered by i.p. route. All analgesic drugs, except gabapentin, were administered i.p. Gabapentin was provided by Zhejiang Chiral Medicine Chemicals (China) and was administered at 100 mg/kg by oral (p.o.) route. E-52862 was synthesized by Laboratories Esteve (Spain), pregabalin by Mercachem (The Netherlands), and morphine was provided by the General Directorate of Pharmacy and Drugs (Spanish Ministry of Health; Madrid, Spain). Drugs were dissolved in saline, 0.5% hydroxypropyl methylcellulose (HPMC; Sigma Aldrich) or carboxymethylcellulose (CMC; Sigma Aldrich) as indicated. All drugs were

administered at a volume of 10 ml/kg except in the IoN experiments —volume used 5 ml/kg.

2.3. Experimental models

2.3.1. Chronic constriction of the infraorbital nerve (IoN)-induced trigeminal neuropathy

Surgical procedure

The infraorbital part of the nerve was exposed and ligated as described by Vos *et al.*[19]. Briefly, rats were anesthetized with pentobarbital (Nembutal, 60 mg/ml) at 60 mg/kg, i.p. followed by a fixed dose of 0.1 ml atropine. The head of the rat was fixed in a stereotaxic frame and a mid-line scalp incision was made, exposing skull and nasal bone. The IoN was exposed using a surgical procedure similar to that described previously[27,28]. The edge of the orbit, formed by the maxillary, frontal, lacrimal, and zygomatic bones, was dissected free. The orbital contents were gently deflected with a cotton-tipped wooden rod, thus the IoN was dissected free at its most rostral extent in the orbital cavity. Two chronic catgut ligatures (5-0, Ethicon; Johnson and Johnson, Belgium) were loosely tied around the IoN (2 mm apart). To obtain the desired degree of constriction, a criterion proposed by Bennet and Xie[29] was applied: the ligatures reduced the diameter of the nerve by a just noticeable amount and slowed but did not interrupt the circulation through the superficial vasculature. The scalp incision was closed using polyester sutures (4-0, Ethicon; Johnson and Johnson). In sham-operated rats, the IoN was exposed on one side using the same procedure, but the exposed IoN was not ligated.

Evaluation of mechanical allodynia

The responsiveness to mechanical stimulation of the IoN territory was measured using a series of five von Frey hairs (Stoelting Co): 0.015 g, 0.127 g, 0.217 g, 0.745 g and 2.150 g. Following a 10-min habituation, the different von Frey hairs were applied at every designated time to the ipsilateral side of the IoN territory. A mean score for the filaments was determined. Baseline data were obtained one day before surgery and on postoperative days 5, 15 and 25 (Fig. 1A). The scoring system described by Vos *et al.* (1994) was used to evaluate the reaction of the rats to the stimulation. The response of an animal was analyzed according to different response categories: 0: no response, 1: detection, 2: withdrawal reaction, 3: escape/attack and 4: asymmetric face grooming. Lower scores indicated a weak responsiveness to mechanical stimulation, whilst higher scores indicated a strong responsiveness. Rats were injected i.p. twice daily (b.i.d.) with compounds dissolved in 0.5% CMC. Behavioural testing was performed 30 min following injection.

2.3.2. Streptozotocin-induced diabetic neuropathy

Development of diabetes

Diabetes was induced in rats through chemical pancreatectomy by a single i.p. injection of STZ (75 mg/kg body weight) dissolved in saline, whereas control rats received saline alone[30]. Blood glucose levels were assessed before behavioural testing. Blood was extracted from the tail vein four days after STZ administration to calculate blood sugar levels by means of glucometer (Cholestech LDX). Upon diabetes induction (blood sugar levels above 240 mg/dl), food and water consumption, urinary volume and glucose levels increased in diabetic *versus* normal animals.

Evaluation of mechanical hyperalgesia

Mechanical hyperalgesia threshold was quantified using the Randall-Selitto test[31] by means of a commercially available apparatus (Ugo Basile, Italy). The response to increasing pressure applied on the dorsum of the animal's hind paw was measured. The nociceptive threshold was defined as the pressure, in grams, at which the rat withdraws its paw.

The mechanical sensitivity of naïve rats was first tested before STZ treatments and again three weeks after STZ administration in both left and right hind paws in order to select those animals showing a nociceptive mechanical threshold lower than 270 g. Pharmacological treatments were initiated 21 days after STZ injection, immediately after baseline determination, with vehicle (0.5% HPMC), morphine (10 and 20 mg/kg), pregabalin (80 mg/kg) and E-52862 (40 and 80 mg/kg) by i.p. route (Fig. 2A). Mechanical hyperalgesia was assessed 1 hour and 15 min after reference compounds and E-52862 administration, respectively. To determine the effects of repeated dosing on the hypersensitivity developed by STZ-treated rats, separate groups of STZ-treated rats were dosed b.i.d. for 7 consecutive days (from day 21 to 27) with 40 mg/kg of E-52862 (cumulative dose per day 80 mg/kg) or its solvent (0.5% HPMC). Mechanical sensitivity following repeated 7-day administration was assayed on day 28 before (pre-dose effect; washout period of approximately 16 hours after the last administration on day 27) and after additional dosing on day 28 (post-dose effect).

2.3.3. Chemotherapy-induced neuropathy after oxaliplatin (OX) treatment

Acquisition of neuropathy

Peripheral neuropathy was induced by repetitive i.p. injections of OX (3 mg/kg, i.p.) 3 times a week for 2 weeks (7 injections; cumulative dose = 21 mg/kg, i.p.)[32].

Distilled water injections were used to reproduce the non-neuropathic condition in a control (baseline) group (Fig. 3A).

Evaluation of cold allodynia

Cold allodynia was assessed using the acetone test. In this test, the intensity of hind paw withdrawal was measured upon application of a drop of acetone to the plantar surface of both hind paws by using a score. Responses to acetone were graded to the following 4-point scale: 0 (no response), 1 (quick withdrawal, flick of the paw), 2 (prolonged withdrawal or marked flicking of the paw), 3 (repetitive flicking of the paw with licking or biting). The cumulative cold score is defined as the sum of the 6 scores for each rat, the minimum score being 0 (no response to any of the 6 trials) and the maximum score being 18 (repetitive flicking and licking or biting of paws on each of the six trials). Cold allodynia was assessed by measuring the responses to acetone on days 8, 15 and 16.

In order to assess the treatment effect of E-52862 on the expression of OX-induced neuropathic pain ("curative" protocol once pain has developed) (Fig. 3A), the compound was administered i.p. (20, 40 and 80 mg/kg) in a repeated dosing paradigm from day 8 until day 15, and its effect on cold allodynia was assessed on day 8 (first day of administration), day 15 (last day of administration) and after treatment completion on day 16. On testing days 8 and 15, E-52862 and vehicle (0.5% HPMC) were administered 30 min before the test. Gabapentin (100 mg/kg, p.o.) was used as a positive reference compound. Animals from the gabapentin-treated group were dosed 120 min before testing on days 8, 15 and 16.

In order to assess a possible preventive effect of E-52862 on the development of OX-induced neuropathic pain (Fig. 4A), E-52862 was co-administered with OX. For this purpose, E-52862 was administered repeatedly b.i.d. at 40 mg/kg i.p., starting 2

days before the first injection of OX (3 mg/kg; i.p.) and throughout the OX treatment period (last OX injection on day 15). On the day of OX treatment, animals were dosed with the compound E-52862 30 min before OX injection. The acetone test was performed on days 8, 15 and 16 (24 h after the last administration).

2.4. Statistical analysis.

All data are presented as mean ± SEM. When several means were compared, all values were subjected to one-way analysis of variance (ANOVA) followed by post hoc Newman-Keuls test. To evaluate the development of diabetes, the comparison between baseline and post-STZ (day 4) glucose values was analyzed using the Student's t-test. GraphPad Prism software (version 5.0; GraphPad Software Inc., La Jolla, CA, USA) was used. In all cases, the criterion for statistical significance was established at a p value less than 0.05.

3. Results

3.1. Development of mechanical allodynia in the neuropathic pain model of constriction injury of the infraorbital nerve (IoN)

Baseline values were obtained one day before surgery, setting the normal response to von Frey filaments (Fig. 1A). Chronic constriction of the IoN induced significant changes in response to mechanical stimulation of the territory innervated by the ligated ipsilateral IoN (Fig. 1B). Initially, 5 days after surgery, the response score dropped significantly, indicating hyposensitivity, but this was followed by a robust hypersensitivity to von Frey filament stimulation on days 15 and 25 after IoN surgery, and hypersensitivity was maintained at least for 32 days after IoN constriction ($F_{3,212}$ = 585.6, $p < 0.001$, ANOVA; $p < 0.001$ for days 5, 15 and 25 *vs.* baseline).

3.2. Effect of acute administration of E-52862, morphine and pregabalin on mechanical allodynia developed after chronic constriction injury of the IoN

Drugs were administered when mechanical allodynia fully developed, on day 25 post-surgery. Single i.p. administration of E-52862 significantly inhibited mechanical allodynia at 40 mg/kg, but not at 20 mg/kg. Similarly, morphine (5 mg/kg) exerted an antiallodynic effect ($F_{5,124}$ = 8.5, $p < 0.001$, ANOVA; $p < 0.01$ for E-52862 40 mg/kg *vs*. vehicle; $p < 0.001$ for morphine 5 mg/kg *vs.* vehicle) but pregabalin (40 mg/kg) was ineffective (Fig. 1C).

3.3. Effect of repeated administration of E-52862 and morphine on mechanical allodynia developed after chronic constriction injury of the IoN

To evaluate the effect of repeated administrations, E-52862 and morphine were administered b.i.d. for 7 days (from day 25 to day 32 post-surgery) (Fig. 1A). After 7

days of administration, E-52862 exerted antinociceptive effect (antiallodynic; reduction of the response score) at both 20 and 40 mg/kg. In contrast, morphine (5 mg/kg) was devoid of antinociceptive effects when assayed after repeated administration for 7 days ($F_{3,49} = 17.4$, $p < 0.001$, ANOVA; $p < 0.001$ and $p < 0.01$ for E-52862 40 and 20 mg/kg, respectively, *vs.* vehicle).

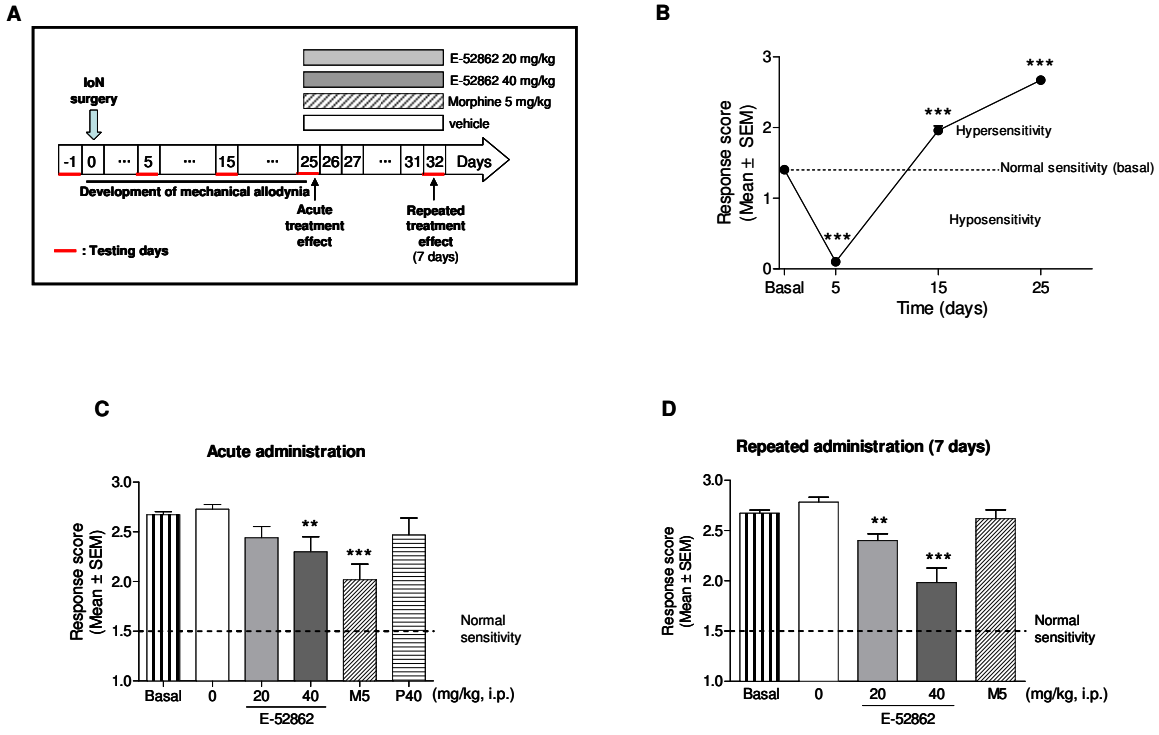

Figure 1. Trigeminal neuropathic pain model of chronic constriction injury of the IoN.

A) Experimental protocol. The acute treatment effects of the drugs were evaluated on day 25 after IoN surgery. The repetitive administrations of the drugs were evaluated on day 32 after 7 days of repeated daily administration. B) Time-related course of mechanical allodynia after IoN ligation. Evaluation was performed one day before surgery (baseline) and on postoperative days 5, 15 and 25 (black circles). C) Effect of a single i.p. administration of E-52862 (10, 20 and 40 mg/kg, i.p.; grey bars), morphine (5 mg/kg; diagonal dashed bars) and pregabalin (40 mg/kg; horizontal dashed bars) on mechanical allodynia. D) Effect of repeated i.p. administration of E-52862 (20 and 40 mg/kg; grey bars) and morphine (5 mg/kg; diagonal dashed bars) on mechanical allodynia. Each point and vertical line represent the mean ± S.E.M. of the values obtained in at least 10 rats per treatment and baseline groups. *** p <0.001 *vs.* pre-surgery in B. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ *vs.* vehicle group in C and D (one-way ANOVA followed by Newman–Keuls test).

3.4. Development of mechanical hyperalgesia in the STZ-induced neuropathic pain model

Rats showed increased glucose levels 4 days after STZ injection (453.3 ± 9.0 mg/dl *vs.* 115.7 ± 1.8 mg/dl, $p < 0.001$; Fig. 2B) and exhibited a robust reduction of paw withdrawal thresholds in response to noxious pressure stimulation (mechanical hyperalgesia) by 3-4 weeks after the injection of STZ (from 418.9 ± 5.4 to 220 ± 5.6 grams, $F_{2,162} = 354.4$, $p < 0.001$, ANOVA; $p < 0.001$ for days 21 and 28 post-STZ *vs.* baseline) (Fig. 2B).

3.5. Effect of acute administration of E-52862, morphine and pregabalin on mechanical hyperalgesia in the STZ-induced neuropathic pain model

Systemic acute administration of 80 mg/kg —but not 40 mg/kg— of E-52862 significantly decreased mechanical hypersensitivity in STZ-treated rats by 44% ($F_{6,122} = 20.9$, $p < 0.001$, ANOVA; $p < 0.001$ for E-52862 80 mg/kg *vs.* vehicle; Fig. 2C). Morphine at 20 mg/kg reversed mechanical hypersensitivity in diabetic rats. In contrast, morphine at 10 mg/kg and pregabalin at 80 mg/kg failed to show any significant effect (Fig. 2C).

3.6. Effect of repeated administration of E-52862 on mechanical hyperalgesia developed in the STZ-induced neuropathic pain model

The effect of repeated dosing b.i.d. for 7 consecutive days (from day 21 to day 27 after STZ injection) with 40 mg/kg of E-52862 (cumulative dose per day 80 mg/kg) on mechanical hypersensitivity developed in STZ-treated rats was evaluated (Fig. 2D). As expected, STZ administration induced hyperalgesia to mechanical stimulation of hind paws ($F_{7,112} = 37.4$, $p < 0.001$, ANOVA; $p < 0.001$ for baseline *vs.* day 21). On day

28, the day after completion of the 7-day treatment period (16 hours after the last E-52862 administration) and before treatment (pre-dose), mechanical hypersensitivity was decreased in STZ-treated rats that received E-52862 (from 237.8 ± 10.5 g in HPMC-treated rats to 311.7 ± 30.8 g in E-52862-treated rats). The administration of one additional dose of 40 mg/kg of E-52862 on day 28 (post-dose) failed to significantly change the mechanical threshold respect to the pre-dose value (hyperalgesia was reduced as compared to 7-day vehicle-treated rats and similar to pre-dose, but additional dosing was unable to further reduce hyperalgesia) ($F_{7,112} = 37.4$, $p < 0.001$, ANOVA; $p < 0.001$ for E-52862 40 mg/kg *vs.* HPMC at both pre- and post-dose assessments on day 28).

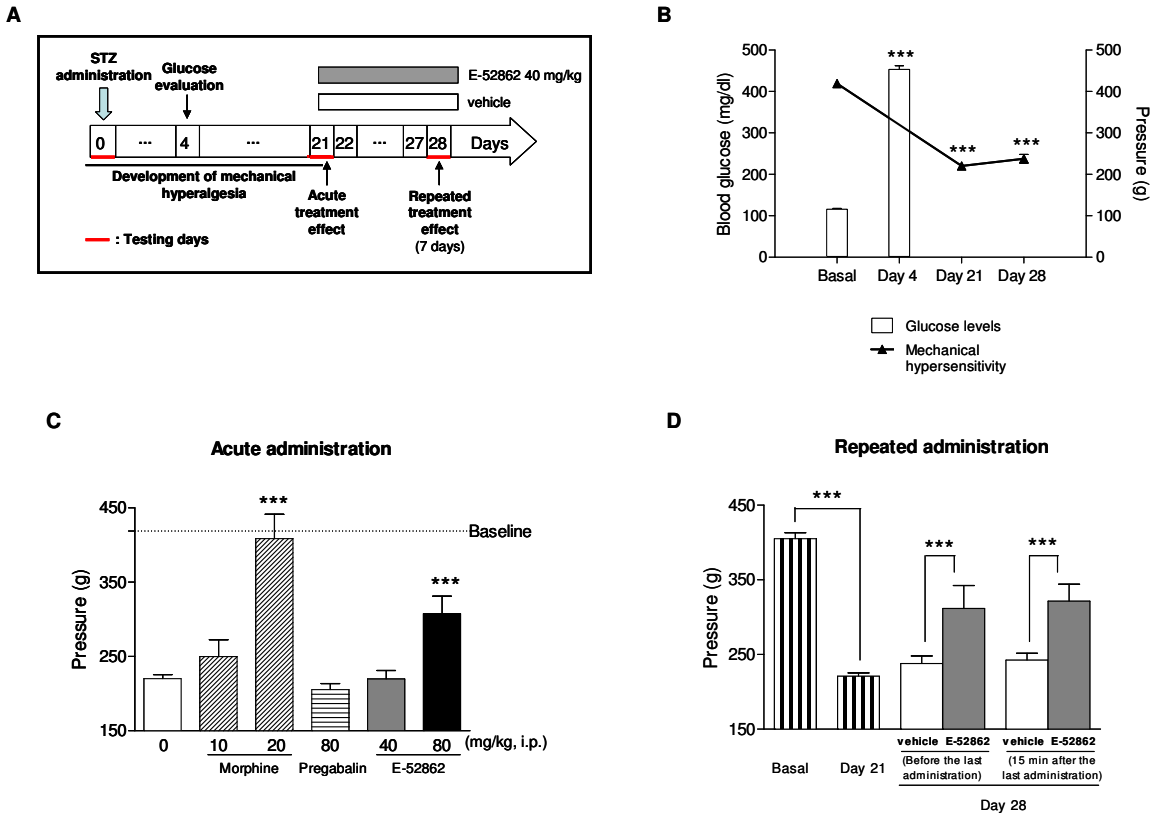

Figure 2. Diabetic neuropathic pain model after STZ injection.

A) Experimental protocol. The acute treatment effects of the drugs were evaluated on day 21 after the STZ injection. The repeated administrations of the drugs were evaluated on day 28 after 7 days of daily administration. B) Time-related course of mechanical hyperalgesia after IoN ligation (black triangles) and blood glucose levels (white bars) evaluated 4 days post-STZ injection. C) Effects of a single i.p. administration of E-52862 (40 and 80 mg/kg; grey and black bars), morphine (10 and 20 mg/kg; diagonal dashed bars) and pregabalin (80 mg/kg; horizontal dashed bars) on mechanical hyperalgesia. D) Effect of repeated i.p. administration of E-52862 (40 mg/kg; grey bars) on mechanical hyperalgesia. Rats were evaluated before and 15 min after the last administration. Each point and vertical line represents the mean ± S.E.M. of the values obtained from at least 8 animals per treatment group. Difference between pre- and post-STZ in glucose levels and mechanical hyperalgesia: *** $p < 0.001$ (Student's t-test for glucose values and one-way ANOVA followed by Newman–Keuls test for hyperalgesia development of); *** $p < 0.001$ *vs.* vehicle group (HPMC 0.5%) (one-way ANOVA followed by Newman–Keuls test).

3.7. Development of cold allodynia in the OX-induced neuropathic pain model

OX administration (Fig. 3A) induced cold allodynia, i.e., increased cumulative cold score in the acetone test respect to HPMC (vehicle)-treated animals on days 8, 15 and 16 ($F_{3,36} = 10.1$, $p < 0.001$ for the cumulative cold score; Fig. 3B).

3.8. Effect of acute administration of E-52862 and gabapentin on cold allodynia in the OX-induced neuropathic pain model

Neuropathy-related cold allodynia was already statistically significant as compared to the non-neuropathic group on day 8 after initiating OX administration (1.1 ± 0.4 *vs.* 5 ± 0.8, respectively; $p < 0.01$ and $p < 0.001$; Fig. 3B). In the acute treatment experiments (day 8), E-52862 exerted a dose-dependent antiallodynic effect on the cumulative cold score (Fig. 3C). The lowest tested dose (20 mg/kg) did not significantly reduce cold allodynia but E-52862 exerted a significant antinociceptive effect when administered at 40 mg/kg ($F_{5,54} = 10.2$, $p < 0.001$, ANOVA; $p < 0.05$ for E-52862 40 mg/kg *vs.* vehicle) and reversed cold allodynia back to normal baseline values at 80 mg/kg. As a clinically validated analgesic drug, the acute effect of gabapentin was also evaluated. Gabapentin orally administered at 100 mg/kg on day 8 reversed OX-induced cold allodynia as evidenced by a significantly decreased cumulative score in the acetone test.

3.9. Effect of repeated administration of E-52862 on the expression of cold allodynia in the OX-induced neuropathic pain model

To evaluate the effect of repeated administration, E-52862 was dosed from day 8 to 15 (Fig. 3A). On day 15 (last day of administration), cold allodynia was significantly inhibited by E-52862 at all three doses (20, 40 and 80 mg/kg) and in a dose-dependent

manner ($F_{4,45}$ = 8.5, $p < 0.001$, ANOVA; $p < 0.001$, $p < 0.01$ and $p < 0.05$ for E-52862 at 80, 40 and 20 mg/kg, respectively, *vs.* vehicle; Fig. 3D). Interestingly, reduced cold allodynia was still noticeable after treatment completion on day 16, 24 h after last administration ($F_{4,45}$ = 8.0, $p < 0.001$, ANOVA; $p < 0.01$ for E-52862 at 40 and 80 mg/kg *vs.* vehicle; Fig. 3D).

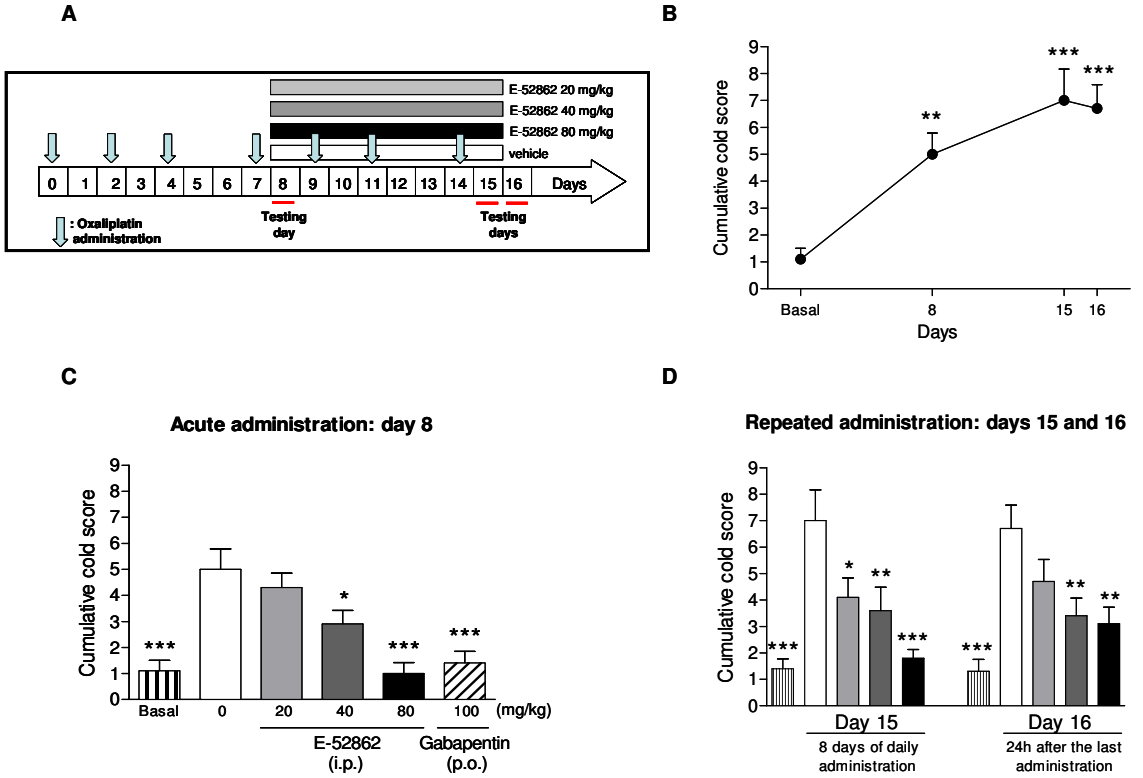

Figure 3. Chemotherapy-induced neuropathic pain after OX treatment.

A) Experimental protocol. The acute treatment effects of the drugs were evaluated on day 8 after four OX administrations. The repeated administration of drugs was evaluated on day 15 after 7 days of daily administration. The cumulative cold scores in response to acetone were evaluated on days 8, 15 and 16 after the baseline reading (testing days). B) Time-related course of cold allodynia after OX treatment evaluated as cumulative cold score. C) Effects of a single i.p. administration of E-52862 (grey and black bars) and gabapentin (diagonal dashed bars) at 40 and 100 mg/kg, respectively, on cold allodynia. D) Dose-response effects of repeated administration of E-52862 (20, 40 and 80 mg/kg) and response to cold stimulus one day after the last administration (day 16). Each point and vertical line represents the mean ± S.E.M. of the values obtained from 10 animals per group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ *vs.* each vehicle group and # $p < 0.05$ *vs.* baseline (one-way ANOVA followed by Newman–Keuls test).

3.10. Effect of repeated administration of E-52862 on the development of cold allodynia in the OX-induced neuropathic pain model (preventive protocol)

Unlike other neuropathic pain conditions, chemotherapy administration to cancer patients is a planned/scheduled procedure and thus neuropathic pain can be awaited (and anticipated) in a short term frame following administration of the cytostatic. Thus, it makes sense in this case to investigate preventive approaches that could attenuate this undesired effect of antineoplastics.

Therefore, given the potential benefit of co-administering patients with antineoplastic drugs and drugs counteracting antineoplastic's undesired effects, the possible preventive effect of E-52862 on the development of OX-induced neuropathic pain (i.e., cold allodynia) was also investigated. For this purpose, E-52862 was administered i.p. b.i.d at 40 mg/kg starting 2 days before the first injection of OX and throughout the OX treatment period (last OX injection on day 15) (Fig. 4A). On day 8, rats co-treated with OX and E-52862 b.i.d. at 40 mg/kg exhibited significantly lower cumulative cold scores as compared to the OX + vehicle group ($p < 0.01$ for comparisons between E-52862 40 mg/kg *vs.* vehicle; Fig. 4B). One week later, on day 15, the antiallodynic effect of the treatment was maintained ($F_{2,25} = 13.5$, $p < 0.001$, ANOVA; $p < 0.05$ E-52862 40 mg/kg *vs.* vehicle). Interestingly, 24 hours after E-52862 treatment completion (day 16) animals showed reduced cold allodynia ($F_{2,25} = 16.4$, $p < 0.001$, ANOVA; $p < 0.01$ E-52862 40 mg/kg *vs.* vehicle) compared to the vehicle-treated OX-treated group (3.5 ± 0.7 *vs.* 6.7 ± 0.9), although cumulative pain scores were higher than baseline values in non-neuropathic animals that did not receive OX ($F_{2,25} = 16.4$, $p < 0.001$, ANOVA; $p < 0.05$ for comparisons between E-52862 40 mg/kg *vs.* baseline).

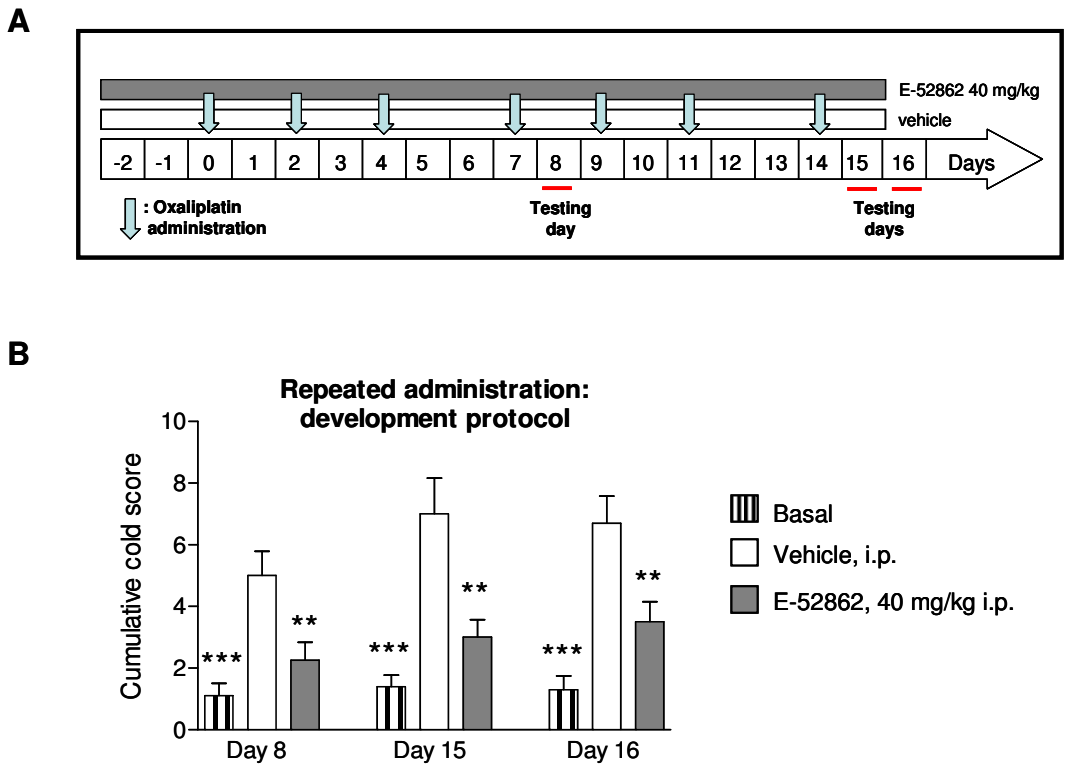

Figure 4. Effect of repeated administration of E-52862 on cold allodynia in a preventive paradigm in OX-induced neuropathy in rats.

A) Experimental preventive protocol. In this case, the administration of E-52862 started two days before the first OX injection. The preventive effect was evaluated on days 8, 15 and 16 after the first injection of OX (testing days). The evaluation on day 16 was performed one day after the last administration of E-52862. B) Effects of the repeated administration of E-52862 at 40 mg/kg (grey bars) in the preventive protocol on cold allodynia evaluated as cumulative cold score. Each point and vertical line represents the mean ± S.E.M. of the values obtained from 10 animals per group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ *vs.* vehicle group (one-way ANOVA followed by Newman–Keuls test).

4. Discussion

A rather compelling role of σ_1R in pain has been proposed from preclinical studies in models of different types of pain, but most studies have used mice as experimental subjects, peripheral nerve surgery as experimental model and acute treatment approaches. In the present study, these findings were broadened by using rats in three different models of neuropathic pain with translational value to measure disease-related pain processes of diverse aetiology and huge unmet need for treatment. In this sense, trigeminal neuralgia[23], diabetic painful polyneuropathy[24], and chemotherapy-induced neuropathic pain[25] are important clinical pain conditions often refractory to current pharmacotherapies. Not only single (acute) but also repeated (subchronic/chronic) treatment with E-52862[7], a selective σ_1R antagonist, was investigated to find out if sustained pharmacological blockade of σ_1R induces tolerance and if repeated administrations could interfere with the expression of neuropathic pain. Different evoked mechanical and thermal readouts were measured to monitor pain development and the antinociceptive effect of drug treatments. The main findings were that E-52862 exerted antinociceptive effects across the different models of neuropathic pain in rats and that its effects were not only maintained but increased following repeated administration. The present data extend recent evidence that σ_1R antagonism constitutes a new mechanism of analgesia the spectrum of which may also encompass chronic treatment of both cephalic (trigeminal) and extra-cephalic neuropathic pain.

Most neuropathic pain models are actually models of extra-cephalic neuropathic pain as they rely on the injury of spinal nerves with primary relay involving neurons at the dorsal root ganglia and the dorsal horn of the spinal cord. As opposed to the extra-cephalic one, cephalic neuropathic pain affects cranial nerve territories and involves primary synaptic relay by neurons at cephalic ganglia and brainstem nuclei. Not only

the anatomy but also the pathophysiology and the response to analgesics differ when comparing extra-cephalic and cephalic neuropathic pain, both preclinically and clinically[33-37]. The rat model of chronic constriction injury of the fifth cranial nerve (infraorbital nerve; IoN) has been reported to be a model for cephalic (trigeminal) neuropathic pain (i.e., trigeminal neuralgia) in humans[36-38]. As shown in previous studies[19,39], we found that nociceptive behaviours following IoN injury were characterized by a robust mechanical hypersensitivity preceded by a transient phase of lower responsiveness to mechanical stimulation of the IoN territory. Acute administration of morphine and E-52862 —but not pregabalin— reversed mechanical allodynia observed in this pain model. The acute antinociceptive effect of morphine and the lack of efficacy of gabapentin (another gabapentinoid similar to pregabalin) had been previously reported[40,41]. The antinociceptive effect of E-52862 was the first evidence of efficacy with a σ_1R ligand in this cephalic pain model, where gabapentinoids —that work in extra-cephalic neuropathic pain models— are ineffective but antimigraine drugs such as triptans (sumatriptan and zolmitriptan), dihydroergotamine and olcegepant —that are essentially inactive in extra-cephalic pain— are effective[33,37]. The efficacy of E-52862 in the IoN model is in agreement with some recent literature showing acute inhibitory effects of the σ_1R antagonist BD1047 on the nociceptive activation of the trigeminal nucleus caudalis in the capsaicin-induced headache model in rats[42] and the behavioural nociceptive responses in the orofacial formalin model in mice[43]. In turn, activation of σ_1R by intracisternal administration of the σ_1R agonist PRE084 evoked nociceptive activation of trigeminal nucleus caudalis in rats, which the antagonist BD1047 reversed[44]. Finally, it is important to note that administration of the σ_1R antagonist E-52862 for 7 days did not induce antinociceptive tolerance in a protocol where tolerance to the antinociceptive effect of morphine was

clearly seen. On the contrary, the effect was increased when preceded by previous administrations as efficacy following repeated dosing was significant at 20 mg/kg (the lower dose used), a dose that was insufficient to elicit significant antiallodynic effects after single acute administration.

Diabetic neuropathy is amongst the most frequent long-term complications of diabetes mellitus, and current treatments are only partially effective. STZ was administered to rats as a model of diabetic painful polyneuropathy. In agreement with previous reports[30,45], rats injected with this toxin for pancreatic β-cells exhibited significantly increased plasma glucose and water intake, decreased body weight, and increased sensitivity to noxious pressure stimuli (i.e., mechanical hyperalgesia) as compared to control, non-diabetic rats, thus reproducing symptoms observed in diabetic humans[46,47]. In addition to E-52862, two marketed drugs (morphine and pregabalin) with different mechanism of action were used to treat mechanical hypersensitivity of STZ-treated rats. Single administration of morphine at 10 mg/kg (a rather high dose) did not produce any significant effect but morphine reversed mechanical hyperalgesia when administered at 20 mg/kg (a sharply high dose). This result adds to the evidence that sensitivity to the analgesic effect of morphine is low in diabetic animals[48-50], and it is consistent with clinical reports that morphine lacks efficacy for the symptomatic relief of neuropathic pain in diabetic patients[51,52]. Similarly, the administration of a quite high dose of pregabalin (80 mg/kg) was ineffective in our model. In this sense, pregabalin has shown to provide some degree of pain relief in patients with painful diabetic neuropathy[53], and other studies reported significant antinociceptive effects of this drug on STZ-induced diabetic rats; however, this effect was modest when mechanical hyperalgesia was evaluated[54]. Single administration of E-52862 significantly reduced mechanical hypersensitivity in STZ-treated rats at the dose of 80 mg/kg, but not at 40

mg/kg. This is the first reporting of efficacy of a σ_1R ligand in a model of diabetic neuropathy and thus cannot be discussed against findings in other studies, but it is consistent with the antihyperalgesic effects exerted by E-52862 and other σ_1R antagonists[5,7,15,55] and the "pain-resistant" phenotype of σ_1R KO mice in a spectrum of pain conditions[10,12,13,17]. Just to note that STZ-induced mechanical hyperalgesia seems to be a difficult to treat pain condition, at least in our hands, based on both the lack of efficacy/reduced potency of marketed drugs and the activity of E-52862, lower than in other models/readouts, including the previously described IoN model. Interestingly, the dose of 40 mg/kg, that failed to produce any significant effect in the acute treatment, was effective against mechanical hypersensitivity in diabetic rats when preceded by repeated E-52862 40 mg/kg administration b.i.d. for 7 consecutive days. In fact, hyperalgesia was attenuated already before the last administration (previous injection of 40 mg/kg of E-52862 the day before), which supports not only lack of tolerance but suggests a modifying effect on the underlying baseline pain (or alternatively drug accumulation, as discussed later).

A third model, the OX-induced neuropathy model in rats was used to mimic neuropathic pain observed in cancer patients receiving this chemotherapy agent[21,56]. OX is a third-generation platinum-based antineoplastic drug used for the treatment of colorectal cancer[57,58] that induces painful neuropathy characterized by marked cold sensitivity[21,59]. Consistent with clinical symptoms, OX-treated rats showed cold allodynia already by day 8 after initiating OX treatment. Single oral administration of 100 mg/kg of gabapentin on day 8 reduced this pain-related behaviour, which is in accordance with previous findings in the same neuropathic pain model[21,60,61]. Regarding σ_1R modulation, E-52862 reduced cold allodynia. The effect of acute administration of E-52862 on day 8 was dose-dependent and equivalent in efficacy to gabapentin. Again,

this is the first study describing efficacy of a σ_1R ligand in this model, but in this case our data are supported by findings in a different chemotherapy model and species, the paclitaxel-induced neuropathic pain model in mice. Paclitaxel-induced mechanical and cold allodynia was dose-dependently reverted by E-52862 and BD1063, another σ_1R antagonist, and it did not develop in σ_1R KO mice, which reinforces pharmacological data with the antagonists[17,62]. In this way, it is important to note that antineoplastic drugs produce a painful peripheral neuropathy characterized by mitochondrial alterations in peripheral nerves, that prophylactic treatment of mice with BD1063 prevented not only paclitaxel-induced allodynia but also mitochondrial alterations, and that paclitaxel treatment did not induce mitochondrial abnormalities in σ_1R KO mice[62]. Moreover, as in the two previous models, no tolerance but increased activity respect to the acute treatment was found following repeated E-52862 administration in the OX model (e.g. the dose of 20 mg/kg, ineffective after single treatment, exerted significant antiallodynic effect on day 15, the last day of treatment, when preceded by repeated E-52862 administration for 7 consecutive days). Also, similar to findings in the STZ model, the pain-related behaviour was attenuated in the OX model one day after the last administration of E-52862 (day 16; 24 h washout, in the absence of active treatment) both when administered from day 8 for 7 consecutive days and when co-administered with OX throughout the OX treatment period (E-52862 administered i.p. b.i.d at 40 mg/kg starting 2 days before the first injection of OX and until the last OX injection on day 15), which again suggests a modifying effect on the underlying baseline pain (or alternatively drug accumulation, as discussed below).

Repeated treatments with E-52862 resulted in higher efficacy and potency respect to single/acute treatments consistently in all three neuropathic pain models. That is, higher antinociceptive activity and lower doses were required to reduce the different

pain-related behaviours if the administration the day of the test was preceded by repeated E-52862 daily (7 days, b.i.d.) administrations. This discards pharmacodynamic tolerance phenomena but opens the possibility that repeated treatments could exert a modifying effect on baseline pain (i.e., a sustained pharmacodynamic effect on nociceptive thresholds over time due to the continued action of the compound that results in a progressive attenuation of pain). The observation that pain-related behaviours were reduced the day after repeated treatment completion in E-52862-treated respect vehicle-treated animals further support the possibility of a sustained pharmacodynamic effect. Alternatively, increased antinociceptive effects following repeated administration could be explained by a pharmacokinetic effect due to drug accumulation over time. However, this is highly unlikely due to the pharmacokinetic profile of E-52862 in rodents. The maximum plasma concentration is achieved shortly after its administration to rodents (t_{max} = 15 min after i.p. administration to mice and rats) and is quickly metabolized, having a short half-life ($t_{1/2}$ = 1.4 h after administration to mice and rats). Undetectable plasma levels were found by 6 h after its administration, its metabolites are inactive and it does not accumulate in tissues, including the brain and the spinal cord[7]. All together, pharmacodynamics, but not phamacokinetics (i.e., drug accumulation), can explain the increased antinociceptive effect after repeated administration of E-52862 and also the attenuated hyperalgesia and allodynia found without E-52862 administration the day after treatment completion. Interestingly, it is in line with the mechanism of action of σ_1R and the inhibitory effect attributed to σ_1R antagonism on central sensitization phenomena, as reported at the behavioural, electrophysiological and molecular level. Behaviourally, it is known that a) capsaicin-induced secondary mechanical hypersensitivity (e.g., allodynia) results from central sensitization (i.e., plastic changes at the spinal cord due to the initial intense nociceptive

discharges that follows the capsaicin injection and result in subsequent increased pain sensitivity), that b) σ_1R antagonists block capsaicin-induced mechanical allodynia[7,11,12]; and that c) capsaicin does not induce mechanical allodynia when injected to σ_1R KO mice[12]. Electrophysiologically, it is known that repetitive stimulation of the dorsal root at stimulus intensities activating C fibres produces a typical amplification of the nociceptive signals in the spinal cord (wind-up response). Wind-up is the result of the sensitization of spinal dorsal horn neurons whose increased excitability is evoked by repetitive stimulation of afferent C fibres, and stands for a spinal amplification of the message coming from peripheral nociceptors[63-65]. σ_1R antagonists (i.e., E-52862) dose-dependently inhibit the spinal wind-up phenomenon when trains of nociceptive stimuli (repetitive stimulation of nociceptive afferent fibres) were applied[7,66] and, accordingly, spinal wind-up amplification of the nociceptive signals is highly reduced in spinal cords from σ_1R KO mice[13]. Finally, at the molecular level, increased signalling through the glutamatergic NMDA receptor on dorsal horn neurons is known to be a key mediator of spinal wind-up[65,67], and account for sensitization and pain hypersensitivity[8]. The NMDA receptor itself becomes phosphorylated in dorsal horn neurons following noxious stimulation or nerve injury, and this facilitates NMDA responses and thus central sensitization and pain hypersensitivity. Ligands of σ_1R are postsynaptic regulators of NMDA receptor-mediated synaptic transmission. Activation of σ_1R enhances the NMDA receptor-mediated rise in cytosolic Ca^{2+} concentration and currents[68,69]. In contrast, the NMDA receptor-mediated responses are inhibited and the enhancing effects of σ_1R agonists on NMDA receptors blocked by antagonizing σ_1R[68,70]. Accordingly, activation of spinal σ_1R by intrathecal administration of the σ_1R agonist PRE084 evoked[71] pain concomitant with increased phosphorylation of the NMDA receptor NR1 subunit, and both nociceptive behaviours and increased phosphorylation

of NR1 in the spinal cord were inhibited by antagonizing spinal σ_1R with BD1047[71-73]. From the mechanistic point of view, we now know that σ_1R interact with the NR1 subunit of NMDA receptors and that σ_1R antagonists (E-52862) remove the binding of σ_1R to NR1 subunits, facilitating the entrance of negative regulators of NMDA receptor activity, likely Ca^{2+}/calmodulin, which results in reduced glutamate-dependent NMDA receptor-mediated pain signalling and amplification[74]. Overall, evidence supports a role for σ_1R in modulating nociception by inhibiting augmented excitability secondary to sustained afferent drive as a mechanism underlying its modulatory effect. Attenuation of plastic changes (central sensitization) following nerve injury could thus underlie the sustained pharmacodynamic modifying effect on pain hypersensitivity exerted by E-52862 following its repeated administration.

Preclinical findings herein support a role for σ_1R antagonists (e.g., E-52862) in both the expression (to treat pain once it has developed) and development (to attenuate pain hypersensitivity progression) of neuropathic pain and extend the potential for the use of σ_1R antagonists (e.g., E-52862) to the chronic treatment of cephalic (trigeminal) and extra-cephalic neuropathic pain.

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2. EFFECT OF σ_1R BLOCKADE ON INFLAMMATORY PAIN

2.1. Article 2: *"S1RA, a selective sigma-1 receptor antagonist, inhibits inflammatory pain in carrageenan and complete Freund's adjuvant models in mice".*

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Georgia Gris, Manuel Merlos, José Miguel Vela, Daniel Zamanillo and Enrique Portillo-Salido.

Department of Pharmacology, Drug Discovery & Preclinical Development, ESTEVE Barcelona, Spain.

Summary of the Article 2:

"S1RA, a selective sigma-1 receptor antagonist, inhibits inflammatory pain in carrageenan and complete Freund's adjuvant models in mice".

Background

The sigma-1 receptor (σ_1R) has been related to pain sensitization processes induced by chemicals agents (Cendán *et al.*, 2005a, b; Entrena *et al.*, 2009a, b; Kwon *et al.*, 2009) or by pathological conditions using σ_1R KO mice and selective σ_1R receptor antagonists (de la Puente *et al.*, 2009, Romero *et al.*, 2012). In σ_1R KO mice, both phases of formalin-induced paw licking were clearly reduced (Cendán *et al.*, 2005b) and capsaicin injected intraplantarly failed to induce mechanical allodynia (Entrena *et al.*, 2009a). Regarding neuropathic pain models, cold and mechanical hypersensitivity were strongly attenuated in σ_1R KO mice treated with paclitaxel (Nieto *et al.*, 2012) or exposed to partial sciatic nerve ligation. Similarly, σ_1R antagonists, including E-52862 (S1RA), also inhibited formalin-induced pain (Cendán *et al.*, 2005a, Romero *et al.*, 2012), capsaicin-induced sensitization and pain-related behaviours (Entrena *et al.*, 2009b; Romero *et al.*, 2012) after partial sciatic nerve ligation (Romero *et al.*, 2012) or paclitaxel-induced neuropathic pain (Nieto *et al.*, 2012) in WT mice.

Objectives

The objective of this work was to study the involvement of σ_1R in acute and chronic inflammatory pain for the first time using σ_1R KO mice and selective σ_1R antagonist E-52862. The analgesic effect of E-52862 on pain-related behaviours

induced by carrageenan or CFA was investigated, and its effect was compared with those of other reference compounds (morphine, ibuprofen and celecoxib).

Results

Intraplantar injection of carrageenan caused both mechanical allodynia and thermal (heat) hyperalgesia that peaked at 3 h and reverted to baseline levels 3–4 days after injection in WT mice. Intraplantarly injected CFA caused robust, long-lasting mechanical allodynia (from days 1 to 9), whereas thermal heat hyperalgesia failed to fully develop in WT mice. The development of pain-related behaviours observed after CFA and carrageenan injection were similar in σ_1R KO and WT mice. The pharmacological study revealed that acute systemic administration of E-52862 dose-dependently inhibited mechanical and thermal (heat) hypersensitivity induced by both carrageenan and CFA in WT mice, but not in σ_1R KO mice. As compared to reference compounds, E-52862 showed an efficacy similar to that of ibuprofen and celecoxib in inhibiting inflammatory painful hypersensitivity. E-52862, however, failed to modify paw volume in mice.

Conclusions

Although the genetic inhibition of σ_1R failed to prevent the development of mechanical and heat hypersensitivity in all inflammatory pain models, acute systemic administration of E-52862 in WT mice dose-dependently supressed both mechanical and thermal (heat) hypersensitivity induced by carrageenan, and inhibited mechanical allodynia induced by CFA. E-52862 failed to exert any antihypersensitivity effect in σ_1R KO mice, thus suggesting that the analgesic activity induced by E-52862 in these models was clearly mediated by the interaction with σ_1R.

In summary, the pharmacological blockade of σ_1R by E-52862 represents a new promising therapy for the treatment of inflammatory pain.

226 Research report

S1RA, a selective sigma-1 receptor antagonist, inhibits inflammatory pain in the carrageenan and complete Freund's adjuvant models in mice

Georgia Gris, Manuel Merlos, José M. Vela, Daniel Zamanillo and Enrique Portillo-Salido

The therapeutic potential of S1RA (E-52862), a selective sigma-1 receptor (σ_1R) antagonist, has been explored in experimental neuropathic pain, but not in inflammatory pain models. The present study investigated the effect of the intraperitoneal administration of S1RA on the hind paw withdrawal response to thermal and mechanical stimulation following an intraplantar injection of carrageenan (CARR) and complete Freund's adjuvant (CFA), which are two well-characterized models of acute and chronic inflammatory pain, respectively. S1RA fully reversed both mechanical [dose of drug that produced half of its maximal response (ED_{50}) = 35.9 and 42.1 mg/kg for CARR-induced and CFA-induced pain, respectively] and thermal (ED_{50} = 27.9 mg/kg, CARR) hypersensitivity, whereas ibuprofen (CARR, mechanical allodynia) and celecoxib (CARR, thermal hyperalgesia; CFA, mechanical allodynia) failed to reach maximum efficacy. Morphine also showed maximum efficacy in all tests. Unlike celecoxib and ibuprofen, which decreased paw volume significantly, CARR-induced paw oedema was not reduced by S1RA and morphine, thus suggesting that the antinociceptive effect of S1RA does not involve a major anti-inflammatory (antioedema) action. S1RA was devoid of efficacy when administered to σ_1R knockout mice, thus suggesting the involvement of σ_1R in the antinociceptive effects exerted by S1RA. We conclude that S1RA represents a promising novel analgesic therapy for inflammatory pain. ***Behavioural Pharmacology*** **25:226–235 © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Keywords: allodynia, carrageenan, complete Freund's adjuvant, E-52862, hyperalgesia, hypersensitivity, inflammatory pain, σ_1R knockout mouse, S1RA, sigma-1 receptor

Drug Discovery and Preclinical Development, Esteve, Parc Científic de Barcelona, Barcelona, Spain

Correspondence to Enrique Portillo-Salido, PhD, Drug Discovery and Preclinical Development, Esteve, Parc Científic de Barcelona, Baldiri Reixac 4-8, 08028 Barcelona, Spain
E-mail: eportillo@esteve.es

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Introduction

Pain is a central feature of highly prevalent clinical diseases such as rheumatoid arthritis and osteoarthritis, a diverse group of conditions characterized by inflammation of the joints, pain, deformity and disability (Pisetsky and Ward, 2012). Even with optimal 'disease-modifying' treatment and proper control of disease severity and patient activity, persistent pain occurs secondary to structural joint damage, and analgesic treatment is therefore required in patients with arthritis (Radner *et al.*, 2012; Walsh and McWilliams, 2012). Pain is generally treated with opioids and anti-inflammatories including cyclooxygenase (COX) inhibitors, but their side effects and toxicities prevent both long-term use and dosage levels likely to provide superior analgesia (Van Laar *et al.*, 2012; Whittle *et al.*, 2013).

The sigma-1 receptor (σ_1R) is a unique ligand-regulated molecular chaperone with two transmembrane domains located mainly in the endoplasmic reticulum and the plasma membrane. The intrinsic activity of drugs targeting σ_1R (e.g. agonist vs. antagonist) is based on the regulation of some ion channels (e.g. small conductance calcium-activated potassium channels and voltage-gated calcium channels), G-protein-coupled receptors (e.g. dopamine D1 and μ-opioid receptors), neurotransmission (e.g. glutamatergic), and second messenger systems (e.g. PLC/PKC/InsP3), for which the σ_1R plays a role as a regulatory chaperone protein (Su *et al.*, 2010; Gómez-Soler *et al.*, 2014). For pain modulation, the σ_1R has been shown to be expressed in key areas for pain control such as the dorsal root ganglia, the superficial layers of the dorsal horn, the periaqueductal grey matter, the locus coeruleus, and the rostroventral medulla. Recent behavioural studies using σ_1R ligands and σ_1R knockout (KO) mice have shown a role of this receptor in pain control, particularly under sensitizing conditions (see Zamanillo *et al.*, 2013 for a review). Successful drug targeting of this protein would represent a new therapeutic approach to clinical pain management (Zamanillo *et al.*, 2012).

The in-vitro and in-vivo pharmacological profile of a novel and selective σ_1R antagonist, S1RA, has been reported recently (Romero *et al.*, 2012). This compound has high affinity for human σ_1R (K_i = 17 nmol/l), but no affinity for the sigma-2 receptors. Moreover, this compound does not show significant affinity for other additional 170 molecular targets (receptors, transporters, ion channels and enzymes). S1RA has a good pharmacokinetic profile, crosses the blood–brain barrier and binds to CNS σ_1Rs when administered systemically, as shown by autoradiographic

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ex-vivo binding experiments in mice (Romero *et al.*, 2012). All these properties make S1RA a good pharmacological tool to investigate σ_1R-mediated effects.

Although the therapeutic potential of S1RA has been explored with positive results in animal models of neuropathic pain (Nieto *et al.*, 2012; Romero *et al.*, 2012; Zamanillo *et al.*, 2013), there is a lack of substantial information on its effects in inflammatory pain models. The two types of pain differ in their aetiology, but share some common mechanisms in that they both involve sustained afferent peripheral input that give rise to central sensitization phenomena (Woolf, 2011; Walsh and McWilliams, 2012), which are modulated by σ_1Rs (De la Puente *et al.*, 2009; Drews and Zimmer, 2009) and inhibited by the σ_1R antagonist S1RA (Romero *et al.*, 2012).

Inflammatory pain can be induced experimentally by intraplantar administration of agents such as carrageenan (CARR) or complete Freund's adjuvant (CFA) (Bendele, 2001; Cobos and Portillo-Salido, 2013), and is behaviourally evidenced by both mechanical and thermal (heat) hypersensitivity. CFA is known to induce a more chronic inflammation compared with the transient acute CARR-induced inflammatory response. The present study aimed to investigate the analgesic effect of S1RA in both acute and chronic inflammatory pain models, and compare its effect with those of other relevant analgesics acting through other mechanisms of action: morphine (μ-opioid agonist), ibuprofen (nonselective COX-1 and COX-2 inhibitor) and celecoxib (selective COX-2 inhibitor). We also took advantage of the genetic approach using σ_1R KO mice to determine whether the effect of S1RA was actually because of σ_1R modulation.

Methods

Subjects

Male wild-type CD-1 mice, supplied by Charles River or Harlan Ibérica and aged 6–8 weeks, were used. To obtain CD-1 σ_1R KO mice, homozygous KO mice (Langa *et al.*, 2003) were backcrossed for 10 generations onto the CD-1 background to reduce to less than 1% the genetic material remaining from the original background (Wong, 2002). Mice carrying the mutation were then bred to homozygosity and used in the experiments. Animals were acclimated in our animal facilities for at least 1 week before testing, provided free access to food and water and kept under controlled laboratory conditions with the temperature at 21 ± 1°C and a light–dark cycle of 12 h (lights on at 07:00 h). Behavioural testing was carried out in a soundproof and air-regulated experimental room. All experimental procedures and animal husbandry were conducted according to the ethical principles of the I.A.S.P. for the evaluation of pain in conscious animals (Zimmermann, 1983) and the European Parliament and the Council Directive of 22 September 2010 (2010/63/EU), and were approved by the local Ethical Committee.

CARR-induced and CFA-induced inflammatory pain

Unilateral inflammation was induced by an intraplantar injection of 50 μl of 2.5% (w/v) CARR solution or 20 μl of 1 mg/ml CFA. Injections were administered into the subcutaneous tissue of the plantar surface of the right hind paw of nonanaesthetized mice. CFA was freshly prepared, whereas CARR was dissolved in saline and stored at room temperature for 1–2 days before administration.

To select the optimal time point to evaluate the analgesic effect of drugs, the time courses of hypersensitivity to CARR-induced and CFA-induced thermal and mechanical stimuli were characterized. After baseline measurements of thermal or mechanical sensitivity, mice were injected with the proinflammatory agent, and thermal hyperalgesia or mechanical allodynia was evaluated in both hind paws at 1, 3, 5 and 24 h, and 2, 3 and 4 days (CARR) or at days 1, 2, 3, 4, 7, 9 and 11 (CFA).

Evaluation of thermal hyperalgesia (plantar test)

Thermal (heat) hyperalgesia was assessed using the plantar test analgesia metre [dynamic plantar aesthesiometer; Ugo Basile, Comerio (VA), Italy] by determination of hind paw withdrawal latency in response to a thermal stimulus (radiant heat). The plantar test was performed according to the Hargreaves method (Hargreaves *et al.*, 1988). On the day of the test, mice were placed in Plexiglas compartment enclosures on the surface of the plantar test device and allowed to acclimate for 1 h. The heat source, a mobile infrared photobeam, was then positioned under the plantar surface of the hind paw. The nocifensive withdrawal reflex interrupts the light reflected from the photocell onto the paw and automatically turns off the light and the timer. The intensity of the light beam was adjusted on the basis of preliminary studies to produce baseline response latencies of around 12 ± 2 s in untreated control mice. A digital timer connected to the heat source automatically recorded the response latency for paw withdrawal to the nearest 0.1 s. The mean withdrawal latencies for the ipsilateral and contralateral hind paws were determined from the average of three separate trials for each animal, with intervals of at least one minute between successive measurements.

Evaluation of mechanical allodynia (von Frey test)

Mechanical allodynia was quantified by measuring the hind paw withdrawal response to punctate mechanical stimulation using von Frey filaments (North Coast Medical Inc., San Jose, California, USA). Mice were placed in compartment enclosures in a test chamber with a framed metal mesh floor through which von Frey monofilaments (bending force range from 0.04 to 2 g) were applied onto the plantar surface, and thresholds were measured using the up–down method paradigm. This method was previously described by the non-parametric method of Dixon (1980), and represents the mechanical threshold that produces 50% of responses (Chaplan *et al.*, 1994). Briefly, the 0.4 g filament

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(i.e. middle range) was used first. Then, the strength of the next filament was decreased when the animal responded or increased when the animal did not respond. This up–down procedure was stopped four measures after the first change in animal responding. The application of each filament was perpendicular to the plantar surface and was applied for 2 s at intervals of about 5–10 s between each stimulation. Clear paw withdrawal, shaking or licking was considered as a nociceptive-like response. Both ipsilateral and contralateral (noninjected) hind paws were tested. The mechanical threshold that produced 50% of responses was calculated using the Dixon formula: 50% paw withdrawal threshold (g) $=\left[\left(10^{(X_f+\kappa\delta)}/10\,000\right)\right]$, where X_f is the value (in logarithmic units) of the final von Frey filament used, κ is a fixed tabular value for the pattern of positive/negative responses and δ is the mean difference (in log units) between stimuli.

Evaluation of CARR-induced paw oedema

Hind paw volume was determined using a plethysmometer (Panlab, S.L.U.; Barcelona, Spain). The hind paw was immersed in a conductive solution (15 drops of Triton X-100 in 1 l of 0.2% NaCl solution) and the displaced volume was measured with a resolution of 10 µl. Paw oedema volume was determined by the difference between the values obtained before and after the intraplantar injection of CARR or its solvent. To minimize variations in the readings within each animal, the junction of hairy and glabrous skin was marked with a pen before the first measurement and used as a reference for subsequent determinations. Animals receiving 50 µl of saline in their ipsilateral paw were used as noninflamed controls. Paw volume determinations were performed before and 3 h after the administration of CARR; the time of maximal paw volume increase. Mice were administered pre-emptively with the drugs or vehicle (HPMC) 30 min before CARR injection to allow the detection of possible antioedema effects of the drugs tested. Two or three measurements per paw were performed.

Drugs

CARR (λ-carrageenan, 1% in 0.2 mol/l KCl solution) and CFA (1 mg/ml of heat-killed *Mycobacterium tuberculosis* in 85% paraffin oil and 15% mannide monooleate) were provided by Sigma-Aldrich (St Louis, Missouri, USA).

S1RA (E-52862; 4-[2-[[5-methyl-1-(2-naphthalenyl)-1H-pyrazol-3-yl]oxy]ethyl] morpholine) was used as hydrochloride and doses were expressed as weights of this form. S1RA and celecoxib were synthesized by Laboratories Esteve (Barcelona, Spain) and ibuprofen was supplied by Tci Europe N.V. (Zwijndrecht, Belgium). Morphine was supplied by the General Directorate of Pharmacy and Drugs, Spanish Ministry of Health (Madrid, Spain). All analgesic drugs were suspended in an aqueous solution (0.5% hydroxypropylmethyl cellulose, HPMC; Sigma-Aldrich) and administered by the intraperitoneal route at a volume of 10 ml/kg 30 min before behavioural testing.

Statistical analysis

Data were represented as mean ± SEM. Data for thermal hyperalgesia and mechanical allodynia were obtained by measuring paw withdrawal expressed as latency (s) or 50% threshold (g), respectively. Data obtained in the CARR and CFA models were subjected to repeated-measures two-way analysis of variance (ANOVA) (comparisons vs. contralateral noninjected paws), followed by post-hoc Bonferroni's test when appropriate. The percentage of antiallodynic or antihyperalgesic effect was calculated as follows: % effect = [(PWD − PWV)/(PWN − PWV)] × 100, where PWD and PWV are the paw withdrawal latency (s) or threshold (g) in drug-treated and vehicle-treated animals, respectively, and PWN is the paw withdrawal in naïve animals (12.5 s latency in the plantar test and 1.5 g mechanical threshold in the von Frey test). These percentages were expressed as antihypersensitivity percentage when the effect on both mechanical allodynia and thermal hyperalgesia was shown simultaneously. A dose–response curve was plotted using nonlinear regression analysis, and ED_{50} (dose of drug that produced half of its maximal response) and E_{max} (maximum effect) values were obtained. SEs were calculated on the basis of the best-fit values ± SEs of regression. The differences between WT and KO mice were analysed using Student's *t*-test. One-way ANOVA, followed by the Bonferroni test was used to compare paw volumes. GraphPad Prism software (version 5.0; GraphPad Software Inc., La Jolla, California, USA) was used. The criterion for statistical significance was set at a P value of less than 0.05.

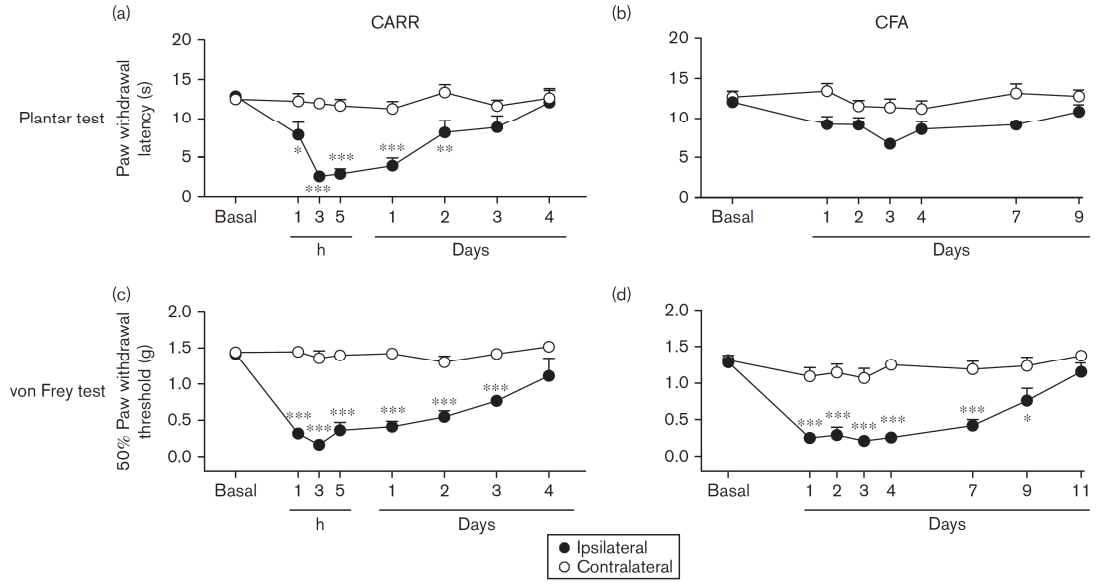
Results

Time course for thermal hyperalgesia and mechanical allodynia induced by CARR and CFA

No significant differences were found in baseline paw withdrawal latencies between the ipsilateral and contralateral paw in the plantar test before CARR injection. Thermal hyperalgesia was evidenced by a decreased withdrawal latency of the ipsilateral paw in response to a noxious heat stimulus (Fig. 1a). Intraplantar injection of CARR caused a marked thermal hyperalgesia from 3 h to 2 days after injection. Repeated-measures ANOVA (time × paw) showed significant effects of time ($F_{7,182} = 9.5$, $P < 0.001$) and paw ($F_{1,182} = 89.3$, $P < 0.001$) and a significant time × paw interaction ($F_{7,182} = 6.5$, $P < 0.001$). However, intraplantar administration of CFA did not produce a clear thermal hyperalgesia (Fig. 1b). A two-way ANOVA showed significant effects of time ($F_{6,152} = 3.5$, $P < 0.01$) and paw ($F_{1,152} = 39.2$, $P < 0.001$), but no significant time × paw interactions ($F_{6,152} = 1.4$, NS).

No significant differences were found between groups in baseline 50% paw withdrawal thresholds in the von Frey test before CARR. Upon intraplantar injection, CARR injection caused significant mechanical hypersensitivity

Fig. 1



Time course of CARR-induced and CFA-induced thermal hyperalgesia and mechanical allodynia in WT mice. Thermal hyperalgesia and mechanical allodynia were found immediately after pain induction. There were significant differences between ipsilateral and contralateral paws under all experimental conditions, except for CFA in the plantar test. Statistically significant differences, compared with the contralateral hind paw for each animal model: ***$P<0.001$; **$P<0.01$; *$P<0.05$ (two-way repeated-measures analysis of variance, followed by the Bonferroni test). Each point and vertical line represent the mean ± SEM of the values obtained in 7–20 animals. The results are an average of two independent experiments for each curve. CARR, carrageenan; CFA, complete Freund's adjuvant.

in the ipsilateral paw from 1 h to day 3 after injection with a maximum at 3 h and recovery to baseline values by day 4 (Fig. 1c). Repeated-measures ANOVA (time × paw) showed significant effects of time ($F_{7,148} = 15.6$, $P < 0.001$) and paw ($F_{1,148} = 341.1$, $P < 0.001$), and a significant time × paw interaction ($F_{7,148} = 11.5$, $P < 0.001$). CFA-induced mechanical allodynia was observed as of day 1 and lasted up to day 9, with recovery to baseline values by day 11 after injection (Fig. 1d). Repeated-measures ANOVA (time × paw) showed a significant effect of time ($F_{7,178} = 13.6$, $P < 0.001$) and paw ($F_{1,178} = 156.2$, $P < 0.001$) and a significant time × paw interaction s ($F_{7,178} = 6.0$, $P < 0.001$).

Thermal hyperalgesia and mechanical allodynia induced by CARR and CFA in σ_1 receptor knockout mice

Baseline responses in the plantar test were not significantly different when comparing σ_1R KO and WT mice subsequently treated with CARR (Fig. 2a). Intraplantar injection of CARR markedly reduced paw withdrawal latency in both WT and σ_1R KO mice 3 h after injection ($F_{3,55} = 77.3$, $P < 0.001$), with no statistically significant differences between genotypes. Similarly, baseline responses in the plantar test of σ_1R KO and WT mice subsequently treated with CFA were not significantly different (Fig. 2b). Four days after the administration of CFA, mice showed nonsignificant (KO) to weak (WT) thermal hyperalgesia, but no statistically significant differences were found in the response to heat stimulus when the two genotypes were compared (Fig. 2b).

Basal mechanical sensitivity was also similar in both genotypes. Intraplantar injection of CARR induced a robust reduction of 50% paw withdrawal threshold 3 h after CARR injection ($F_{3,47} = 106.7$, $P < 0.001$), with no differences in CARR-induced mechanical allodynia between WT and σ_1R KO mice (Fig. 2c). Similarly, CFA-induced mechanical allodynia did not differ when the two genotypes were compared (Fig. 2d). CFA injection clearly decreased the pressure threshold, inducing withdrawal of the ipsilateral paw in both genotypes (from 1.3 ± 0.1 to 0.3 ± 0.04 g and from 1.4 ± 0.04 to 0.3 ± 0.06 g in WT and σ_1R KO mice, respectively).

Effects of S1RA and reference compounds on thermal hyperalgesia and mechanical allodynia in wild-type mice

The effects of drugs were investigated, 30 min after intraperitoneal administration, as the change in thermal and mechanical hypersensitivity expressed as the mean ±

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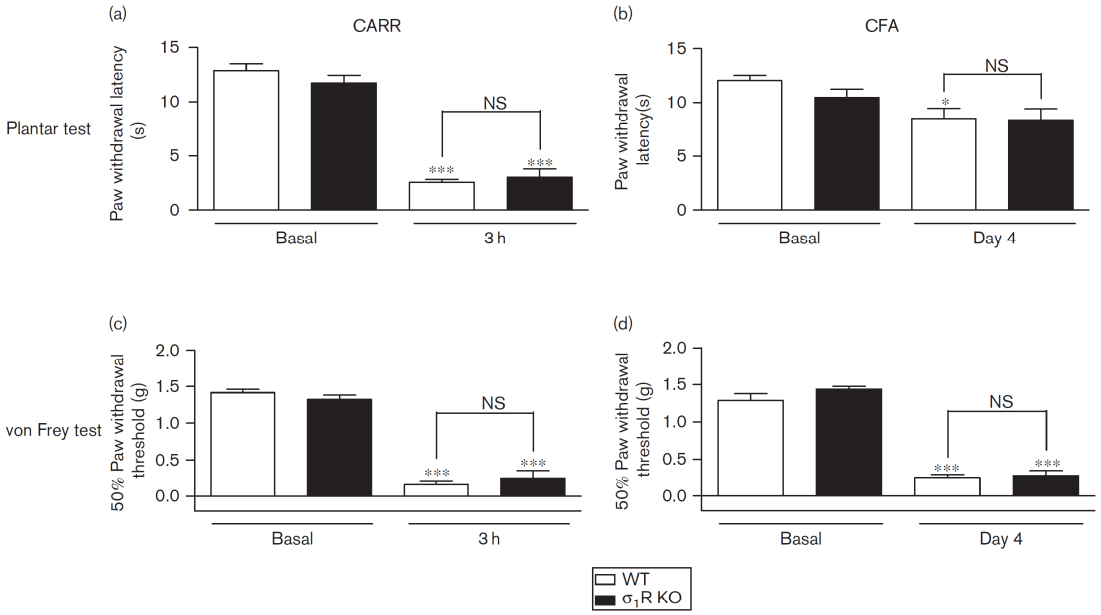
Fig. 2



Inflammation-induced thermal and mechanical hypersensitivity in σ_1 receptor knockout mice. No significant differences in pain sensitivity were observed between WT and σ_1R KO mice. Statistically significant differences as compared with the baseline values for each mouse type: ***$P<0.001$; *$P<0.05$ (one-way analysis of variance, followed by the Bonferroni test). Each point and vertical line represents the mean ± SEM of the values obtained in 8–18 animals. The results are an average of two independent experiments. CARR, carrageenan; CFA, complete Freund's adjuvant.

SEM (Table 1) and as the percentage of antihyperalgesic and antiallodynic effect, respectively (Fig. 3). On the basis of previous data obtained in time-course experiments, the effect of drugs on behavioural hypersensitivity was assessed 3 h (CARR) or 4 days (CFA) after an intraplantar injection of the proinflammatory agent. Administration of S1RA dose dependently inhibited CARR-induced thermal hyperalgesia with maximum efficacy ($E_{max} = 104.0 \pm 15.7\%$) (Fig. 3a). Morphine and ibuprofen, but not celecoxib ($E_{max} = 73.1 \pm 16.4\%$), also reached a maximum effect ($E_{max} = 102.2 \pm 4.3$ and $98.0 \pm 5.0\%$, respectively). The order of potency (ED_{50} values) of all tested drugs was as follows: morphine (2.0 ± 0.2 mg/kg) > ibuprofen (7.5 ± 1.0 mg/kg) > S1RA (27.9 ± 5.2 mg/kg) > celecoxib (47.5 ± 16.3 mg/kg).

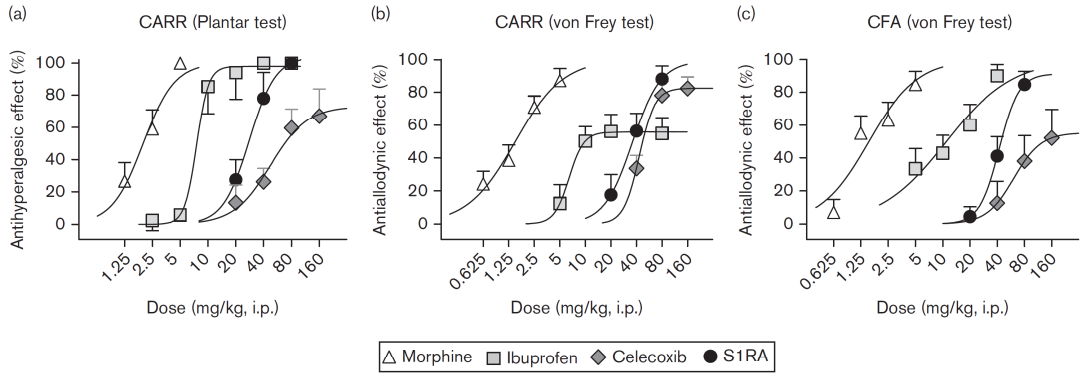
For CARR-induced mechanical allodynia, all analgesic drugs reversed, in a dose–response manner, the 50% withdrawal threshold. S1RA, morphine and celecoxib achieved the maximum possible effect ($E_{max} = 99.1 \pm 23.7$, 102.6 ± 4.4 and $82.6 \pm 7.6\%$, respectively). However, ibuprofen did not exert maximum efficacy ($E_{max} = 55.8 \pm 6.7\%$; Fig. 3b). The order of potency (ED_{50} values) was as follows: morphine (1.5 ± 0.2 mg/kg) > ibuprofen

Table 1 Hind paw withdrawal in response to punctate mechanical stimulation (g) and thermal-heat stimulus (s) in the CARR and CFA models of inflammatory pain expressed as the mean ± SEM for each drug and tested dose

		CARR		CFA
	Dose (mg/kg)	PT (s)	VF (g)	VF (g)
Morphine	0	2.2 ± 0.2	0.2 ± 0.0	0.4 ± 0.1
	0.625	ND	0.5 ± 0.1	0.5 ± 0.1
	1.25	4.9 ± 1.2	0.7 ± 0.1	1.0 ± 0.1
	2.5	8.3 ± 1.2	1.1 ± 0.1	1.1 ± 0.1
	5	13.3 ± 1.5	1.4 ± 0.1	1.4 ± 0.1
Ibuprofen	0	2.8 ± 0.2	0.4 ± 0.0	0.4 ± 0.1
	2.5	3.0 ± 0.6	ND	ND
	5	3.3 ± 0.4	0.5 ± 0.1	0.7 ± 0.1
	10	11.1 ± 1.6	1.0 ± 0.1	0.9 ± 0.1
	20	11.9 ± 1.6	1.0 ± 0.1	1.1 ± 0.1
	40	14.6 ± 1.1	ND	1.4 ± 0.1
	80	13.1 ± 1.0	1.0 ± 0.1	ND
Celecoxib	0	2.8 ± 0.2	0.2 ± 0.0	0.6 ± 0.1
	20	4.1 ± 1.1	ND	ND
	40	5.3 ± 0.8	0.7 ± 0.1	0.7 ± 0.1
	80	8.6 ± 1.1	1.2 ± 0.1	0.9 ± 0.1
	160	9.3 ± 1.7	1.3 ± 0.1	1.1 ± 0.2
S1RA	0	2.3 ± 0.3	0.2 ± 0.0	0.3 ± 0.0
	20	5.1 ± 1.3	0.4 ± 0.2	0.3 ± 0.1
	40	10.3 ± 1.6	0.9 ± 0.1	0.8 ± 0.1
	80	16.1 ± 1.0	1.4 ± 0.1	1.3 ± 0.1

CARR, carrageenan; CFA, complete Freund's adjuvant; ND, not determined; PT (s), plantar test latency (s); VF (g), von Frey filaments threshold (g).

Fig. 3



Effects of S1RA and reference compounds on mechanical allodynia and thermal hyperalgesia. All drugs dose dependently reversed thermal and mechanical hypersensitivity. Each point and vertical line represents the mean ± SEM of the values obtained in 6–10 animals. CARR, carrageenan; CFA, complete Freund's adjuvant.

(6.4 ± 1.3 mg/kg) > S1RA (35.9 ± 9.8 mg/kg) > celecoxib (43.3 ± 4.6 mg/kg).

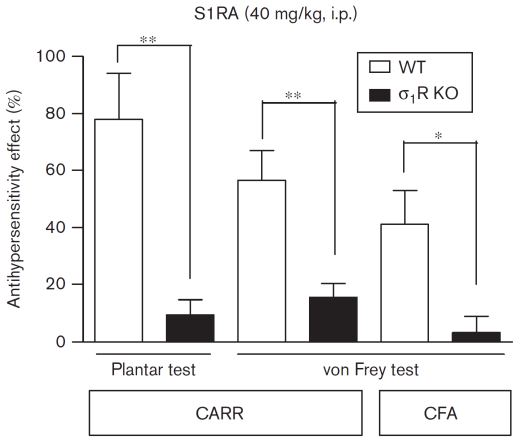
The results observed in CFA-treated mice are shown in Fig. 3c. All the drugs tested inhibited CFA-induced mechanical allodynia in a dose-dependent manner. All drugs reached maximum efficacy (E_{max} = 91.3 ± 18.6, 100.4 ± 5.5 and 106 ± 8.1% for S1RA, morphine and ibuprofen, respectively), except celecoxib, with a maximum percentage of antiallodynic effect of 55.3 ± 27.4%. The order of potency (ED_{50} values) matches that found in the CARR model: morphine (1.5 ± 0.2 mg/kg) > ibuprofen (11.1 ± 2.2 mg/kg) > S1RA (42.1 ± 7.1 mg/kg) > celecoxib (60.9 ± 38.0 mg/kg).

Effects of S1RA on thermal hyperalgesia and mechanical allodynia induced by CARR and CFA in σ_1R knockout mice

To explore whether the analgesic effects exerted by S1RA in both models were specifically mediated by σ_1R, the antiallodynic and antihyperalgesic effects of S1RA at 40 mg/kg (effective dose in WT) were tested in σ_1R KO mice and compared with WT mice (Fig. 4).

S1RA administered intraperitoneally at 40 mg/kg reversed the thermal (heat) and mechanical hypersensitivity induced by the intraplantar injection of CARR (efficacy = 78.0 ± 16.1 and 56.5 ± 10.8% for thermal and mechanical hypersensitivity, respectively; Fig. 4). However, the same dose of S1RA did not reduce thermal and mechanical hypersensitivity in σ_1R KO mice, and the differences versus the effect in WT mice were statistically significant: 78.0 ± 16.1% in WT mice (corresponding to 10.3 ± 1.6 s of paw withdrawal latency) versus 9.4 ± 5.2% in σ_1R mice (3.4 ± 0.5 s), t_{13} = 3.8, $P < 0.01$ and 56.5 ± 10.8% in WT mice (corresponding to 0.9 ± 0.1 g of paw withdrawal response) versus 15.5 ± 4.8% in σ_1R mice (0.4 ± 0.1 g), t_{14} = 3.5, $P < 0.01$, in the plantar test and the von Frey test, respectively. CFA-induced mechanical allodynia was also inhibited by S1RA at 40 mg/kg in WT mice, but not in σ_1R KO mice: 41.1 ± 11.9% in WT mice (0.8 ± 0.1 g) versus 3.3 ± 5.7% in σ_1R mice (0.3 ± 0.1 g), t_{13} = 2.7, $P < 0.02$.

Fig. 4



Antihypersensitivity effect of S1RA in the absence of sigma-1 receptor (σ_1R) by genetic inactivation. S1RA reversed both mechanical allodynia and thermal hyperalgesia with statistically significant differences between mouse types: $^{**}P < 0.01$; $^{*}P < 0.05$ (*t*-test). Each point and vertical line represents the mean ± SEM of the values obtained in 7–8 animals. CARR, carrageenan; CFA, complete Freund's adjuvant.

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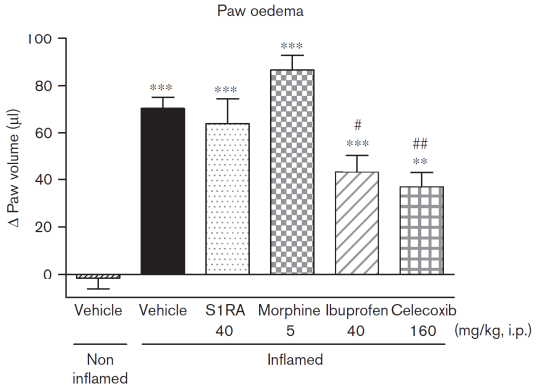
Effect of S1RA and reference compounds on CARR-induced paw oedema

CARR induced a robust increase in paw volume (~70 µl) 3 h after injection ($F_{5,74} = 21.2$, $P < 0.001$, ANOVA; $P < 0.001$ and $P < 0.01$ for comparison between inflamed vs. noninflamed; Fig. 5). As expected, oedema was significantly reduced by preventive treatment (30 min before CARR injection) with the NSAIDs ibuprofen and celecoxib. In contrast, paw volume was not modified by treatment with S1RA or morphine ($P > 0.05$ vs. vehicle).

Discussion

In this study, the analgesic efficacy of the selective σ_1R antagonist S1RA was investigated in acute and chronic inflammatory pain models induced by an intraplantar injection of CARR and CFA to mice. S1RA exerted antinociceptive effects in both experimental models of inflammatory pain. Administration of S1RA failed to reduce CARR-induced paw oedema, thus suggesting that the reduction of thermal and mechanical hypersensitivity elicited by S1RA does not involve major anti-inflammatory action. Finally, S1RA-induced analgesia was abolished in σ_1R knockout mice, thus showing the in-vivo σ_1R-mediated specificity of this compound. CARR and CFA are among the most commonly used substances to induce inflammatory pain in animal models. Because both agents produce chemical sensitization and inflammation that can be pharmacologically inhibited by analgesics, they are used to predict the efficacy of new analgesics for the treatment of inflammatory pain conditions (Bendele, 2001). Our results showed that CARR induced a transient acute inflammatory state, characterized by both mechanical and thermal hypersensitivity, that peaked at 3–5 h and reverted to baseline levels 3–4 days after injection. In contrast, CFA induced a robust and long-lasting mechanical hypersensitivity (not returning to baseline levels until day 11), but no or only mild heat hypersensitivity. This is in agreement with previous studies carried out under similar experimental conditions (i.e. injection of 20 µl of 1 mg/ml CFA solution) where CFA-induced thermal hyperalgesia developed to a lesser extent than mechanical allodynia (Bellavance and Beitz, 1996; Almarestani *et al.*, 2011) or even failed to develop, depending on the mouse strain (Liang *et al.*, 2006). Accordingly, multiple studies support differences in the outcome of the animal model depending on the nature of the painful stimuli (i.e. thermal vs. mechanical) (Bennet *et al.*, 2000; Mansikka *et al.*, 2000; Schepers *et al.*, 2008). Finally, it is worth emphasizing that mechanical and thermal thresholds of the contralateral paw were stable across time (from the beginning to the end of experimental measurements), thus ruling out the possibility of sensitization because of successive nociceptive stimulation in the time-course experiments (Casarrubea *et al.*, 2011; Rahn *et al.*, 2013). Altogether, data obtained in this study are consistent with previous studies in mice and rats (Bellavance and Beitz, 1996; Sammons *et al.*, 2000; Molina and Herrero, 2006; Almarestani *et al.*, 2011), thus suggesting that CARR produces an acute inflammatory state whereas the effect produced by CFA is related more to chronic inflammatory pain conditions.

Fig. 5



Effect of S1RA, morphine, ibuprofen and celecoxib on paw oedema induced by CARR. All CARR-injected mice showed an increase in the paw volume vs. noninflamed paws (vehicle treated): ***P<0.001; **P<0.01 (one-way ANOVA, followed by the Bonferroni test). Celecoxib and ibuprofen partially reversed paw oedema with statistically significant differences vs. the vehicle-treated inflamed group: ##P<0.01; #P<0.05 (one-way ANOVA, followed by the Bonferroni test). Each point and vertical line represents the mean ± SEM of the values obtained in 9–22 animals. ANOVA, analysis of variance.

Mechanical thresholds and thermal latencies were similar in naïve WT and σ_1R KO mice, which indicates that KO mice perceive and respond normally to mechanical and thermal stimuli. This is in agreement with previous studies, where the absence of σ_1R in KO animals has been shown not to interfere with the perception of mechanical and thermal (heat and cold) stimuli applied to the hind paw or with the motor response required for paw withdrawal (Cendán *et al.*, 2005; De la Puente *et al.*, 2009; Nieto *et al.*, 2012; Romero *et al.*, 2012; González-Cano *et al.*, 2013). However, unlike other experimental pain models (formalin, capsaicin and neuropathic pain models), where σ_1R KO mice showed a 'pain-resistant' phenotype (i.e. nociceptive behaviours did not develop or were inhibited vs. WT) (Cendán *et al.*, 2005; De la Puente *et al.*, 2009; Entrena *et al.*, 2009; Nieto *et al.*, 2012), the genetic inactivation of σ_1R did not prevent the acquisition of CARR-induced and CFA-induced pain-related behaviours. Similarly, σ_1R KO mice exposed to partial sciatic nerve ligation showed fully developed heat hypersensitivity, although mechanical allodynia was blocked and cold allodynia was attenuated (De la Puente *et al.*, 2009). As different injuries are known to recruit different pain pathways and mechanisms (Lee *et al.*, 1998;

Dowdall *et al.*, 2005; Li *et al.*, 2013), the different phenotypes observed throughout the various models suggest a distinct involvement of the σ_1R system in the mechanisms underlying hypersensitivity, depending on the pain model and the readout.

The selective σ_1R antagonist S1RA inhibited pain behaviours in mice rendered arthritic following intraplantar CFA or CARR injection. This confirms and extends the spectrum of analgesic activity shown in previous studies, where S1RA was effective in a broad range of acute and chronic pain models (Nieto *et al.*, 2012; Romero *et al.*, 2012; González-Cano *et al.*, 2013). S1RA achieved full efficacy in both pain models, with an ED_{50s} of 27.9, 35.9 and 42.1 mg/kg for CARR-induced thermal hyperalgesia, CARR-induced mechanical allodynia and CFA-induced mechanical allodynia, respectively. These ED_{50} values are well within the range of doses active in other pain models, including chemical sensitization with capsaicin (ED_{50} = 26 mg/kg) or formalin (ED_{50} = 40 mg/kg) and neuropathic pain induced by partial sciatic nerve ligation (ED_{50} = 23 mg/kg) (Romero *et al.*, 2012). To assess the in-vivo selectivity of this compound and to unambiguously attribute the antinociceptive effect of S1RA to σ_1R, we tested whether S1RA shows activity in the absence of its putative target (Petrus *et al.*, 2007; González-Cano *et al.*, 2013; Vidal-Torres *et al.*, 2013). To this end, we took advantage of the fact that σ_1R KO mice show fully developed behavioural hypersensitivity after CARR and CFA. We found that S1RA was devoid of antiallodynic and antihyperalgesic effects in mice lacking σ_1Rs, which suggests that the antinociceptive effect of S1RA in acute (CARR) as well as chronic (CFA) inflammatory pain models is actually mediated by σ_1R. The high selectivity of this compound, which shows no interaction with a wide variety of putative pain as well as inflammation-related targets such as COX-1, COX-2, cytokines, prostanoid and steroid receptors, leukotrienes and others, also supports this conclusion (Romero *et al.*, 2012).

The pharmacological blockade of hypersensitivity to thermal and mechanical stimuli by S1RA in the CARR and CFA models suggests that σ_1R modulates inflammatory pain. However, the finding that σ_1R KO mice normally develop CARR-induced and CFA-induced pain-related behaviours would seem contradictory and deserves discussion. The absence of the modulatory system (as in KO mice) precludes the regulation by ligands, but does not mimic the modulatory (inhibiting) effect elicited by an antagonist acting on σ_1R. This can be explained by the chaperone nature of σ_1R, which exerts its action by physical protein–protein interactions. Accordingly, the absence of the regulatory mechanism in KO mice is not equivalent to the decrease or gain of function promoted by a σ_1R ligand (i.e. S1RA) through conformational changes relating to and affecting the activity of the target protein with which σ_1R interacts. With respect to the intrinsic activity of S1RA, in-vitro data derived from the use of phenytoin confirmed its antagonistic nature. Phenytoin shifts known σ_1R agonists (e.g. dextromethorphan, PRE-084) to significantly higher affinities, whereas σ_1R antagonists (e.g. haloperidol, BD-1063, NE-100) show no shift or a small shift to lower-affinity values (Cobos *et al.*, 2005; Nahas *et al.*, 2008). S1RA produced a small shift to lower-affinity values when incubated in the presence of phenytoin, which indicated antagonist properties at the σ_1R (Romero *et al.*, 2012).

For thermal hyperalgesia (CARR model), the efficacy of S1RA was similar to that of celecoxib and ibuprofen. For mechanical allodynia, S1RA was more effective than ibuprofen in the CARR model and more effective and potent than celecoxib in the CFA model. Morphine was the most potent analgesic in both inflammatory pain models. These results are in agreement with previous data reporting increased paw withdrawal latencies to heat and mechanical stimuli after systemic administration of opioids in the same animal models (Luger *et al.*, 2002; Lähdesmäki *et al.*, 2003; Bileviciute-Ljungar *et al.*, 2006; Liang *et al.*, 2006; Ortiz *et al.*, 2007). The nonselective COX inhibitor ibuprofen showed higher potency than the selective COX-2 inhibitor celecoxib in both inflammatory models. Moreover, ibuprofen showed higher efficacy than celecoxib in the inhibition of CARR-induced thermal hyperalgesia and CFA-induced mechanical allodynia. Studies using CFA but evaluating a different behavioural outcome have shown that celecoxib is less effective than other NSAIDs (Cobos *et al.*, 2012). However, in the present study, celecoxib dose dependently reversed CARR-induced mechanical hypersensitivity more effectively than ibuprofen. Accordingly, limited efficacy of ibuprofen on CARR-induced and CFA-induced mechanical hypersensitivity has been reported (Gould *et al.*, 2004; Rutten *et al.*, 2011).

Finally, prophylactic treatment with S1RA did not reduce CARR-induced paw oedema. As expected, ibuprofen and celecoxib, but not morphine, elicited a significant inhibition of oedema volumes of CARR-injected paws in the same experiments. The lack of anti-inflammatory actions by S1RA indicates that the reduction of hypersensitivity to thermal and mechanical stimuli elicited by antagonizing σ_1Rs is not secondary to an anti-inflammatory effect, but rather results from nociceptive inhibition.

Celecoxib (a selective COX-2 inhibitor) and ibuprofen (a COX-1 and COX-2 inhibitor) were selected as positive controls from a therapeutic class used widely in the clinical management of inflammatory pain. Both COX inhibitors exert potent anti-inflammatory and analgesic effects. However, their COX-inhibiting properties underlie their ulcerogenic potential and cardiovascular risk, which represent a limitation to their therapeutic use. This is of particular importance in arthritic conditions requiring

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chronic treatments, and it has been reported that patients with rheumatoid arthritis are more prone to NSAID-induced gastropathy than other NSAID users (Dijkmans *et al.*, 1995). On this basis, the discovery of drugs with efficacy similar to COX inhibitors but with a better safety profile could be of great clinical value for arthritic patients. Weak opioids such as codeine or tramadol may be effective in the short-term management of inflammatory pain, but the development of tolerance to the analgesic effect, as well as significant and common adverse effects, leads to consideration of alternative painkillers for these patients (Whittle *et al.*, 2013). Safety studies carried out following administration of S1RA to animals showed a good profile (Romero *et al.*, 2012; Vidal-Torres *et al.*, 2013). Furthermore, S1RA has successfully completed single-dose and multiple-dose phase I clinical studies in healthy volunteers, with good safety and tolerability profiles (Abadias *et al.*, 2013), and it is currently undergoing phase II clinical trials.

Conclusion

Acute pretreatment of mice with S1RA produced a full antinociceptive effect in two well-characterized models of acute and chronic inflammatory (CARR and CFA) pain when evaluated by mechanical and heat hypersensitivity in the hind paw. The analgesic activity induced by S1RA was clearly mediated by the interaction with σ_1R and was not because of a reduction in paw oedema. These results support that σ_1R antagonists – particularly S1RA – could be an effective treatment for pain in conditions with underlying inflammatory mechanisms, as an alternative or complementary to NSAIDs or opioids.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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2.2. Article 3: *"Sigma-1 receptor and inflammatory pain".*

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Georgia Gris, Enrique José Cobos, Daniel Zamanillo and Enrique Portillo-Salido.

Department of Pharmacology, Drug Discovery & Preclinical Development, ESTEVE Barcelona, Spain.

Note that this publication is a commentary, a bibliographic review of the topic sigma-1 receptor in inflammatory pain, without adding new experimental results. Therefore, we have considered not necessary to summarize it.

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Inflammation Research

COMMENTARY

Sigma-1 receptor and inflammatory pain

Georgia Gris[1] · Enrique José Cobos[2] · Daniel Zamanillo[1] · Enrique Portillo-Salido[1]

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Abstract
Introduction The sigma-1 receptor (Sig-1R) is a unique ligand-regulated molecular chaperone that interacts with several protein targets such as G protein-coupled receptors and ion channels to modulate their activity. Sig-1R is located in areas of the central and peripheral nervous system that are key to pain control. Previous preclinical studies have suggested a potential therapeutic use of Sig-1R antagonists for the management of neuropathic pain.
Discussion Recent studies using pharmacological and genetic tools have explored the role of Sig-1R in inflammatory pain conditions. Mice lacking the Sig-1R have shown different patterns of phenotypic responses to inflammatory injury. Systemic or peripheral administration of several Sig-1R antagonists, including the selective Sig-1R antagonist S1RA, inhibited both mechanical and thermal hypersensitivity in several preclinical models of inflammatory pain. These recent studies are summarized in the present commentary.
Conclusion Central and peripheral pharmacological blockade of Sig-1R could be an effective option to treat inflammatory pain.

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✉ Enrique Portillo-Salido
eportillo@esteve.es

[1] Drug Discovery and Preclinical Development, ESTEVE, Baldiri Reixach, 4-8, 08028 Barcelona, Spain

[2] Department of Pharmacology and Institute of Neuroscience, Faculty of Medicine, University of Granada, Granada, Spain

Keywords Sigma-1 receptor · Inflammatory pain · Analgesic · Arthritis · Carrageenan · Complete Freund's adjuvant

Introduction

The sigma-1 receptor (Sig-1R) is a unique ligand-regulated target class with chaperoning functions over different molecular targets. In its role as a molecular chaperone, Sig-1R modulates downstream signalling pathways activated upon the stimulation of other systems [1, 2]. The function of Sig-1R can be modulated by agonists and antagonists that differentially affect the ability of Sig-1R to interact with different proteins. It has been postulated that Sig-1R ligands have no effects by themselves, but are able to modulate signalling pathways under pathological conditions [1]. Sig-1R is distributed in peripheral organs and in different areas of the central nervous system (CNS) involved in memory, emotions, and sensory and motor functions [3]. In particular, it is expressed in key areas for pain control, such as the dorsal root ganglia, the superficial layers of the dorsal horn, the periaqueductal grey matter, the locus coeruleus, and the rostroventral medulla [4, 5]. Sig-1R was first linked to analgesia by Chien and Pasternak 20 years ago. These authors described Sig-1R as an endogenous anti-opioid system because they found that Sig-1R agonists counteracted opioid receptor-mediated analgesia, while Sig-1R antagonists potentiated it [6]. At the preclinical level, several recent studies using knockout (KO) mice (genetic approach) and selective Sig-1R antagonists (pharmacological approach) have suggested a potential use of Sig-1R antagonists for the treatment of (particularly, neuropathic) pain in the absence of opioids [2]. Indeed, the selective Sig-1R antagonist S1RA is

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currently undergoing phase II clinical trials in neuropathic pain patients. Different laboratories using similar preclinical studies have recently added a further piece to the puzzle by looking at inflammatory pain conditions [7–10].

Insights from Sig-1R knockout mice

The generation of a Sig-1R KO mouse was a key milestone in establishing the role of Sig-1R in pain modulation. Behavioural baseline responses of normal, unlesioned Sig-1R KO mice to mechanical and thermal stimuli were found to be undistinguishable from those of wild-type (WT) mice, thus suggesting that normal sensory thresholds are not affected by the absence of the gene. Sig-1R KO mice, however, exhibited a reduced pain phenotype under some pain conditions. Pain behaviours elicited by intraplantar (i.pl.) administration of formalin and capsaicin are attenuated in Sig-1R KO mice [11, 12]. In the case of neuropathic pain, either induced by sciatic nerve ligation or after treatment with the antineoplastic agent paclitaxel, cold and mechanical allodynia did not develop or were strongly attenuated in Sig-1R KO mice [13, 14]. In contrast, thermal (heat) hyperalgesia developed to the same extent as in WT mice following nerve injury [13] (Table 1).

New studies have evaluated the role of Sig-1R receptors in inflammatory pain [7–10]. Inflammatory pain can be induced experimentally by i.pl. injection of agents such as carrageenan (CARR) or complete Freund's adjuvant (CFA) and is behaviourally evidenced by both mechanical and thermal (heat) hypersensitivity. CARR produces an acute inflammatory state, whereas the effect produced by CFA better models chronic inflammatory pain conditions [15]. Unlike neuropathic pain models without mechanical allodynia, the genetic inactivation of Sig-1R failed to prevent the development of CARR-induced and CFA-induced mechanical allodynia when von Frey filaments were applied [7]. In contrast, pain-like responses evoked by a blunt mechanical stimulus (different from the "allodynic" punctate stimulation elicited by von Frey filaments) were inhibited in the CARR-sensitized Sig-1R KO mice [10]. Finally, Sig-1R KO mice developed heat hyperalgesia to the same extent as WT mice in the CARR-induced acute inflammation model (Table 1). Taken together, these data indicated that the lack of Sig-1R did not impact enough on the development of behavioural hypersensitivity induced by peripheral inflammation. Because neuropathic and inflammatory pain is known to involve different pathways, the different phenotypes observed in Sig-1R KO mice suggest—depending on the pain model and the readout—a distinct involvement of the Sig-1R system in the mechanisms underlying hypersensitivity [7].

Insights from selective Sig-1R antagonists

Many studies in the Sig-1R field led to conclusions that were often blurred by the non-selective nature of many of the compounds used [16, 17]. The recent discovery of S1RA (E-52862), a selective Sig-1R antagonist, is a step forward in studying the role of Sig-1R in nociception and pain [18]. S1RA shows high affinity for Sig-1R (Ki = 17 nM) and a good Sig-1R/Sig-2R selectivity ratio (>550). Moreover, it is selective over a panel of 170 molecular targets. It behaves as an antagonist, penetrates the blood–brain barrier, and binds to Sig-1R in the CNS [18]. As a practical in vivo proof of Sig-1R selectivity and

Table 1 Summary of pain behaviours shown by Sig-1R KO mice in several models of neuropathic and inflammatory pain

Pain type	Animal model	Readout	Method (units)	Sig-1R KO response	References
Neuropathic pain	Partial sciatic nerve ligation-induced neuropathy	Mechanical allodynia	von Frey test (pressure, g)	Attenuation	De la Puente et al. [13]
		Thermal (cold) allodynia	Cold plate test (score)	Attenuation	
		Thermal (heat) hyperalgesia	Plantar test (latency, s)	Similar to WT	
	Chemotherapy (paclitaxel)-induced neuropathy	Mechanical allodynia	von Frey test (pressure, *g*)	Attenuation	Nieto et al. [14, 19]
		Thermal (cold) allodynia	Acetone test (latency, s)	Attenuation	
Inflammatory pain	Carrageenan-induced acute inflammation	Mechanical allodynia	von Frey test (pressure, *g*)	Similar to WT	Gris et al. (2014)
		Thermal (heat) hyperalgesia	Plantar test (latency, s)	Similar to WT	Gris et al. [7]; Tejada et al. [10]
		Mechanical hyperalgesia	Paw pressure test (latency, s)	Attenuation	Tejada et al. [10]
	CFA-induced chronic inflammation	Mechanical allodynia	von Frey test (pressure, *g*)	Similar to WT	Gris et al. [7]

Sigma-1 receptor and inflammatory pain

Table 2 Summary of the effects of pharmacological blockade of Sig-1R on different end points in inflammatory pain models

Pain type	Readout	Method (units)	Animal model	Treatment protocol	Sig-1R antagonist	Effect	References
Inflammatory pain	Mechanical allodynia	von Frey test (pressure, *g*)	Carrageenan (2.5 %, 50 µl) in male mice	Curative	S1RA (i.p.)	Attenuation	Gris et al. [7]
			CFA (1 mg/ml, 20 µl) in male mice	Curative	S1RA (i.p.)	Attenuation	
			Carrageenan (2 %, 100 µl) in male rats	Preventive	(−)-MRV3 and (+)–MR200 (s.c.)	Attenuation	Parenti et al. [8, 9]
	Mechanical hyperalgesia	Paw pressure test (latency, s)	Carrageenan (1 %, 50 µl) in female mice	Curative	BD-1063 and S1RA (s.c. and i.pl.)	Attenuation	Tejada et al. [10]
	Thermal (heat) hyperalgesia	Plantar test (latency, s)	Carrageenan (2.5 %, 50 µl) in male mice	Curative	S1RA (i.p.)	Attenuation	Gris et al. [7]
			Carrageenan (1 %, 50 µl) in female mice	Curative	S1RA (s.c. and i.p.)	Attenuation	Tejada et al. [10]
			Carrageenan (2 %, 100 µl) in male rats	Preventive	(−)-MRV3 and (+)–MR200 (s.c.)	Attenuation	Parenti et al. [8, 9]
	Paw oedema	Plethysmometer (volume, µl)	Carrageenan (2.5 %, 50 µl) in male mice	Preventive	S1RA (i.p.)	No effect	Gris et al. [7]
			Carrageenan (1 %, 50 µl) in female mice	Preventive	BD-1063 and S1RA (s.c.)	No effect	Tejada et al. [10]
			Carrageenan (2 %, 100 µl) in male rats	Preventive	(−)-MRV3 and (+)–MR200 (s.c.)	Attenuation	Parenti et al. [8, 9]

CFA complete Freund's adjuvant, *i.p.* intraperitoneal, *s.c.* subcutaneous, *i.pl.* intraplantar

unequivocal involvement in its antinociceptive effects, Gris et al. [7] took advantage of the fact that Sig-1R KO mice developed behavioural hypersensitivity in inflammatory pain models and found that S1RA was devoid of antiallodynic and antihyperalgesic effects in mice lacking the Sig-1R. In turn, Tejada et al. [10] proved the in vivo selectivity of S1RA (and BD1063, another known Sig-1R antagonist) by means of a pharmacological strategy, i.e., using the Sig-1R agonist PRE-084 to revert its antihyperalgesic effects.

Systemically administered S1RA provided efficacy similar to that of ibuprofen and celecoxib in inhibiting inflammation-induced (CARR or CFA) hypersensitivity. Unlike anti-inflammatory agents, its activity was purely antinociceptive—the CARR-induced oedema remained unaffected in Sig-1R KO mice or after treatment with S1RA or BD1063 in WT mice [7, 10]. Interestingly, systemically administered Sig-1R antagonists inhibited all pain-related behaviours evaluated in WT mice, including those developed by Sig-1R KO mice (mechanical allodynia and thermal hyperalgesia) (Table 1). This brings out the difference between the effect of genetics (i.e., the absence of the receptor and adaptive changes) and the pharmacological blockade of Sig-1R (i.e., the modulatory effect of a ligand at the time of the test) [2].

By administering other Sig-1R antagonists such as MRV3 and (+)–MR200 before CARR i.pl. injection (preventive protocol), Parenti et al. [8, 9] also showed a dose-dependent inhibition of mechanical allodynia and thermal hyperalgesia. They, however, found a reduction of paw oedema which was not observed in either the Gris or the Tejada studies (Table 2). An effect of MRV3 and (+)–MR200 on molecular targets other than Sig-1R—by modulating inflammatory reactions—cannot be ruled out. To confirm this possibility, the selectivity profile of MRV3 and (+)–MR200 should be thoroughly investigated. Alternatively, whether these drugs do or do not maintain their antioedematous activity in Sig-1R KO mice could also be studied. If these drugs still had an effect even in the absence of their purported pharmacological target, off-target effects responsible for the anti-inflammatory effect should be considered.

Thus, all the above results suggest that the use of selective Sig-1R antagonists could be a valid approach for the treatment of inflammatory pain, thereby extending the spectrum of analgesic activity shown for this drug class in previous studies.

Insights from the periphery

Increasing evidence suggests that activity from the periphery is essential to not only initiate, but also maintain painful symptoms. Targeting the peripheral nervous system would overcome the typical side effects related to central nervous system actions. While the role of peripheral Sig-1R on pain modulation has not been extensively studied

[10], the fact that the inhibition of peripheral Sig-1R potentiates opioid antinociception was recently reported. The peripheral role of Sig-1R is also supported by its high density in peripheral nervous tissues such as dorsal root ganglia [19]. The peripheral mechanism for Sig-1R modulation in inflammatory pain has been recently addressed by Tejada et al., who first described the role played by peripheral Sig-1R in inflammatory pain conditions in vivo and brought out the possibility of targeting peripheral Sig-1R to ameliorate inflammatory hyperalgesia. These authors used a behavioural approach where i.pl. administration of Sig-1R agonist PRE-084 abolished the systemic antinociceptive effect of selective Sig-1R antagonists in the CARR pain model. Moreover, i.pl. administration of S1RA in the inflamed paw was sufficient to completely reverse hyperalgesia.

Previous studies focussing on neuropathic pain have shown that the inhibition of Sig-1R leads to decreased amplification of pain signalling within the spinal cord (central sensitization) [13, 18]—this supporting the use of centrally acting Sig-1R compounds. Today, recent data of the study by Tejada et al. [10] in inflammation-induced pain suggest that the effects exerted by Sig-1R antagonists at the periphery contribute to their antinociceptive effect, and perhaps not only in inflammatory conditions but also in other pain conditions involving peripheral sensitization, including neuropathic pain.

Underlying anti-inflammatory pain mechanisms of Sig-1R

Inflammatory pain is characterized by a pronounced enhancement of nociceptor responsiveness (peripheral sensitization) in response to mediators released at the inflammation site. Due to its pleiotropic chaperoning nature Sig-1R could, by acting downstream to the activation of different receptors and channels, modulate the intracellular signalling of a variety of algesic mediators. In fact, bradykinin and nitric oxide (NO) are key mediators released during inflammation that contributes to peripheral sensitization [20] and Sig-1R activation is known to enhance both bradykinin-induced Ca^{2+} signalling [21] and NO signalling [22]. In addition, pain sensitization after peripheral inflammation involves plastic changes mediated by an increase in spinal excitatory neurotransmission together with activation of kinases, including $ERK_{1/2}$, which are known to be modulated by Sig-1R [13, 23–27]. The antinociceptive effects induced by Sig-1R antagonists could be reasonably attributed to an inhibition of such intracellular cascades.

Final remarks

Sig-1R is being cited as an example of great interest from a translation perspective and because it represents a potentially new therapeutic approach to pain management [28]. In particular, a rather compelling role for Sig-1R in neuropathic pain has been suggested by preclinical models of several different forms of pain [2]. Now, the systemic effects of several structurally different compounds including S1RA, a selective compound for this receptor, have been tested in preclinical models of acute and chronic inflammatory pain with positive results [7–10]. The analgesic effect in the inflammatory pain models was also observed after local application of selective Sig-1R antagonists to inflamed tissue, which suggests a role for Sig-1R in peripherally mediated analgesia. Thus, both central and peripheral pharmacological blockade of Sig-1R could be an effective option to treat inflammatory pain.

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2.3. Annex 1: *"Spinal modulation of pain-related molecular markers by genetic inactivation of σ_1R in inflammatory models".*

Annex 1 details complementary experiments that support and extend some of the conclusions obtained from Articles 2 and 3. Article 2 described for the first time the role of σ_1R in inflammatory pain using either genetic (σ_1R KO mice) or pharmacological (E-52862) tools. Article 3 reviewed these results and other recent data published on inflammatory pain and σ_1R, and compared them with previous results published on neuropathic pain.

Changes in the spinal cord (SC) were addressed here, as it is a prime site of action for analgesia where σ_1R plays an important role by facilitating central sensitization mechanisms both electrophysiologically (wind-up phenomenon) and biochemically (pERK enhancement) (De la Puente *et al.*, 2009; Nieto *et al.*, 2012; Romero *et al.*, 2012). The spinal expresion of several key molecular markers was specifically studied by using the inflammatory pain models induced by intraplantar injection of carrageenan and CFA in WT and σ_1R KO mice. The evaluation of the spinal expression was performed 3 h and 4 days following injection of carrageenan and CFA, respectively (Article 2), using two different approaches:

- Study of the spinal expression of pERK by western blot.

- Study of the spinal expression of c-Fos, GFAP, nNOS, SP and NPY by immunohistochemistry.

Study of the spinal expression of pERK by western blot.

Several studies have reported the involvement of σ_1R in the activation of the extracellular signal-regulated kinase (ERK) in the SC sensitization process in neuropathic pain models such as chronic constriction compression of the DRG, partial sciatic nerve ligation, and paclitaxel-induced neuropathic pain (De la Puente *et al.*, 2009; Son and Kwon, 2010; Nieto *et al.*, 2012). In particular, ERK phosphorylation within the SC has been associated with mechanical and cold allodynia in animal models of neuropathic pain: ERK phosphorylation in the SC was found in neuropathic WT but not σ_1R KO mice, and σ_1R KO mice exhibited reduced cold allodynia and did not develop mechanical allodynia as compared to WT mice (De la Puente *et al.*, 2009; Nieto *et al.*, 2012) (Table 1).

The **objective** of this part of the present Annex was to investigate whether the changes in the activation (phosphorylation) of spinal ERK related to both acute and chronic inflammatory pain models are affected in σ_1R KO mice.

The **experimental approach** was basically performed according to Nieto *et al.*, 2012, with minor modifications as detailed in the Methods section. Sample processing was performed separately for WT and σ_1R KO mice.

Western blot experiments using antibodies against total ERK and phosphorylated ERK (pERK) identified two bands with molecular weights of 44 and 42 KDa , which correspond to the ERK subunits 1 and 2, respectively. As expected, the GAPDH antibody identified a band with a molecular weight of 37 KDa. pERK immunodetection in SC was low in baseline (naïve) conditions, as previously described (Borges *et al.*, 2015), but it was higher in WT than in σ_1R KO mice, which could reflect actual differences in the baseline threshold for ERK activation but methodological differences due to separate processing of WT and σ_1R KO mice

samples cannot be discarded. No significant changes in the intensity of pERK immunoreactive bands were found in carrageenan- and CFA-treated mice as compared to the control (naïve) group in both mouse types (WT and σ_1R KO) (Fig. 1A and 1B). Therefore, the pain-related hypersensitivity observed in WT mice 3 hours or 4 days after carrageenan and CFA injection, respectively, was not accompanied by a selective increase of ERK phosphorylation within SC at the time of behavioural evaluation. As mentioned above, the phosphorylation of ERK is a key process involved in the sensitization of pain pathways, and the increased pERK levels in the dorsal SC during neuropathy were attenuated by σ_1R inhibition. However, no apparently increased pERK levels in the dorsal horn of the SC were observed after either carrageenan or CFA administration. This not only suggests the involvement of different mechanisms in the sensory hypersensitivity of experimental models of inflammatory and neuropathic pain, but also in the different mechanisms in which σ_1R is involved (Table 1 and Figure 2) (Gris *et al.*, 2015).

We hypothesized that spinal pERK is up-regulated in neuropathic pain conditions in WT mice and that the lack of σ_1R in KO mice prevents such an increase in pERK and attenuates the development of mechanical and cold allodynia (De la Puente *et al.*, 2009). This is supported by studies showing that spinal up-regulation of pERK and mechanical hypersensitivity in neuropathic animals (e.g., streptozocin-induced diabetic neuropathy) are both suppresed by spinal inhibition of ERK phosphorylation (e.g., intrathecal administration of the selective MAPK/ERK-kinase inhibitor PD 198306) (Ciruela *et al.*, 2003). In contrast, these new results suggest that pERK at the SC might not be a key mediator in the modulation by σ_1R of pain hypersensitivity either in acute or chronic inflammatory processes, which is consistent

with the observation that σ_1R KO mice develop mechanical allodynia to the same extent as WT mice (Figure 2 and Table 1).

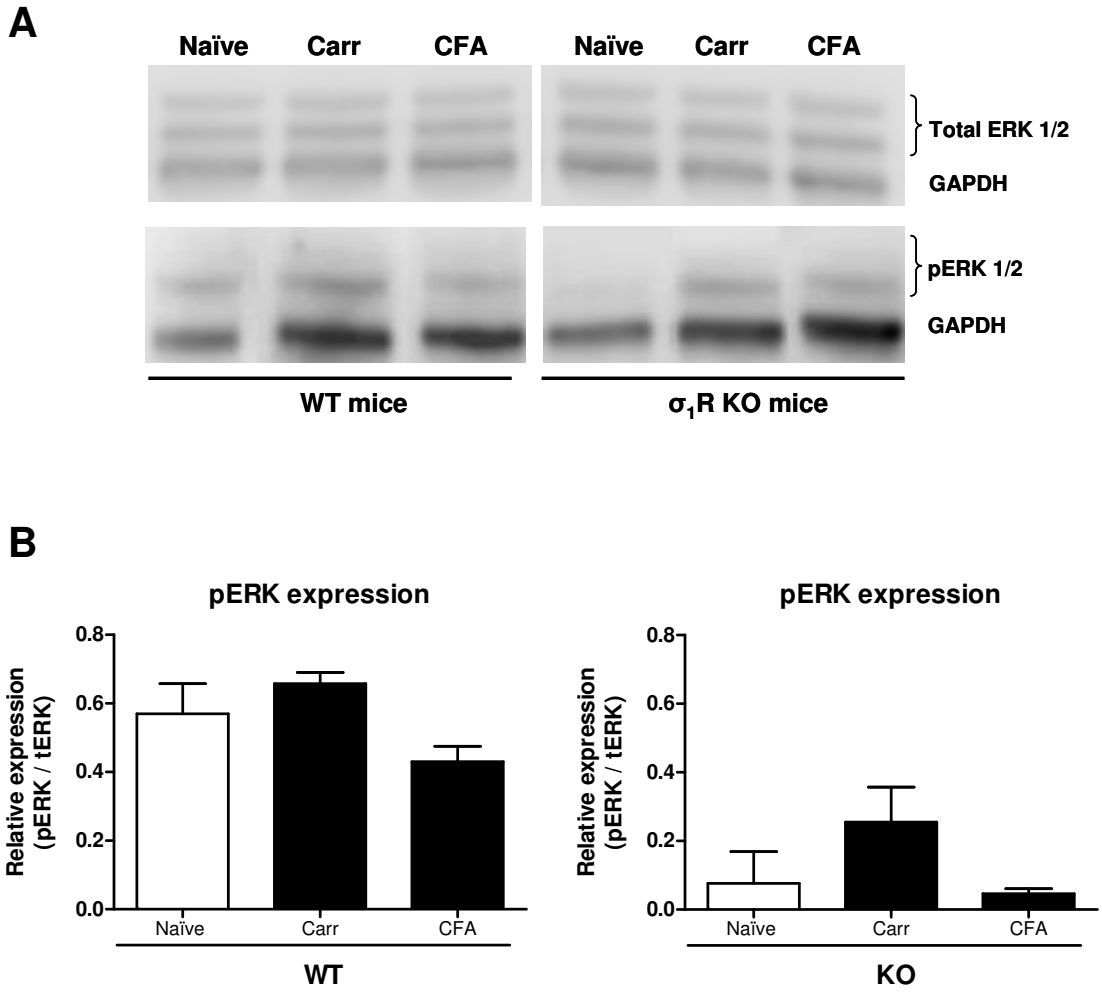

Fig. 1. Total ERK and pERK expression in the ipsilateral dorsal horn of the SC secondary to carrageenan and CFA intraplantar injection in WT and σ_1R KO mice. (A) Representative immunoblots of total ERK and pERK in WT and σ_1R KO mice. GAPDH was used as loading control. (B) Quantification of immunoblotting for pERK in WT and σ_1R KO mice. The intensity of pERK bands was relativized to those of their corresponding loading control GAPDH bands, and then normalized respect to the mean control group intensity. pERK was finally normalized to total ERK protein. No significant differences *versus* corresponding control group (one-way ANOVA followed by Bonferroni test) were found. Each bar and vertical line represents the mean ± SEM of the densitometric values obtained from 3 pools of 4 animals each.

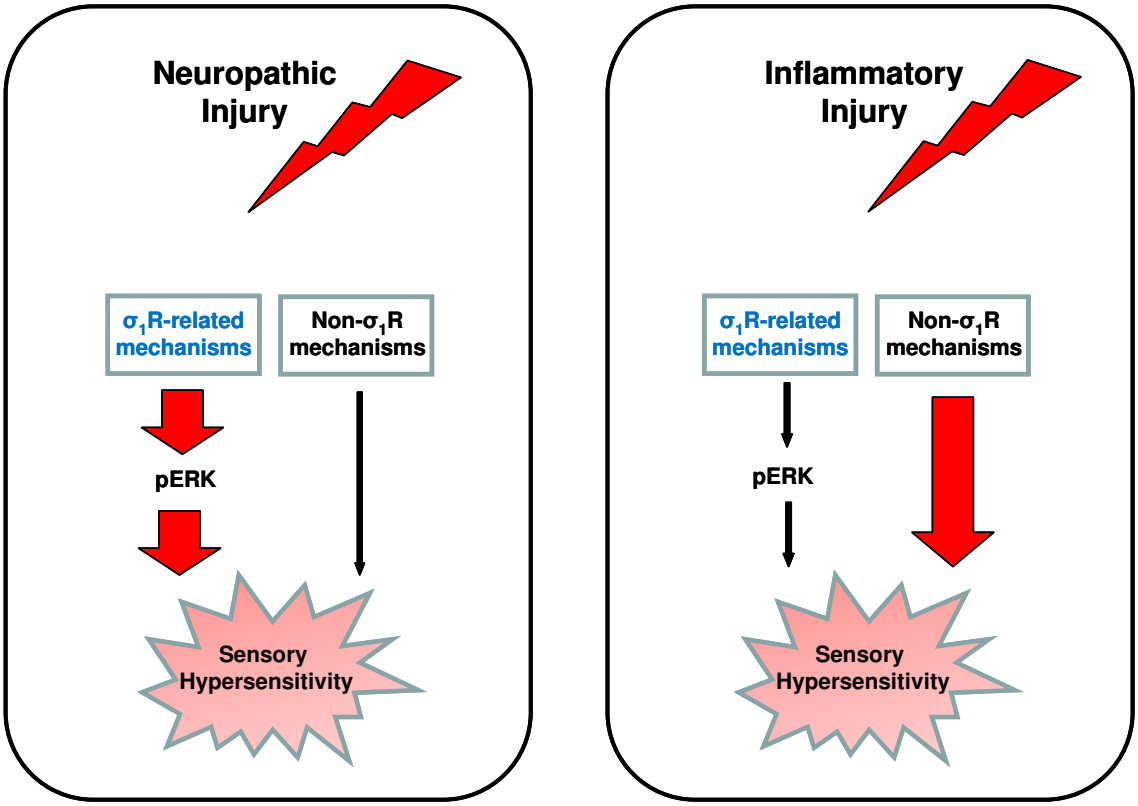

Fig. 2. Schematic diagram suggesting the different role of σ_1R in the development of pain hypersensitivity in neuropathic and inflammatory processes. The left and right panels represent the relative importance of spinal σ_1R-related ERK activation on sensory hypersensitivity after inflammatory or neuropathic injury, respectively. The absence of σ_1R in KO mice prevents ERK activation in the SC and the development of sensory hypersensitivity after neuropathic injury (De la Puente *et al.*, 2009; Nieto *et al.*, 2012). In contrast, spinal pERK does not seem to be a key component in the development of sensory hypersensitivity after inflammatory injury.

Table 1. Summary of pain behaviours and pERK expression shown by WT and σ_1R KO mice in several models of neuropathic and inflammatory pain.

		WT mice		σ_1R KO mice		
Pain Type	**Animal model**	**Pain expression**	**pERK expression**	**Pain expression**	**pERK expression**	**Reference**
Neuropathic pain	Partial sciatic nerve ligation-induced neuropathy	Allodynia	Up-regulation	Attenuation	Attenuation	De la Puente *et al.*, 2009
	Chemotherapy (paclitaxel)-induced neuropathy	Allodynia	Up-regulation	Attenuation	Attenuation	Nieto *et al.*, 2012, 2014
Inflammatory pain	Carrageenan-induced acute inflammation	Allodynia	No up-regulation	Similar to WT	Similar to WT	Gris *et al.*, 2014
	CFA-induced chronic inflammation	Allodynia	No up-regulation	Similar to WT	Similar to WT	Gris *et al.*, 2014

Study of the spinal expression of c-Fos, GFAP, nNOS, SP and NPY by immunohistochemistry.

As mentioned, σ_1R plays an important role in facilitating central sensitization mechanisms in the dorsal horn of the SC after peripheral injury, where several pain-related molecular markers such as c-Fos, GFAP, nNOS, SP and NPY are involved:

- The immediate early gene c-Fos has been related to neuronal activation (Gao and Ji, 2009). In fact, the activation of nociceptive afferent neurons by noxious heat, mechanical stimuli or chemical stimuli results in the rapid appearance of c-Fos immunoreactivity in the superficial layers of the dorsal horn. The pharmacological blockade of σ_1R has shown to attenuate the c-Fos expression in several pain models, such as the formalin test or the migraine models induced by either formalin or capsaicin (Kim *et al.*, 2006; Kwon *et al.*, 2009; Roh and Yoon, 2014).
- GFAP is an intermediate filament component distributed in the cytoplasm of some glial cells. While its expression is low in the intact adult CNS, it increases after astrocytic activation due to synaptic remodeling in chronic pain states (Stephenson and Byers, 1995; Sun *et al.*, 2005). It has been recently discovered that σ_1R activates astrocytes via p38 MAPK phosphorylation, thus leading to mechanical allodynia in a peripheral neuropathic pain model (Moon *et al.*, 2014).
- The enzyme nNOS plays a key role in the nociceptive processes that regulate NO production in laminae I and II of the SC (Sardella *et al.*, 2011). The spinal activation of σ_1R induced sensitization that is mediated by an increase in nNOS activity, which in turn is associated with a NO-induced increase in PKC-dependent NR1 expression (Roh *et al.*, 2011).

- Neuropeptides such as NPY and SP are released after injury, are widely distributed in the CNS and PNS, and play an important neuromodulatory role in primary sensory neurons (Cuesta *et al.*, 1999; Yalamuri *et al.*, 2013). While no clear evidence has related σ_1R to these neuropeptides in pain models (Ohsawa *et al.*, 2011), it is known that NPY-induced increases in hippocampal dopamine can be mediated by σ_1R (Meurs *et al.*, 2007).

The **objective** of this second part of the present Annex was to investigate the role of σ_1R in the expression of spinal c-Fos, GFAP, nNOS, SP and NPY in inflammatory pain models. To this end, the expression of these proteins in σ_1R KO and WT mice was compared by immunohistochemistry after acute and chronic inflammatory pain processes.

The **material and methods** are the same than those described in Article 4 (immunohistochemistry methods) and are also detailed in the Methods section. The main points are briefly summarized below.

The changes in protein immunoreactivity in the spinal hemicord (ipsilateral and contralateral) segments L4-S1 were assessed by quantifying laminae I and II in two different ways:

- c-Fos, nNOS and NPY expression were evaluated by counting the number of immunopositive cells.
- GFAP and SP expression were determined by evaluating the mean grey intensity of the immunoreactive area.

In order to maintain a constant threshold for each image and to compensate for subtle immunostaining variability, only immunoreactivity at least 70% darker than the

average grey level of each image after background substraction was quantified for each antibody (Choi *et al.*, 2012).

The control group corresponds to naïve animals from the same experimental set. Four experimental groups were run in parallel for immunostaining purposes: naïve WT, injured WT (carrageenan or CFA), naïve σ_1R KO, and injured σ_1R KO (carrageenan or CFA). The values obtained from 4 mice of at least 4 sections for each mice were averaged for each experimental group.Results are expressed in two ways:

- Absolute values of immunopositive neurons (cFos, nNOS and NPY) or mean grey intensity values (GFAP and SP) of the ipsilateral dorsal horn (B and C of Figures 3, 4, 5, 6, 7).
- Percentages of change *versus* control animals for each hemicord (contralateral and ipsilateral side) segments (D and E of Figures 3, 4, 5, 6, 7).

In all experimental conditions, **c-Fos** was mainly expressed in laminae I–II of the SC. In the control groups (WT and σ_1R KO), similar number of c-Fos positive neurons (13-20) were detected in both the ipsilateral and contralateral sides of the lumbar SC. No significant differences were found between WT and KO naïve groups (Fig. 3A). Consistent with previous studies (Chapman *et al.*, 1995; Morgenweck *et al.*, 2010), the c-Fos expression in the ipsilateral dorsal horn was markedly increased in WT mice 3 h after carrageenan injection ($p < 0.05$, 2.1-fold increase *versus* control). A similar increase was also found in the ipsilateral dorsal horns of σ_1R KO mice (1.8-fold increase *versus* control; Fig. 3B and 3D). No statistically significant differences between genotypes were found when the ipsilateral dorsal horns were compared after carrageenan injection. No changes in c-Fos-immunopositive cells following injection of carrageenan were found in the contralateral WT and σ_1R KO dorsal horns (Fig. 3D).

Unlike observations in the acute inflammatory pain model, no significant changes were found 4 days after CFA intraplantar injection in WT mice. This is consistent with the fact that c-Fos is an immediate early gene that is rapidly activated at the beginning of a potentially harmful stimulus (i.e. inflammatory injury). Conversely, σ_1R KO mice with inflamed paw showed a significant ipsilateral increase of positive cells expressing c-Fos ($p < 0.05$ *versus* control, Fig. 3C). No changes were observed in c-Fos-immunopositive cells following CFA injection in WT and σ_1R KO contralateral dorsal horns (Fig. 3E).

In summary, c-Fos was selectively expressed in the superficial dorsal horn of the ipsilateral SC after carregeenan injection in both WT and σ_1R KO mice. In contrast, c-fos expression in the ipsilateral dorsal horn 4 days after CFA injection was found in σ_1R KO mice, but not in WT mice, thus suggesting a prolonged activation of some SC neurons after chronic inflammation in the absence of σ_1R.

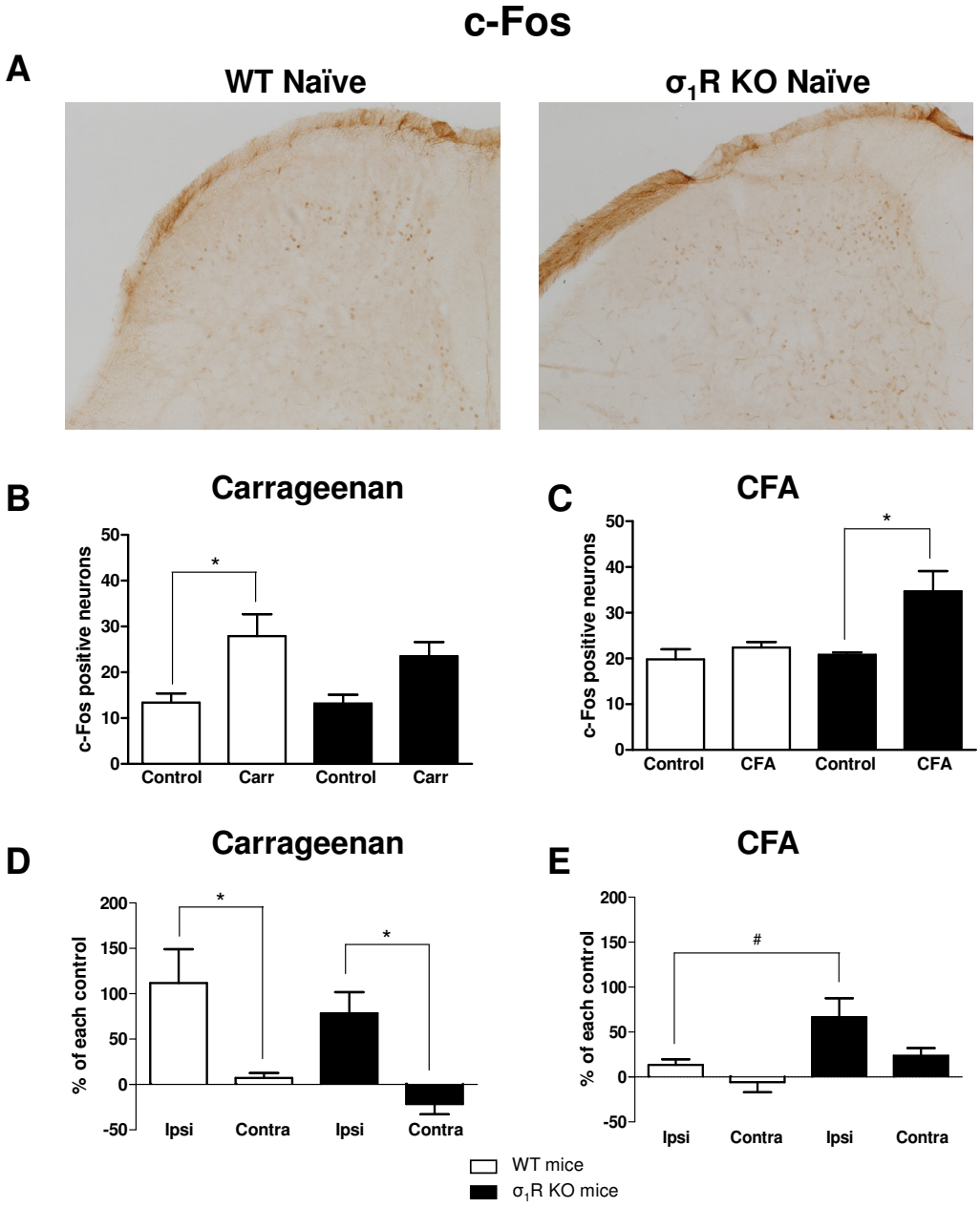

Fig. 3. c-Fos expression in the dorsal horn of the SC secondary to carrageenan and CFA intraplantar injection in WT and σ_1R KO mice. Representative slices of c-Fos immunostaining in the dorsal horn of SC in WT and σ_1R KO naïve mice (A). c-Fos quantification as the number of immunoreactive c-Fos neurons in the ipsilateral dorsal horn of the SC secondary to carrageenan (B) and CFA (C) intraplantar injection in WT and σ_1R KO mice. Note that the control group corresponds to naïve animals within the same experimental set. Percentage of c-Fos immunoreactivity *versus* control following carrageenan (D) or CFA (E) injection in the ipsilateral (ipsi) and contralateral (contra) dorsal horn of WT and σ_1R KO mice. Statistically significant differences obtained by comparing each control group in WT and σ_1R KO mice: * $p < 0.05$; and by comparing WT and σ_1R KO mice: # $p < 0.05$ (two-way ANOVA followed by Bonferroni test). Each bar and vertical line represents the mean ± SEM of the values obtained in 4 animals.

Expression of **GFAP** within laminae I and II of the SC was found in all experimental groups. Both naïve groups (WT and σ_1R KO) showed similar GFAP immunostaining patterns, thus suggesting similar baseline expression of this astrocytic marker (mean grey intensity values around 140; Fig. 4A). GFAP immunostaining in the superficial layers of the SC in WT and σ_1R KO was not significantly modified as compared to the respective control groups 3 hours after carrageenan intraplantar injection. This result is consistent with previous reports where intraplantar injection of carrageenan failed to produce early, acute significant change in spinal GFAP immunoreactivity (Schreiber *et al.*, 2008; Choi *et al.*, 2015). Accordingly, no statistically significant increase (of 5%) in GFAP immunostaining was observed in the ipsilateral and contralateral sides of both WT and σ_1R KO mice following intraplantar carrageenan injection (Fig. 4B and 4D), but these differences were not statistically significant. This is consistent with studies describing that microglial activation occur during the early phase of peripheral inflammation (Choi *et al.*, 2015), whereas astrocytic reaction is delayed and occurs a few days after injury. Therefore, 3 hours after carrageenan may be too early to observe GFAP changes in both WT and σ_1R mice. CFA injection also failed to produce any noticeable change in GFAP expression in the ipsilateral WT dorsal horn of the SC (Fig. 4C). While other studies have shown an enhanced GFAP expression in rats several days after CFA injection (Zhang *et al.*, 2008; Meng *et al.*, 2013; Wu *et al.*, 2014), our results are consistent with another report performed in mice where no differences in the morphology or level of immunoreactivity of astrocytes were found 4 days after CFA injection (Rabchevsky *et al.*, 1999). However, in the absence of σ_1R in KO mice, GFAP expression slightly increased in the SC 4 days after CFA injection in both the ipsilateral and contralateral sides, being statistically significant ($p < 0.01$; Fig. 4E) only when the contralateral

slices were compared ($p < 0.01$; Fig. 4E). This is the first time that a σ_1R-related change of GFAP expression has been described in inflammatory pain conditions. A previous report related σ_1R to astrocytic reaction in a neuropathic pain model induced by chronic constriction injury. In contrast to findings here using σ_1R KO mice in the CFA model of inflammatory pain, this report showed that blocking this receptor inhibited the pathological activation of astrocytes in the SC 3 days after injury (Moon *et al.*, 2014).

In summary, the absence of σ_1R did not change the pattern of spinal expression of GFAP in the acute carrageenan-induced inflammatory pain model. However, the lack of σ_1R slightly increased GFAP expression in the contralateral dorsal horn 4 days in the chronic CFA-induced inflammatory pain model.

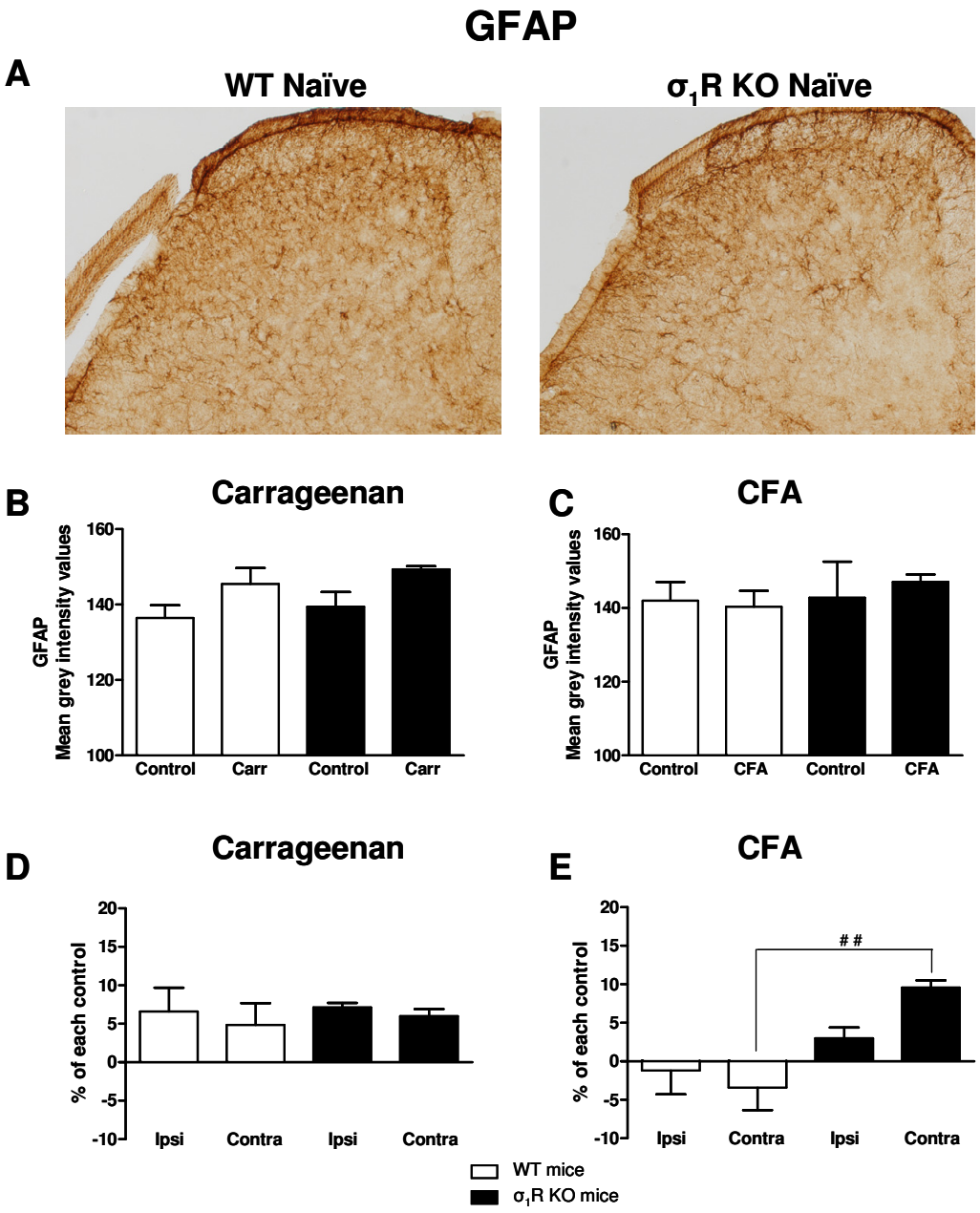

Fig. 4. GFAP expression in the dorsal horn of the SC secondary to carrageenan and CFA intraplantar injection in WT and σ_1R KO mice. Representative slices of GFAP immunostaining in a dorsal horn of the SC in WT and σ_1R KO naïve mice (A). Quantification of GFAP immunoreactivity (expressed as mean grey intensity values) in the ipsilateral dorsal horn of the SC secondary to carrageenan (B) and CFA (C) intraplantar injection in WT and σ_1R KO mice. Note that the control group corresponds to naïve animals within the same experimental set. Percentage of GFAP immunoreactivity *versus* control following carrageenan (D) or CFA (E) injection in the ipsilateral (ipsi) and contralateral (contra) dorsal horn of WT and σ_1R KO mice. Statistically significant differences comparing WT and σ_1R KO mice: ## $p < 0.01$ (two-way ANOVA followed by Bonferroni test). Each bar and vertical line represents the mean ± SEM of the values obtained in 4 animals.

nNOS was mainly expressed in dendrites and axons forming a dense plexus in the superficial dorsal horn, particularly in laminae I and II. nNOS-immunoreactive cell bodies were also detected in this region. No main differences were found between WT and KO naïve groups (Fig. 5A). In WT mice, nNOS-immunopositive cells were slightly increased after carrageenan injection *versus* control in both sides of the SC (increase of approximately 7 immunopositive cells; Fig. 5B and 5D). Similarly increased nNOS expression was observed in σ_1R KO mice —statistically significant differences obtained at both the ipsilateral side when comparing to control group ($p < 0.05$; Fig. 5A) and the contralateral side *versus* corresponding group in WT mice ($p < 0.05$; Fig. 5D). Indeed, the lack of σ_1R did not modify oedema and hyperalgesia (Gris *et al.*, 2014), and thus the similar spinal increment of nNOS in carrageenan-inflamed paws of both mouse types would suggest a similar σ_1R-independent involvement of NO in the molecular pathways triggered by carrageenan (Omote *et al.*, 2001). Regarding chronic inflammatory pain, no significant changes in the number of nNOS-immunoreactive cells were found between groups in both WT and KO mice 4 days after CFA injection (Fig. 5C and 5E). An earlier nNOS up-regulation can not be discarded, as it has been previously described 24h after intraplantar injection of CFA (Chu *et al.*, 2005), but it seems that nNOS is not primarily regulated by σ_1R in inflammatory acute and chronic pain conditions.

In conclusion, the lack of σ_1R failed to change the pattern of spinal expression of nNOS in the acute and chronic inflammatory pain model induced by carrageenan and CFA, respectively.

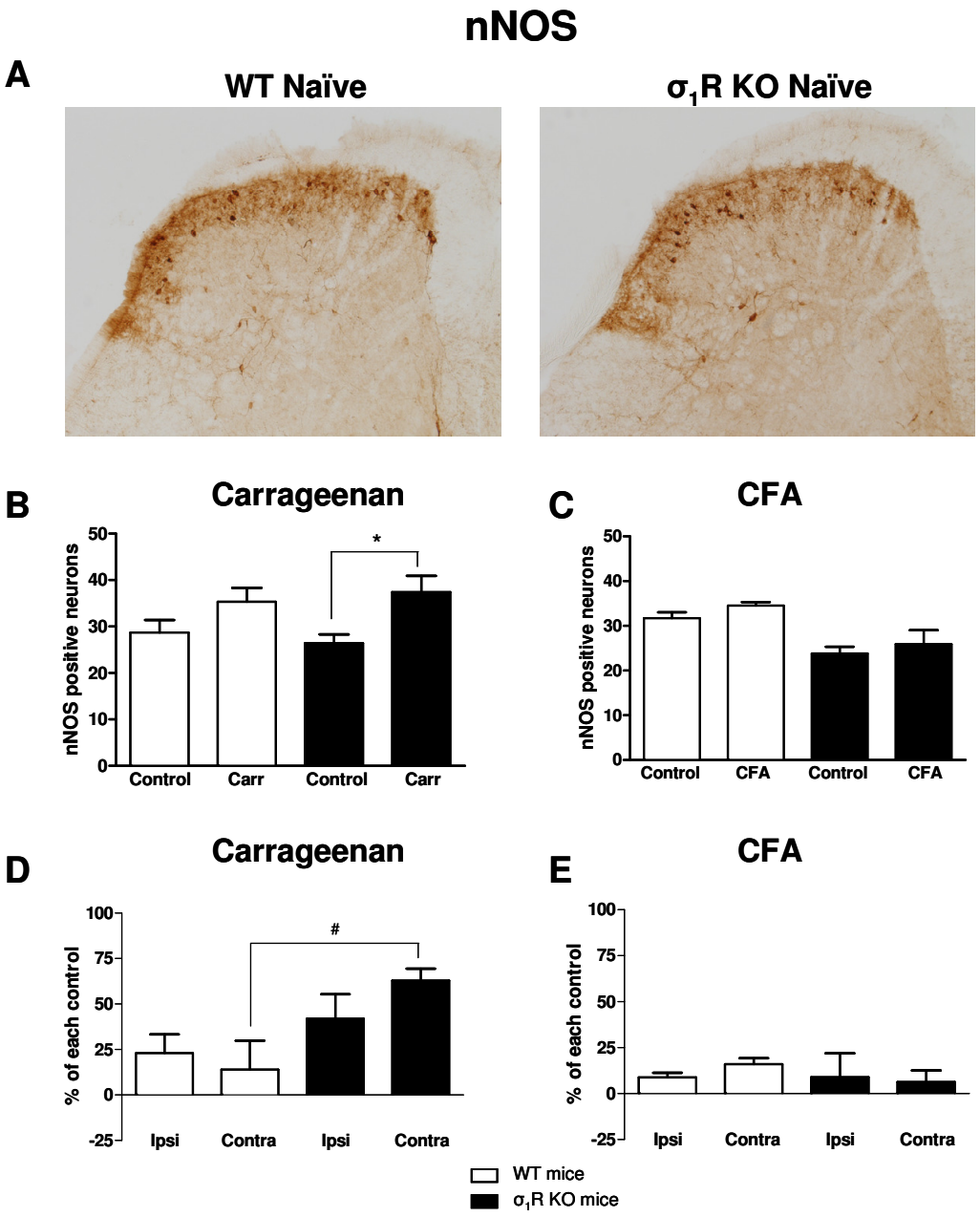

Fig. 5. nNOS expression in the dorsal horn of the SC secondary to carrageenan and CFA intraplantar injection in WT and σ_1R KO mice. Representative slices of nNOS immunostaining in a dorsal horn of the SC in WT and σ_1R KO naïve mice (A). Quantification of nNOS as number of immunoreactive nNOS-neurons in the ipsilateral dorsal horn of the SC secondary to carrageenan (B) and CFA (C) intraplantar injection in WT and σ_1R KO mice. Note that the control group corresponds to naïve animals within the same experimental set. Percentage of nNOS immunoreactivity *versus* control following carrageenan (D) or CFA (E) injection in the ipsilateral (ipsi) and contralateral (contra) dorsal horn of WT and σ_1R KO mice. Statistically significant differences *versus* each control group in WT and σ_1R KO mice: * $p < 0.05$; and between WT and σ_1R KO mice: # $p < 0.05$ (two-way ANOVA followed by Bonferroni test). Each bar and vertical line represents the mean ± SEM of the values obtained in 4 animals.

SP immunoreactivity was mainly concentrated in neurons of lamina I of the superficial dorsal horn. Both naïve groups (WT and σ_1R KO) showed comparable baseline expressions (Fig. 6A). Carrageenan-induced acute inflammatory pain in WT mice was unable to promote any significant change in the spinal expression of SP in either the ipsilateral or the contralateral dorsal horn ($p > 0.05$ *versus* WT control mice; Fig. 6B and 6D). This result is consistent with a previous report in rats which also failed to observe any significant change in SP immunoreactivity 3 h and 24 h after carrageenan injection (Ma and Eisenach, 2003), and it is in line with the fact that SP is synthesized in DRG and mainly released from primary afferent neurons at the periphery in the acute phase of inflammation (Ma and Eisenach, 2003). Similarly, carrageenan injected intraplantarly to σ_1R KO mice failed to modify spinal SP immunostaining. In chronic inflammation (CFA) laminae I and II of the ipsilateral (Fig. 6C and 6E) and contralateral (Fig. 6E) sides were less immunostained with SP antibodies than control slices in WT mice. This result is consistent with a previous report showing a decrease in SP immunoreactivity in the DRG 5 days after CFA injection (Calzà *et al.*, 1998). In contrast, no significant changes in SP expression in the chronic condition induced by CFA were found in either side of the SC in the absence of σ_1R in KO mice.

In summary, SP expression did not change in the superficial dorsal horn of the ipsilateral SC after carrageenan injection in both WT and σ_1R KO mice. In contrast, WT mice experienced a slight down-regulation in SP expression 4 days after CFA injection, whereas this expression remained unchanged in the ipsilateral dorsal horn in the absence of σ_1R.

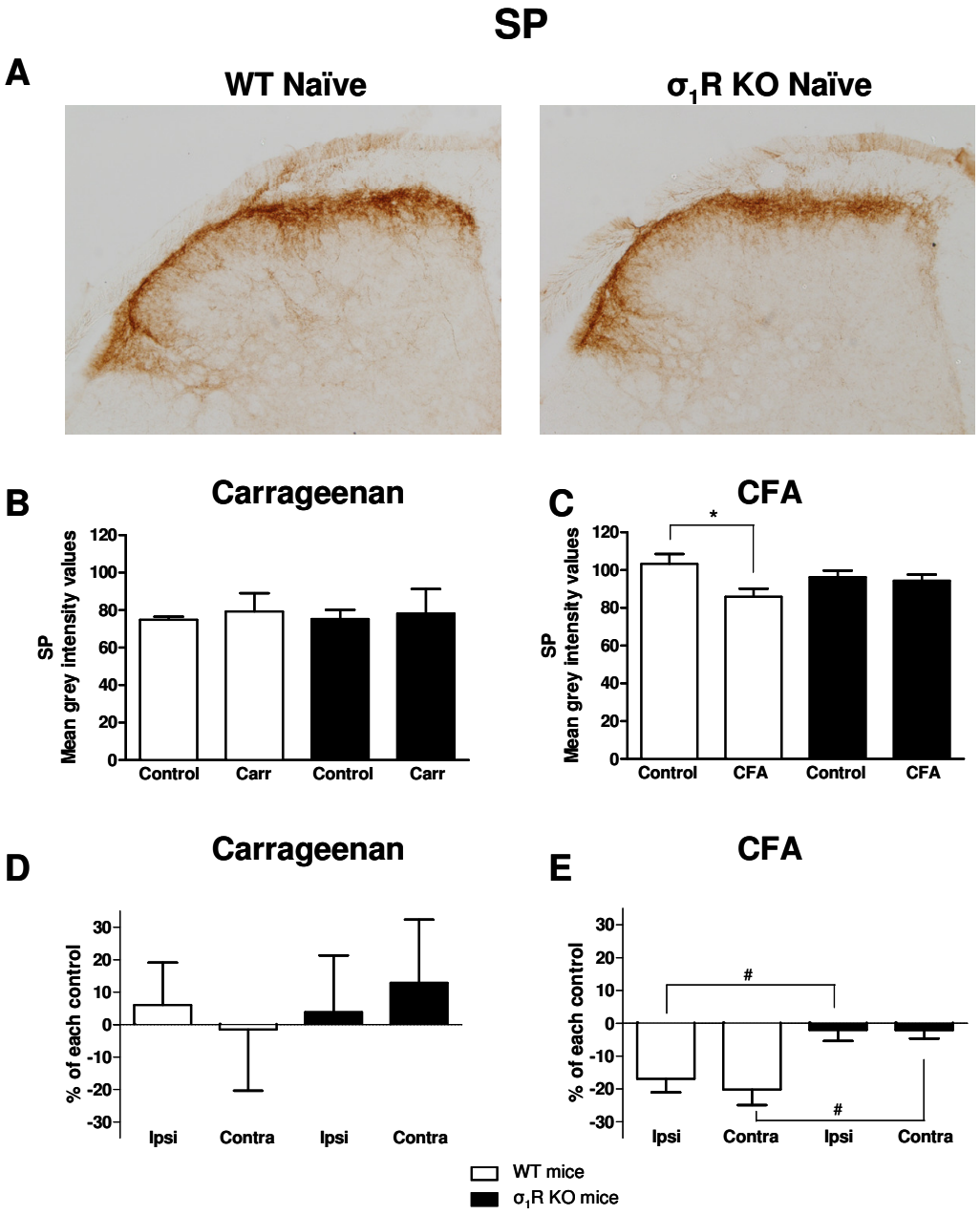

Fig. 6. SP expression in the dorsal horn of the SC secondary to carrageenan and CFA intraplantar injection in WT and σ_1R KO mice. Representative slices of SP immunostaining in a dorsal horn of the SC in WT and σ_1R KO naïve mice (A). Quantification of SP immunoreactivity (expressed in mean grey intensity values) in the ipsilateral dorsal horn of the SC secondary to carrageenan (B) and CFA (C) intraplantar injection in WT and σ_1R KO mice. Note that the control group corresponds to naïve animals within the same experimental set. Percentage of SP immunoreactivity *versus* control following carrageenan (D) or CFA (E) injection in the ipsilateral (ipsi) and contralateral (contra) dorsal horn of WT and σ_1R KO mice. Statistically significant differences comparing each control group in WT and σ_1R KO mice: * $p < 0.05$; and between WT and σ_1R KO mice: # $p < 0.05$ (two-way ANOVA followed by Bonferroni test). Each bar and vertical line represents the mean ± SEM of the values obtained in 4 animals.

NPY positive neurons were found in laminae I and II, and no differences were found between naïve mice (WT and σ_1R KO) (Fig. 7A). No significant changes were found in NPY expression in either side of the SC after carrageenan injection in WT mice (Fig. 7B and 7D). Similarly, the number of NPY positive neurons in the dorsal horn of the ipsilateral SC did not change in the absence of σ_1R in KO mice in acute inflammatory pain. Like in the acute condition, in the case of chronic inflammatory pain (CFA), WT mice did not experience any significant change in NPY immunoreactive cells at either side of the SC (Fig. 7C and 7E). To this point, it is important to note that NPY has been described to act at neuropeptide Y1 receptors in the dorsal horn to decrease nociception by inhibiting SP release (Taylor *et al.*, 2014). Thus, the reduction of SP (Figure 6), but not NPY, found in the CFA model could indicate that peptide expression in the SC shows distinct and individual temporal patterns: when peptide expression at the SC was evaluated, NPY expression restored to control level but the effect of reduced SP expression was still present. However, the absence of σ_1R in KO mice seems to lead to a different temporal pattern of expression for NPY and SP: while CFA injection led to a slightly reduced NPY expression, SP reached the level of expression of the control group in the SC of σ_1R KO.

In conclusion, the absence of σ_1R failed to significantly alter the pattern of expression of NPY in the carrageenan-induced acute inflammatory pain model. In contrast, the lack of σ_1R led to significant down-regulation of NPY immunostainning 4 days after CFA injection.

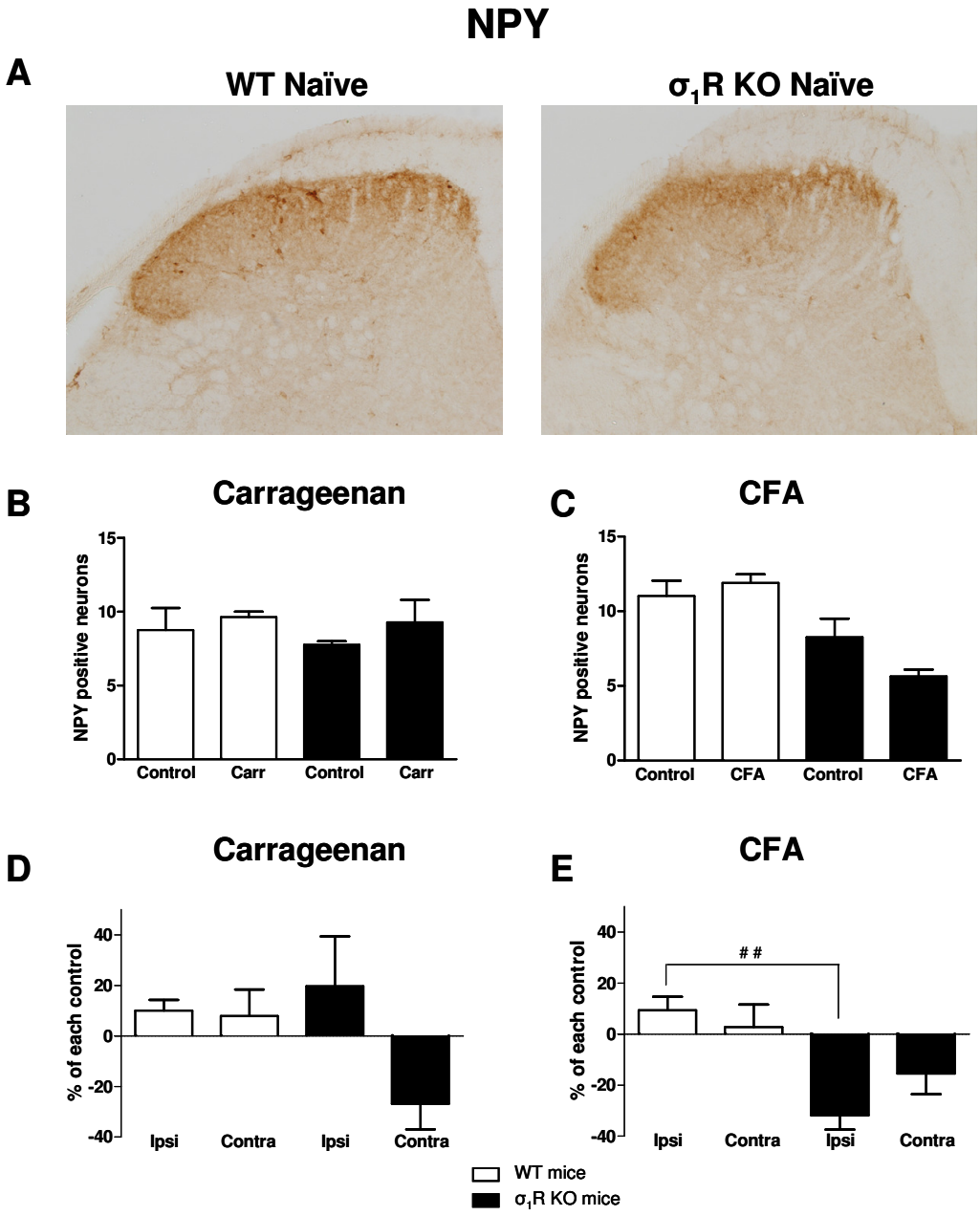

Fig. 7. NPY expression in the dorsal horn of the SC secondary to carrageenan and CFA intraplantar injection in WT and σ_1R KO mice. Representative slices of NPY immunostaining in a dorsal horn of the SC in WT and σ_1R KO naïve mice (A). Quantification of NPY immunoreactivity (expressed in mean grey intensity values) in the ipsilateral dorsal horn of the SC secondary to carrageenan (B) and CFA (C) intraplantar injection in WT and σ_1R KO mice. Note that the control group corresponds to naïve animals within the same experimental set. Percentage of NPY immunoreactivity *versus* control following carrageenan (D) or CFA (E) injection in the ipsilateral (ipsi) and contralateral (contra) dorsal horn of WT and σ_1R KO mice. Statistically significant differences comparing WT and σ_1R KO mice: ## $p < 0.01$; ### $p < 0.001$ (two-way ANOVA followed by Bonferroni test). Each bar and vertical line represents the mean ± SEM of the values obtained in 4 animals.

A summary of the spinal expression changes of the pain-related molecular markers analysed in acute and chronic inflammatory models, and how the lack of σ_1R could interfere in the expression pattern, is shown in Table 2. The expression changes of pain markers observed in WT and KO mice are different when acute and chronic models of inflammation are compared.

- A similar expression pattern in WT and σ_1R KO mice was found in carrageenan-induced acute inflammatory pain , thus suggesting that σ_1R would not have an impact on the expression of these pain-related molecular markers.
- While CFA-induced chronic inflammatory pain did not lead to significant changes *versus* the naïve condition in WT mice, σ_1R KO mice showed a different expression pattern, thus suggesting that the lack of σ_1R would alter the expression of the molecular markers under pain conditions.

Table 2. Summary of the spinal expression of the pain-related molecular markers used in this study in WT and σ_1R KO mice in the carrageenan and CFA models of inflammatory pain. Significant protein up-regulation is shown in dark green, slightly increased or tendency to protein up-regulation (no significant) is shown in light green, and significant protein down-regulation is shown in orange, according to our previous discussion for each target. The lack of σ_1R seems to differentially affect the expression pattern in the acute and chronic inflammatory models.

Spinal changes after carrageenan

	pERK	c-Fos	GFAP	nNOS	SP	NPY
WT						
KO						

Spinal changes after CFA

	pERK	c-Fos	GFAP	nNOS	SP	NPY
WT						
KO						

Decrease
No change
Slight increase
Increase

3. EFFECT OF σ_1R BLOCKADE ON POSTOPERATIVE PAIN

3.1. Article 4: *"Role of the sigma-1 receptor in the expression and development of postoperative pain".*

(Manuscript to be submitted)

Georgia Gris, Enrique Portillo-Salido, Alicia Carceller, Ricard Sanchez-Arroyos, José Miguel Vela, Manuel Merlos, Daniel Zamanillo.

Department of Pharmacology, Drug Discovery & Preclinical Development, ESTEVE Barcelona, Spain.

Summary of the Article 4:

"Role of the sigma-1 receptor in the expression and development of postoperative pain".

Background

Sigma-1 receptor (σ_1R) is a unique ligand-regulated molecular chaperone that is expressed in areas that are key for pain control. genetic inactivation of σ_1R caused a marked attenuation of pain responses in some sensitizing conditions: formalin-induced pain, capsaicin-induced hypersensitization and some neuropathic pain models, such as the chemotherapy-induced neuropathic model or the sciatic nerve ligation model (De la Puente *et al.*, 2009; Nieto *et al.*, 2012). The genetic ablation of σ_1R, however, failed to prevent the acquisition of some inflammatory pain-related behaviours in mice. Carrageenan- and complete Freund adjuvant-induced pain models in mice were equally developed in WT and σ_1R KO mice (Gris *et al.*, 2014). In contrast, the pharmacological antagonism of σ_1R was effective as analgesic under all these sensitizing conditions, including the inflammatory pain models (Gris *et al.*, 2014; Tejada *et al.*, 2014). The mechanisms of post-incisional pain and chronic postsurgical pain are complex and have inflammatory and neuropathic components. Acute postoperative pain remains a significant medical problem —most treatments for postoperative pain have only minimal analgesic effects.

Objectives

The general objective of this work was to study the involvement of σ_1R in the expression and development of postoperative pain for the first time, using the model of plantar incision. The development of pain-related behaviours as well as the spinal

expression of some pain-related molecular targets in wild-type (WT) and σ_1R knockout (KO) mice were compared, and the analgesic effects of selective σ_1R antagonist E-52862 were evaluated.

Results

Firstly, the development of mechanical allodynia and thermal hyperalgesia following plantar incision was observed. Interestingly, the genetic inactivation of σ_1R produced a marked attenuation of mechanical hypersensitivity on days 1, 3 and 4 as compared to WT mice. However, thermal hyperalgesia developed to a similar extent in both mouse types, from 4 hours to 4 days post-surgery.

Secondly, the spinal protein expression of key molecular pain markers was studied in both WT and σ_1R KO mice by western blot and immunohistochemistry. pERK, c-Fos, GFAP and NPY —but not nNOS and SP— were up-regulated 4 hours post-incision in the ipsilateral dorsal horn of the spinal cord in WT mice, whereas they were attenuated in σ_1R KO mice.

Finally, the pharmacological study was also evaluated 4 h after paw incision. E-52862 failed to exert any antihypersensitivity effect in σ_1R KO mice, thus suggesting that the antinociceptive effects of E-52862 were mediated by σ_1R. In contrast, E-52862 was able to dose-dependently attenuate both mechanical allodynia and thermal hyperalgesia with better efficacy than other marketed drugs (morphine, ibuprofen and celecoxib) in WT mice.

Conclusions

The genetic inhibition of σ_1R failed to prevent the development of heat hypersensitivity in postoperative pain and showed an attenuation of mechanical

allodynia and some molecular markers in the spinal cord. E-52862 dose-dependently supressed both mechanical and thermal (heat) hypersensitivity induced by paw incision but failed to exert any antihypersensitivity effect in σ_1R KO mice, thus suggesting that the analgesic activity induced by E-52862 in this model was clearly mediated by the interaction with σ_1R. In summary, these results represent the first evidence of the role of σ_1R in postoperative pain. Also, the pharmacological blockade of σ_1R by E-52862 represents a new promising therapy for the treatment of postoperative pain.

Article 4: ***"Role of the sigma-1 receptor in the expression and the development of postoperative pain".***

ABSTRACT

Genetic inactivation of sigma-1 receptors (σ_1R) produced a marked attenuation of pain responses in several sensitizing conditions, but did not prevent the acquisition in others, such as the inflammatory pain-related behaviours in mice. However, the pharmacological antagonism of σ_1R was effective as analgesic in all these sensitizing conditions, including the inflammatory pain models. The present study was aimed to compare the development of pain-related behaviours and the spinal expression of several pain-related molecular markers in the postoperative pain model of paw incision in wild-type (WT) and σ_1R knockout (σ_1R KO) mice, and to evaluate the analgesic effects of the selective σ_1R antagonist E-52862 (S1RA). Maximum mechanical and thermal hypersensitivity were observed 4 h after injury, and evidenced for at least 4 days in WT mice. No differences were observed in WT and σ_1R KO mice in the acquisition of thermal hyperalgesia after plantar incision, whereas σ_1R KO mice showed a faster recovery in mechanical hypersensitivity. Increased phosphorylated extracellular signal-regulated kinase (pERK), c-Fos, glial fibrillary acidic protein (GFAP) and neuropeptide Y (NPY) expression in the dorsal horn of the spinal cord was observed in operated *versus* control (sham-operated) WT mice 4 h after surgery, which was attenuated in σ_1R KO mice. E-52862 produced a dose-dependent antinociceptive effect on mechanical allodynia (Emax = 70%) and thermal hyperalgesia (Emax = 58%) 4 h after surgery, whereas ibuprofen and celecoxib failed to produce any significant effect on thermal hyperalgesia. E-52862 was devoid of efficacy when administered to σ_1R KO mice, thus confirming the involvement of σ_1R in E-52862-mediated effects. These

findings suggest that σ_1R contribute to the sensitization to noxious stimuli induced by plantar incision in mice.

Keywords

Allodynia, E-52862, hyperalgesia, hypersensitivity, postoperative pain, sigma-1 receptor

1. Introduction

The mechanisms underlying acute pain after surgery and its evolution to chronic postoperative pain are complex and still not completely understood. Chronic postoperative pain is a relatively frequent pain condition which seriously affects quality of life. Patients with severe postoperative pain are at greater risk of developing chronic pain (Perkins and Kehlet, 2000). After surgery, patients experience ongoing pain and are sensitive to normally non-painful stimuli. Surgery may cause nerve damage, as well as the release of inflammatory mediators that activate Aδ- and C- fibre nociceptors, which trigger changes in the spinal cord. These changes lead to a sensitization process that is essentially responsible for the allodynia and hyperalgesia observed in patients. Postoperative pain is experimentally induced by a surgical incision in the plantar surface of mice or rats (Brennan *et al.* 1996) that causes evoked pain behaviours and spontaneous activity leading to the establishment and maintenance of nociceptive-induced plasticity in the spinal cord. Nitric oxide (NO), protein kinase C, mitogen-activated protein kinases (MAPKs), glial fibrillary acidic protein (GFAP) and neuropeptides, among others, are key molecular markers involved in these changes at the spinal cord level (Chu *et al.*, 2007; Campillo *et al.*, 2010). Increased phosphorylation of the extracellular signal-regulated kinase (ERK) levels associated with c-Fos expression in dorsal horn neurons is also evidenced after postoperative pain (Kawasaki *et al.*, 2004; Obata *et al.*, 2004).

The sigma-1 receptor (σ_1R) is a neuromodulatory chaperone protein of 223 amino acids found in the endoplasmic reticulum and the plasma membranes (Zamanillo *et al.*, 2013) and present in areas of the central and peripheral nervous system important in pain control, including the dorsal horn of the spinal cord (Alonso *et al.*, 2000; Kitaichi *et al.*, 2000). Genetic and pharmacological studies using σ_1R knockout (σ_1R

KO) mice and σ_1R antagonists have shown the involvement of σ_1R in the modulation of pain-related behaviours. Pain behaviours elicited by intraplantar (i.pl.) administration of formalin and capsaicin are attenuated in σ_1R KO mice (Cendán *et al.*, 2005; Entrena *et al.*, 2009). Mechanical and cold allodynia were strongly attenuated in σ_1R KO mice in neuropathic pain conditions induced by partial sciatic nerve ligation (PSNL) or by treatment with the antineoplastic agent paclitaxel, respectively (De la Puente *et al.*, 2009; Nieto *et al.*, 2012). In contrast, thermal (heat) hyperalgesia developed to the same extent as in WT mice following nerve injury (PSNL) (De la Puente *et al.*, 2009). On the contrary, the genetic inactivation of σ_1R failed to prevent the development of carrageenan- and complete Freund adjuvant-induced mechanical allodynia when von Frey filaments were applied (Gris *et al.*, 2014).

The selective σ_1R antagonist E-52862 shows high affinity for σ_1R (Ki = 17 nM), good σ_1R / σ_2R selectivity ratio (550), and selectivity over a panel of 170 molecular targets. E-52862 was effective in several experimental models of acute and chronic pain (Nieto *et al.*, 2012; Romero et al., 2012b; González-Cano *et al.*, 2013), even in the pain-related behaviours (inflammatory pain or PSNL-induced heat hyperalgesia) where σ_1R failed to sufficiently influence the development of behavioural hypersensitivity in σ_1R KO mice (Gris *et al.*, 2015 for review).

The purpose of the present study was to investigate the role of σ_1R in postoperative pain by using the paw incision model. The development of mechanical and thermal hypersensitivity and the spinal expression of several key molecular markers, including phosphorylated ERK (pERK), c-Fos, GFAP, neuronal NO synthase (nNOS), substance P (SP), and neuropeptide Y (NPY), after surgical incision in WT and σ_1R KO mice and the effect of the pharmacological blockade of σ_1R compared to the

analgesic effect of marketed drugs such as morphine, ibuprofen and celecoxib were investigated.

2. Material and methods

2.1. Animals

Male WT CD-1 mice supplied by Charles River and aged 6 to 8 weeks were used in the experiments. σ_1R KO mice were generated on a CD-1 background as previously described (Langa *et al.*, 2003). Animals were acclimatized in our animal facilities for at least 1 week before testing, supplied with food and water ad libitum, and kept under controlled laboratory conditions at a temperature of 21 ± 1ºC and a light-dark cycle of 12 h (lights on at 7:00 a.m.). Experimental behavioural testing was carried out in a soundproof, air-regulated experimental room. All experimental procedures and animal husbandry were conducted according to the ethical principles of the I.A.S.P. for the evaluation of pain in conscious animals (Zimmermann, 1983) and the European Parliament and the Council Directive of 22 September 2010 (2010/63/EU), and were approved by the local Ethics Committee.

2.2. Drugs and drug administration

E-52862 (4-[2-[[5-methyl-1-(2-naphthalenyl)-1H-pyrazol-3-yl]oxy]ethyl] morpholine) was used as hydrochloride, and doses were expressed as weight of the base. E-52862 and celecoxib were synthesized by Laboratories Esteve (Barcelona, Spain). Ibuprofen was supplied by Tci Europe N.V. (Zwijndrecht, Belgium). Morphine was supplied by the General Directorate of Pharmacy and Drugs, Spanish Ministry of Health (Madrid, Spain). All drugs were dissolved in aqueous solution (0.5% hydroxypropylmethyl cellulose, HPMC; Sigma–Aldrich) and were administered by intraperitoneal (i.p.) route at a volume of 10 ml/kg. All treatments were performed in independent groups of mice and were evaluated 30 min after administration and 4 h after surgery.

2.3. Plantar incision-induced postoperative pain model

The postoperative pain model was adapted to mice according to the previously described plantar incision method in rats by Brennan (1996). Animals were placed in the prone position under anesthesia with isoflurane 2.5% (v/v). Anesthesia was maintained throughout surgery by means of a tube that conducted isoflurane vapors to the animal's nose. A 0.7-cm longitudinal incision was made with a number 11 blade through the skin, fascia and muscle of the right hind paw, starting 0.3 cm from the proximal edge of the heel and extending towards the toes. The underlying plantaris muscle was exposed and incised longitudinally, keeping the muscle origin and insertion intact. After hemostasis with slight pressure, the skin was closed with two simple sutures of braided silk (ref. 6/0, B. Braun). Finally, the wound was wiped with povidone-iodine antiseptic ointment (Topionic; Esteve) to prevent infection. After surgery the animals were allowed to recover in cages provided with sterile beddings. Control animals (sham-operated mice) underwent a sham procedure consisting in the administration of isoflurane under identical conditions.

2.4. Evaluation of mechanical allodynia (von Frey test)

Mechanical allodynia was quantified by measuring the hind paw withdrawal response to a punctate mechanical stimulation using von Frey filaments (North Coast Medical, Inc., San Jose, CA, USA). Mice were placed into compartment enclosures in a test chamber with a framed metal mesh floor through which von Frey monofilaments (bending force range of 0.04 to 2 g) were applied onto the plantar surface, and thresholds were measured using the up-down method paradigm (Chaplan *et al.*, 1994).

This method was based on Dixon's non-parametric method in 1980 and measures the mechanical threshold that produces 50% of responses (Dixon, 1980). Briefly, the (medium range) 0.4 g filament was first used. Then, the strength of the next filament was decreased when the animal responded or increased when the animal failed to respond. This up-down procedure was stopped four measurements after the first change in animal response. Each filament was applied perpendicular to the plantar surface for 2 s at intervals of 5-10 s between each stimulation. The threshold producing 50% of responses was calculated by means of an up-down spreadsheet. Clear paw withdrawal, shaking or licking was considered as a nociceptive-like response. Both ipsilateral and contralateral (non-operated) hind paws were tested.

2.5. Evaluation of thermal hyperalgesia (plantar test)

Thermal (heat) hyperalgesia was assessed using the plantar test apparatus (IITC Life Science Inc, model 390G, Los Angeles, CA, USA) by determination of the hind paw withdrawal latency in response to a thermal stimulus (radiant heat). The plantar test was performed according to the Hargreaves method with slight modifications (Hargreaves *et al.*, 1988). On the day of the test, mice were placed into Plexiglas compartment enclosures and upon a tempered glass surface of the plantar test device, and were allowed to habituate to their environment for 1 h. The heat source —a mobile infrared photobeam— was then positioned under the plantar surface of the hind paw. The nocifensive withdrawal reflex interrupts the light reflected from the photocell onto the paw and automatically turns off the light and the timer. The intensity of the light beam was adjusted based on preliminary studies to produce baseline response latencies of around 12 ± 2 s in untreated control mice. The digital timer connected to the heat source automatically recorded the response latency for paw withdrawal to the nearest

0.1 s. The mean withdrawal latencies for the ipsilateral and contralateral hind paws were determined from the average of two or three separate trials for each animal, with at least a one-minute interval between successive measurements.

2.7. Western Blotting

The lumbar enlargement (L4-S1) of the spinal cord was removed from WT and σ_1R KO mice 4 hours after plantar surgery, and ipsilateral hemicord segments were dissected, frozen immediately in dry ice, and stored at -80ºC. Tissue was homogenized by sonication in RIPA buffer containing a 0.5% protease and 1% phosphatase inhibitor cocktail (all supplied by Sigma-Aldrich). The homogenate was centrifuged at 16,000 g for 10 min at 4 ºC and the supernatant was used. The protein concentration in the homogenate was measured using the Lowry protein assay (DC assay from Biorad). Equal amounts of protein (30 μg) were fractionated by 10% (w/v) SDS–PAGE and transferred onto a polyvinylidene difluoride membrane blocked with 5% non-fat dry milk in Tris–Tween 20-buffered Saline (T–TBS) for 1 h. Membranes were then incubated in 1% non-fat dry milk in T–TBS overnight at 4 ºC with rabbit primary polyclonal antibodies recognizing the mitogen-activated protein kinase (MAPK, total ERK 1/2) or with mouse monoclonal antibodies recognizing the activated MAPK (diphosphorylated MAPK, pERK 1/2) at a 1:40,000 and a 1:1,000 dilution, respectively. Mouse monoclonal anti-GAPDH antibody (1:80,000) or rabbit polyclonal anti-GAPDH antibody (1:10,000) was used as loading control. All antibodies were purchased from Sigma-Aldrich. Blots were washed four times with T–TBS for 15 min and then incubated for 1 h with horseradish peroxidase–conjugated goat anti-rabbit IgG (1:4,000) or goat anti-mouse IgG (1:2,000), supplied by Sigma-Aldrich. Immunoreactive bands were detected by a peroxidase reaction using an enhanced

chemiluminescence method (WesternSure® PREMIUM Chemiluminescent Substrate, Li-cor) and by a C-DiGit® Blot Scanner (Li-cor). The densitometric analysis of immunoreactive bands was performed using the Image studio lite software and normalized with respect to the intensity of the corresponding GAPDH immunoreactive bands. pERK was finally normalized with respect to total ERK protein.

2.8. Immunohistochemistry

Protein expression was estimated 4 h after plantar incision as c-Fos-, nNOS-, and NPY-immunopositive cells or immunoreactive area of GFAP and SP in the dorsal horn (laminae I + II) of the spinal cord. Animals were not exposed to nociceptive testing before tissue extraction (non-stimulated). Four mice per group were deeply anesthetized with isoflurane 2.5% (v/v) and then intracardially perfused with 200 ml of saline solution followed by 250 ml of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. After perfusion, the lumbar region of the spinal cord was dissected out, postfixed for 4 h in 4% paraformaldehyde, and cryopreserved in 30% sucrose solution at 4 °C for 48 h. The section from L4 to S1 of the spinal cord was selected and then embedded in frozen section medium (Neg-50, Thermo scientific, MA, USA), sliced in 40 μm sections on a cryostat (Leica Model CM3050 S, Madrid, Spain), and collected in phosphate-buffered saline (PBS) to be processed immunohistochemically as free-floating sections. Sections were pre-incubated with 0.3% H_2O_2 in PBS for 30 min to block endogenous peroxidase activity and, after washing three times in PBS, with normal goat pre-immune serum diluted 1,5:100 in PBS for 1 h at room temperature (RT) to prevent unspecific staining. Sections were then incubated for 48 h at 4 ºC with the primary antibody: rabbit polyclonal anti-c-Fos (diluted 1:6,000 in PBS; Santa Cruz Biotechnology, CA, USA), rabbit polyclonal anti-GFAP (diluted 1:4,000 in PBS; Dako, Glostrup, Denmark), rabbit

polyclonal anti-nNOS (diluted 1:3,000 in PBS; Cayman chemical, MI, USA), rat monoclonal anti-SP (diluted 1:750 in PBS; AbD Serotec, Oxford, UK), or rabbit polyclonal anti-NPY (diluted 1:10,000 in PBS; Peninsula Laboratories LLC, CA, USA). After washing three times in PBS for 10 min, sections were incubated with the appropiate secondary biotinylated antibodies from Vector Laboratories Inc. (Burlingame, CA, USA) diluted 1:200 in PBS for 1 h at RT. After washing the sections three times in PBS, an avidin–biotin–peroxidase complex (Vector Laboratories, Inc.) was applied diluted 1:100 in PBS for 1 h at RT. The sections were washed again in PBS and placed in a chromogen solution containing 0.05% 3,3'-diaminobenzidine and 0.01% H_2O_2 in PBS for 5 min. The immunostained sections were placed on slides and coverslipped with glycergel mounting medium (DakoCytomation, Inc., Barcelona, Spain) for microscopic observation and photography.

2.9. Quantification of immunohistochemistry

Changes in protein immunoreactivity in the dorsal horn were assessed by quantifying positive profiles of immunopositive cells (relative size and circularity) or immunoreactive areas (measured in terms of mean grey intensity values). Sixteen to eighteen images were analysed for each experimental group, and fields containing ipsilateral and contralateral dorsal horn were digitized using a video camera (Olympus, model DP70, Madrid, Spain) connected to a microscope (Olympus, model BX61, Madrid, España) and interfaced to a computer. Individual immunodensity values were corrected by subtracting the background for each section. In order to maintain a constant threshold for each image and to compensate for subtle immunostaining variability, only immunoreactivity at least 70% darker than the average grey level of each image after background substraction was counted for each antibody. The boundary of the dorsal

horn (laminae I + II) was traced and the immunopositive c-Fos, nNOS and NPY expressing cells or the immunoreactive density area of GFAP and SP were counted based on the inverse computer grayscale (from 0 = white to 255 = black) by means of the National Institutes of Health (NIH) Image J software. Immunoreactivity values were compared using WT and σ_1R KO at the ratio between sham and operated mice.

2.10. Statistical analysis

Data were represented as means ± SEM. In the assessment of mechanical allodynia and thermal hyperalgesia, data were obtained by measuring paw withdrawal expressed as 50% threshold (g) or latency (s), respectively. The mechanical threshold that produced 50% of responses was calculated using Dixon's formula: 50% paw withdrawal threshold (g) = $[(10^{(Xf+\kappa\delta)}/10000)]$, where Xf = value (in logarithmic units) of the final von Frey filament used, κ = tabular value for the pattern of positive/negative responses, and δ = mean difference (in log units) between stimuli. Data obtained in the development of postoperative pain model were subjected to two-way repeated measures ANOVA (paw and genotypes as between factors of variation; time and pain-related behaviour as within group levels) followed by post hoc Bonferroni test. The percentage of antiallodynic or antihyperalgesic effect was calculated as follows: % effect = [(PWD-PWV)/(PWN–PWV)]x100, where PWD and PWV are the paw withdrawal threshold (g) or latency (s) in drug- and vehicle-treated animals, respectively, and PWN is the paw withdrawal threshold in naïve animals (1.51 g and 12.5 s for the von Frey test and the plantar test, respectively). These data were subjected to one-way ANOVA followed by post hoc Bonferroni comparison. These percentages were expressed as antihypersensitivity percentage when the effect on both mechanical allodynia and thermal hyperalgesia was shown simultaneously, and a two-way ANOVA followed by

post hoc Bonferroni test was applied to compare both pain-related behaviours. Similarly, the comparisons of the E-52862 antiallodynic and antihyperalgesic effects between WT and KO mice were analysed using two-way ANOVA followed by Bonferroni comparison. In the immunohistochemistry studies, percentages between sham (mice exposed to anesthesia with isoflurane only) and operated mice were calculated to obtain the increment of protein expression under pain conditions. Thus, immunoreactivity for several antibodies in Western blots and immunohistochemistry was performed using two-way ANOVA followed by Bonferroni test. GraphPad Prism software (version 5.0; GraphPad Software Inc., La Jolla, CA, USA) was used. The criterion for statistical significance was established at a p value below 0.05.

3. Results

3.1. Time course of mechanical allodynia and thermal hyperalgesia induced by plantar incision

No significant differences between groups were found in the paw withdrawal threshold in the von Frey test before surgery (Fig. 1A). The contralateral hind paws of both genotypes (WT and KO) were similar at the different time points. A significantly decreased paw withdrawal threshold (mechanical allodynia) was observed in the ipsilateral (injured) paw of both mouse types 4 h after surgery, lasting up to 3 and 4 days in σ_1R KO and WT mice, respectively. Repeated measures ANOVA (time x genotype) showed a significant effect of time ($F_{6,219} = 59.6$, $p < 0.001$), genotype ($F_{1,219} = 26.2$, $p < 0.001$), and interaction between these two factors ($F_{6,219} = 3.6$, $p < 0.001$). A significant increase in 50% paw withdrawal threshold was found in σ_1R KO mice as compared to WT mice at 1, 3 and 4 days after surgery.

Baseline values before surgery did not reveal significant differences between genotypes, either contralaterally or ipsilaterally, in paw withdrawal latencies in the plantar test (Fig. 1B). Surgical incision caused a marked thermal hyperalgesia in the ipsilateral hind paws from 4 h to day 4 in both mouse types. Repeated measures ANOVA (time x genotype) showed a significant effect of time ($F_{6,153} = 53.8$, $p < 0.001$) and genotype ($F_{1,153} = 5.8$, $p < 0.05$), but no interaction between these two factors ($F_{6,153} = 0.9$, $p = 0.5$). Therefore, no differences were found between WT and σ_1R KO in the time course of thermal hypersensitivity.

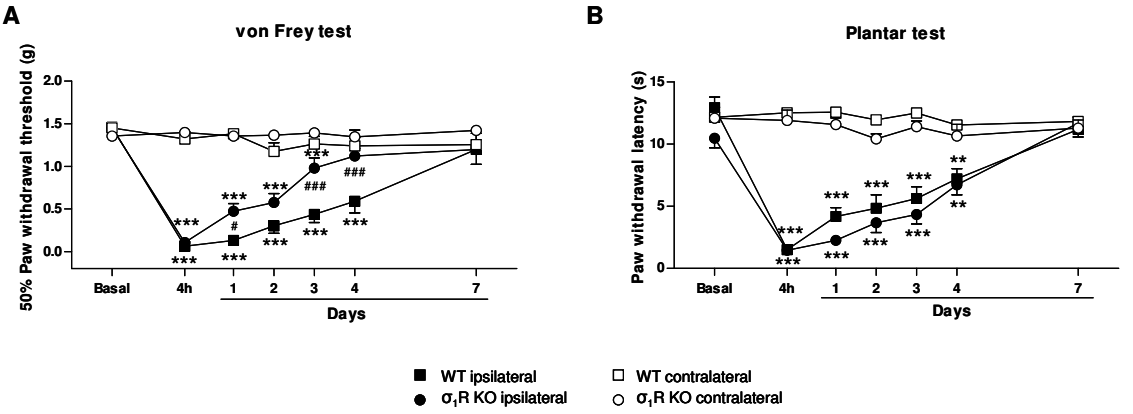

Fig. 1. Time course of mechanical allodynia and thermal hyperalgesia induced by paw incision. Mechanical allodynia and thermal hyperalgesia were found 4 h after surgery (A and B). Statistically significant differences as compared to contralateral values for each time point: ** $p < 0.01$; *** $p < 0.001$; and between WT and σ_1R KO mice at the same time point: # $p < 0.05$; ### $p < 0.001$ (two-way repeated measures ANOVA followed by Bonferroni test). No statistically significant differences in the contralateral hind paw were observed between both groups. Each point and vertical line represents the mean ± S.E.M of the values obtained from at least 6 animals.

3.2. pERK and c-Fos expression in σ_1R KO and WT mice exposed to plantar incision

Western blot experiments with total ERK and phosphorylated ERK antibodies identified two bands with a molecular weight of 44 and 42 kDa corresponding to the ERK subunits 1 and 2, respectively. As expected, GAPDH antibody identified a band of 37 KDa of molecular weight. Sham WT and σ_1R KO mice showed similar ERK1/2 levels. No significant changes in total ERK protein were found in WT or σ_1R KO mice when plantar-injured and sham-operated mice were compared (Fig. 2A). Phosphorylated ERK1/2 (pERK1/2) was significantly higher in σ_1R KO mice (1.8-fold; $p < 0.05$). However, while ERK phosphorylation was notably increased (3.0-fold; $p < 0.01$) in the ipsilateral spinal cord of WT mice exposed to plantar incision as compared to sham-operated WT mice, this increase was not found in σ_1R KO animals (1.6 ± 0.3

and 1.6 ± 0.2 units of relative pERK expression for sham and operated σ_1R KO mice, respectively; $p > 0.05$).

c-Fos immunoreactivity from histological sections was mainly observed in laminae I and II of the ipsilateral dorsal horn. No differences were found between WT and KO sham groups. Similarly to pERK, c-Fos expression was significantly higher in the ipsilateral spinal cord of WT mice exposed to plantar incision as compared to sham-operated WT mice (216% higher than in the control (sham) group; $p < 0.01$) whereas c-Fos immunopositive neurons were not significantly changed in σ_1R KO mice after surgery (Fig. 2B). Two-way ANOVA (dorsal horn × genotype) showed significant effects of dorsal horn ($F_{1,12} = 13.9$, $p < 0.01$) and genotype ($F_{1,12} = 7.1$, $p < 0.05$) after surgery, but no significant interaction between these two factors ($F_{1,12} = 2.0$, $p > 0.05$). In summary, the expression of pERK and c-Fos was clearly and selectively up-regulated in the ipsilateral dorsal horn of the spinal cord of WT mice 4 h after surgery, and these changes were attenuated in σ_1R KO mice.

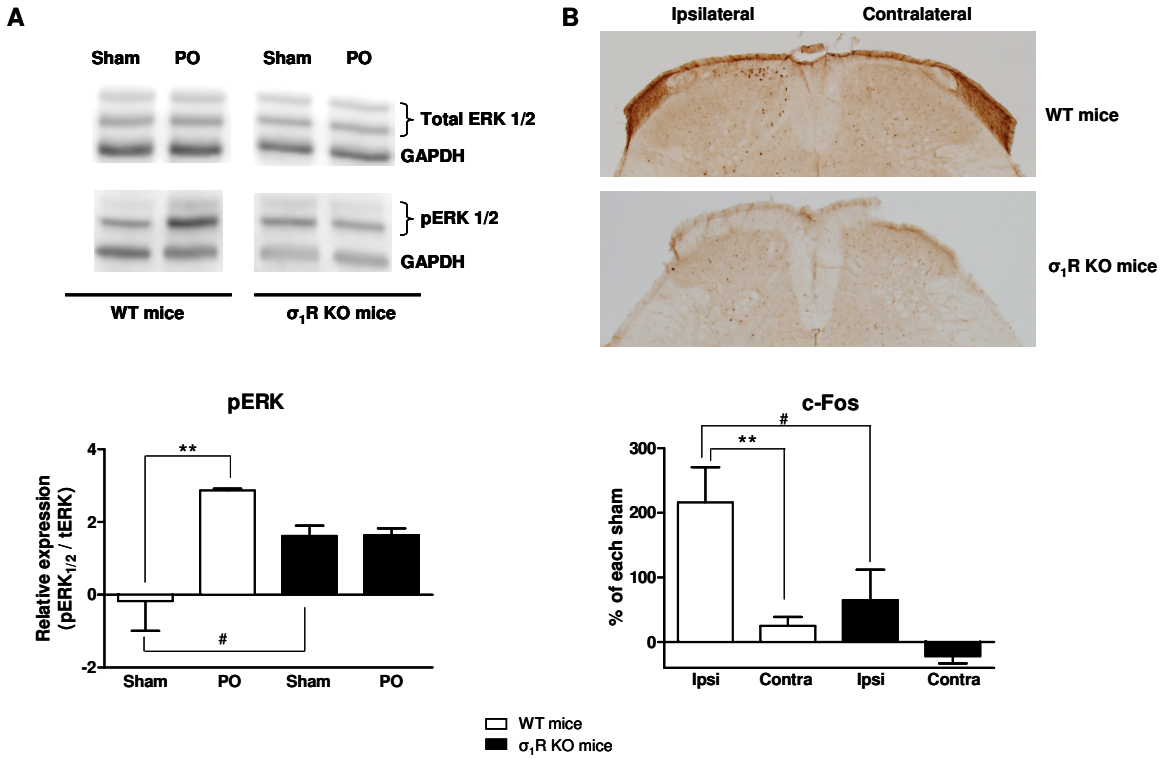

Fig. 2. pERK and c-Fos expression in the spinal cord secondary to plantar incision injury in WT and σ_1R KO mice. Note that pERK and c-Fos are selectively up-regulated in the ipsilateral dorsal horn of WT mice, but not of KO mice 4 hours after surgery. Representative immunoblots and quantification of total ERK and pERK, and GAPDH as loading control in the spinal cord of WT and σ_1R KO mice. (A). Statistically significant differences operated (PO) *versus* sham: ** $p < 0.01$; and between WT and σ_1R KO sham mice: # $p < 0.05$ (two-way ANOVA followed by Bonferroni test). Each bar and vertical line represents the mean ± SEM of the densitometric values obtained from 2-3 pools of 4 animals each. (B) Representative slices and quantification of c-Fos immunopositive cells in ipsilateral (ipsi) and contralateral (contra) dorsal horn of the spinal cord of operated WT and σ_1R KO mice. Statistically significant differences comparing % of each sham in operated WT: ** $p < 0.01$; and between WT and σ_1R KO operated mice: # $p < 0.05$ (two-way ANOVA followed by Bonferroni test). Each bar and vertical line represents the mean ± SEM of the values obtained in 4 animals.

3.3. GFAP, nNOS, SP and NPY expression in σ_1R KO and WT mice exposed to plantar incision

No differences were found in the spinal expression of all pain-related molecular markers when comparing both sham groups (WT and σ_1R KO mice).

Increased GFAP expression was observed 4 h after paw incision surgery in both sides of the spinal cord (laminae I-II and deep laminae) of WT mice (approximately 15% *versus* corresponding side of sham mice; Fig. 3A). However, GFAP up-regulation in σ_1R KO mice was almost not found (increase in 3.5% above the sham condition). Two-way ANOVA (dorsal horn × genotype) showed a non-significant effect of dorsal horn after plantar incision ($F_{1,12} = 0.3$, $p > 0.05$), with genotype effect ($F_{1,12} = 10.6$, $p < 0.01$) and without significant interaction between these two factors ($F_{1,12} = 0.2$, $p > 0.05$). Hence, the comparison between both sides of the spinal cord failed to show any significant difference in GFAP expression following plantar incision in any group ($p > 0.05$). Conversely, a significant difference was found between genotypes when both ipsilateral dorsal horns were compared (from 16.5 ± 3.4 to 3.7 ± 2.6% of each sham in WT and σ_1R KO operated mice, respectively; $p < 0.05$; Fig. 3A).

The expression of nNOS-immunoreactive neurons 4 h after plantar incision was mainly found in laminae I and II of the ipsilateral dorsal horn (Fig. 3B). The plantar incision did not significantly modify the number of nNOS-immunoreactive cells among groups, either in WT or σ_1R KO mice.

SP immunostaining was primarily observed in lamina I of the lumbar spinal cord (Fig. 3C). SP expression in the dorsal horn of WT mice was increased as compared to the WT control group (increase in 19.0 ± 5.0% and 8.8 ± 5.1% in the ipsilateral and contralateral sides, respectively, *versus* the corresponding side of the sham group), although no significant differences were found. No changes in SP expression were observed in the dorsal horns of σ_1R KO mice after paw incision.

Four hours after paw incision, ipsilateral and contralateral NPY immunopositive cell numbers were higher in WT operated mice as compared to the sham group (32% and 94% in the ipsilateral and contralateral side, respectively; Fig. 3D). Conversely, the

spinal cord dorsal horn of σ_1R KO operated mice expressed less NPY-immunopositive cells than in sham σ_1R KO animals (around 40%), in both contralateral and ipsilateral sides. A significant difference was found between genotypes when both contralateral dorsal horns were compared ($p < 0.05$; Fig. 3D).

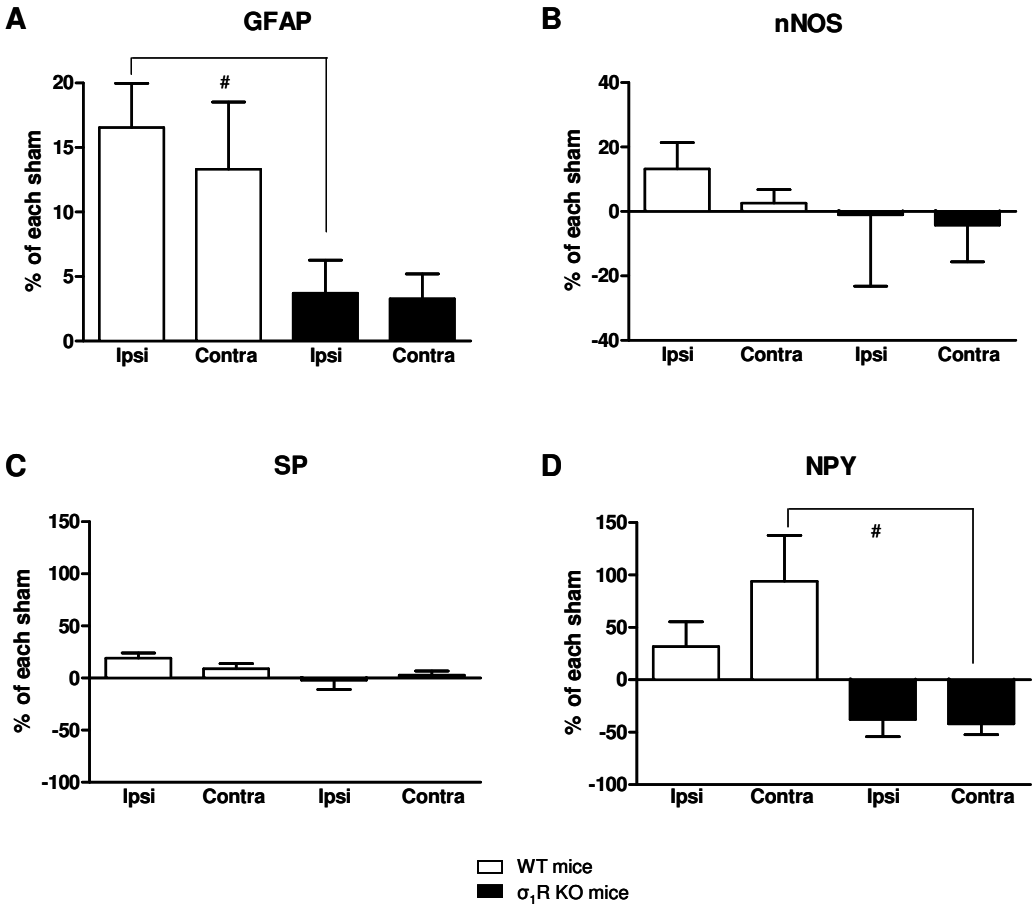

Fig. 3. GFAP, nNOS, SP and NPY expression in the spinal cord secondary to plantar incision injury in WT and σ_1R KO mice. Quantification of GFAP (A), nNOS (B), SP (C) and NPY (D) immunoreactivity in the ipsilateral (ipsi) and contralateral (contra) dorsal horn of the spinal cord of WT and σ_1R KO operated mice. Statistically significant differences comparing % of each sham in operated WT and σ_1R KO mice: # $p < 0.05$ (two-way ANOVA followed by Bonferroni test). Each bar and vertical line represents the mean ± SEM of the values obtained in 4 animals.

3.4. Effects of E-52862 and reference compounds on mechanical allodynia and thermal hyperalgesia in WT mice

The i.p. administration of E-52862 (40-160 mg/kg) and morphine (1.25-8 mg/kg) dose-dependently inhibited plantar incision-induced mechanical allodynia (Emax = 69.6 ± 12.6 and 51.0 ± 8.8%, respectively). On the contrary, ibuprofen and celecoxib showed reduced efficacy (Emax = 22.5 ± 5.0 and 25.7 ± 4.5%, respectively; Fig. 4A).

Regarding thermal hyperalgesia, E-52862 reached a maximum efficacy of around 60%, while morphine, ibuprofen and celecoxib did not exceed 25% (Fig. 4B). Morphine was only able to produce a mild antihyperalgesic effect at the dose of 8 mg/kg ($F_{4,40} = 3.2$, $p < 0.05$; Emax = 22.7 ± 9.3%). Higher doses were not investigated due to the well known opioid-induced increase in locomotor activity in mice. The response of the contralateral hind paw to von Frey filaments and plantar test was unchanged after the drug treatments (data not shown). In order to compare the E-52862 effect on both pain-related behaviours, the antiallodynic and antihyperalgesic curves were plotted in the same chart (Fig. 4C). Two-way analysis of variance (ANOVA) showed a significant effect of the dose ($F_{3,86} = 35.4$, $p < 0.001$), but no effect of pain-related behaviours ($F_{1,86} = 0.5$, $p = 0.5$) and interaction between these two factors ($F_{3,86} = 1.4$, $p = 0.2$), thus suggesting that both curves had a similar slope. The comparison between treatments revealed a similarly significant increase of paw withdrawal threshold and latency after E-52862 administration at 80 and 160 mg/kg ($p < 0.001$) *versus* the vehicle group in both experimental tests.

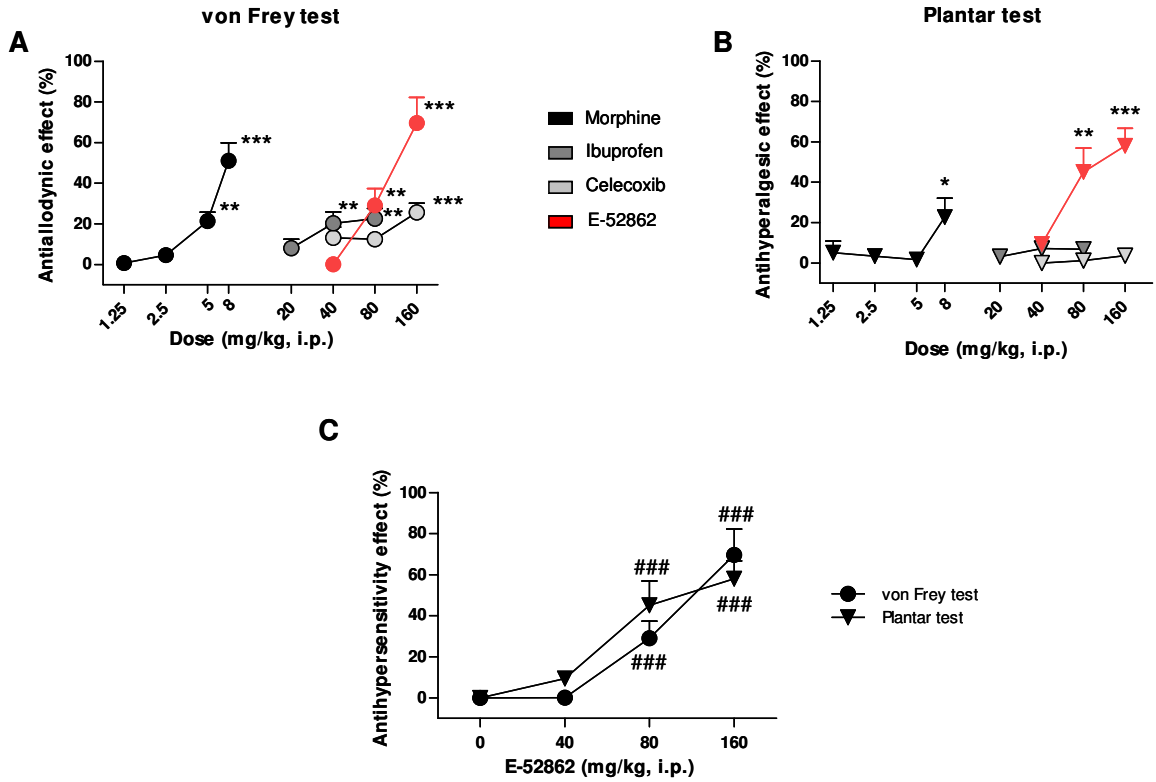

Fig. 4. Effects of E-52862 and reference compounds on mechanical allodynia and thermal hyperalgesia. Antiallodynic and antihyperalgesic effect of morphine, ibuprofen, celecoxib and E-52862 in the von Frey (A) and plantar test (B), respectively. Statistically significant differences compared to vehicle group: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (one-way ANOVA followed by Bonferroni test). Comparison of the antihypersensitivity effect of E-52862 in the von Frey and plantar test (C). Statistically significant differences compared to vehicle group: ### $p < 0.001$ (two-way ANOVA followed by Bonferroni test). Each point and vertical line represents the mean ± S.E.M. of the values obtained in 6-16 animals.

3.5. Effects of E-52862 on mechanical allodynia and thermal hyperalgesia induced by plantar incision in σ_1R KO mice.

In order to study whether the analgesic effects exerted by E-52862 were specifically mediated by σ_1R, the antiallodynic and antihyperalgesic effects of E-52862 were tested in σ_1R KO mice and compared with WT mice (Fig. 5). No antihypersensitivity effects were found in σ_1R KO mice treated with E-52862 in response to either mechanical or thermal stimulation of the operated hind paw, whereas

WT mice showed a dose-dependent response after E-52862 administration in both pain-related behaviours (29.1 ± 8.3% at 80 mg/kg and 69.6 ± 12.6% at 160 mg/kg in WT mice) (Fig. 5A and 5B). Two-way ANOVA showed a significant effect of dose ($F_{3,85}$ = 17.6, $p < 0.001$), genotype ($F_{1,85}$ = 34.8, $p < 0.001$), and interaction between these two factors ($F_{3,85}$ = 15.2, $p < 0.001$). The comparison between treatments revealed a significantly increased paw withdrawal threshold after E-52862 administration at 80 and 160 mg/kg in WT mice, but not in σ_1R KO mice ($p < 0.001$).

E-52862 was unable to reverse thermal hyperalgesia in σ_1R KO mice at 40 and 80 mg/kg, thus demonstrating its *in vivo* specificity (Fig. 5B). The maximum tested dose (160 mg/kg) caused a mild antihyperalgesic effect in σ_1R KO mice that failed to achieve statistical significance *versus* the vehicle. Two-way ANOVA revealed a significant effect of dose ($F_{3,61}$ = 22.1, $p < 0.001$), genotype ($F_{1,61}$ = 18.5, $p < 0.001$), and interaction between these two factors ($F_{3,61}$ = 4.5, $p < 0.01$). The comparison between treatments revealed a significantly increased paw withdrawal latency after E-52862 administration at 80 and 160 mg/kg in WT mice, but not in σ_1R KO mice ($p < 0.001$ and $p < 0.05$, respectively).

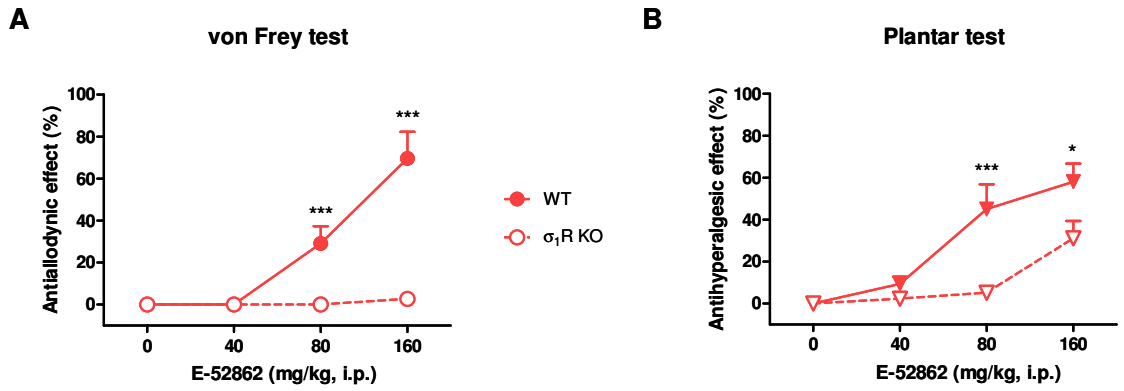

Fig. 5. Effects of E-52862 on mechanical allodynia and thermal hyperalgesia induced by plantar incision in WT and σ_1R knockout mice. Effect of E-52862 in the von Frey (A) and plantar test (B) in WT and σ_1R KO mice. Statistically significant differences compared to σ_1R KO mice: * $p < 0.05$; *** $p <$

0.001 (two-way ANOVA followed by Bonferroni test). Each point and vertical line represents the mean ± S.E.M. of the values obtained in 8-16 animals.

4. Discussion

The present study has evaluated for the first time the role of σ_1R in postoperative pain by using two experimental strategies. First, σ_1R KO mice were used to study the effect of the absence of σ_1R on the development of pain-related behaviours (mechanical and thermal hypersensitivity) and on changes in spinal cord pain-related molecular markers (pERK, c-Fos, GFAP, SP, NPY, and nNOS) in the postoperative pain model of paw incision. Second, pharmacological modulation with E-52862 was used to selectively block the σ_1R function in the same model.

Paw incision model has proved to be a useful tool for studying the neurobiological mechanisms of pain after surgery (Brennan *et al.*, 1996). Although the postoperative pain model was first characterized and widely used in rats, several studies in mice have also been published (Pogatzki and Raja, 2003; Célérier *et al.*, 2006; Cabañero *et al.*, 2009). The main advantage of using this species is that the role of a specific receptor can be studied by using genetically modified mice (Pogatzki and Raja, 2003). In our study, paw withdrawal responses in the contralateral side of mice were unchanged by surgery, and the behavioural responses were stable over time after successive nociceptive stimulation. Consistent with previous data reported by other groups, mechanical allodynia and thermal hyperalgesia were evidenced by strong hypersensitivity immediately after surgery (4 h in our study), with normal thresholds being gradually restored in WT mice on day 7 post-surgery (Célérier *et al.*, 2006; Cabañero *et al.*, 2009). Mechanical and thermal sensitivity were similar in WT and σ_1R KO mice before surgery, thus suggesting that basic mechanisms for transduction, transmission and perception of sensory inputs are intact in mice lacking σ_1R. In our

study, although maximal mechanical allodynia was reached at 4 h after surgery in σ_1R KO mice, an attenuation of mechanical allodynia was observed at 24 h after surgery. Baseline sensitivity recovery was also faster in σ_1R KO compared to WT mice. On the contrary, the time course of thermal hyperalgesia was similar in σ_1R KO and WT mice, thus suggesting that σ_1R plays a more determinant role in the development of mechanical allodynia as compared to thermal hyperalgesia. No differences between WT and σ_1R KO mice have also been reported when thermal hyperalgesia induced by carrageenan (Gris *et al.*, 2014; Tejada *et al.*, 2014) or by PSNL (De la Puente *et al.*, 2009) was evaluated.

Tissue injury activates nociceptors that induce plastic changes, which include the release of inflammatory and other mediators, changes in gene expression, receptor translocation to the cell membrane, and sustained activation of glial cells in the spinal cord that result in central sensitization and ultimately in pain hypersensitivity (Voscopoulus *et al.*, 2010; Brennan, 2011; Romero *et al.*, 2012a). Accordingly, 4 h after peripheral surgery of WT mice (at maximum mechanical and thermal hypersensitivity), selective up-regulation of pERK and c-Fos expression in the ipsilateral dorsal horn of WT mice was observed, thus suggesting a concomitant action leading to a central sensitization process. The absence of σ_1R attenuated the ipsilateral increase of both pERK and c-Fos immunoreactivity 4 hours after injury, coinciding with a faster recovery of mechanical allodynia to baseline. As in the present study, the absence of σ_1R prevented the increase in pERK and attenuated the development of mechanical allodynia in the PSNL model (De la Puente *et al.*, 2009) and cold allodynia in the paclitaxel model (Nieto *et al.*, 2012). pERK is up-regulated in neuropathic pain processes underlying the development of mechanical and cold allodynia in WT mice (Romero *et al.*, 2012b). Campillo and coworkers (2010) reported partial colocalization

of pERK and c-Fos in the superficial laminae of the dorsal horn in the same model, both regulating prodynorphin mRNA levels, which roughly correlated with the time course of postoperative pain sensitization. c-Fos is an immediate-early gene whose expression in the spinal cord has been extensively used as a marker of peripheral noxious stimulation and "early" pain (Jinks *et al.*, 2002; Coggeshall 2005; Campillo *et al.*, 2010). It has been widely reported to be increased in several animal models of nociception, including paw inflammation (carrageenan and complete Freund's adjuvant models), postoperative pain, neuropathic injury, and cancer pain (Coggeshall, 2005; Campillo *et al.*, 2010; Romero *et al.*, 2012a; Arun *et al.*, 2013; Hossaini *et al.*, 2014). In the present study, the immunohistochemistry images in operated WT mice showed a specific spot (medial zone of the dorsal horn of the spinal cord) in the c-Fos-immunoreactive neurons where they were preferentially located. Interestingly, a previous report in which the tibial branch of the sciatic nerve was ligated (Corder *et al.*, 2010) showed that the topography (distal and central termination sites) of the protein expression was located in the same area as in our study. Thus, although no dissection of the nerve ending was performed in our plantar incision model, adjacent areas of the tibial nerve might have been altered. c-Fos was clearly induced in ipsilateral laminae I-II of the dorsal horn of the spinal cord of WT mice, consistent with that previously observed (Sun *et al.*, 2004). In our study, no differences in c-Fos expression between both control (sham) groups (WT and σ_1R KO mice) were observed. However, c-Fos immunoreactivity was ipsilaterally attenuated in σ_1R KO spinal cords 4 h after surgery as compared to WT slices, thus agreeing with a previous work by Yamada *et al.* (2006) where σ_1R agonists induced c-Fos expression in the CNS.

Expression changes in the spinal cord of GFAP, nNOS and neuropeptides SP and NPY were also studied. Consistent with previous studies where an increased GFAP

expression following plantar incision was observed and associated with the initiation and maintenance of mechanical hypersensitivity (Romero-Sandoval *et al.*, 2008), an up-regulation of GFAP in both sides of the spinal cord 4 h after surgical incision was observed. The induction of spinal GFAP following chronic constriction injury has been recently related to σ_1R in both astrocytric expression and pain-related behaviours (Moon *et al.*, 2014). Consistently, a significant attenuation in GFAP immunoreactivity in σ_1R KO mice *versus* WT mice was found in the present study.

It has been reported that NO produced by nNOS in the spinal cord participates in the early induction (Levy and Zochodne, 2004) and the maintenance (Xu *et al.*, 2007; Roh *et al.*, 2011) of neuropathic pain. However, in our study, no significant changes in nNOS expression 4 h after plantar incision in WT mice were observed, and nNOS expression was unchanged by the absence of σ_1R. A possible explanation to these observations could be that changes in NO production are not only linked to nNOS expression in the spinal cord, but also to the relationship between the phosphorylated and non-phosphorylated forms of nNOS. At this regard, intratecal administration of PRE-084, a σ_1R agonist, produces mechanical and thermal hypersensitivity which is linked to a decreased ratio of phosphorylated nNOS to nNOS expression. Both hypersensitivity and changes in nNOS phosphorylation were prevented by the σ_1R antagonist BD-1047 (Roh *et al.*, 2011).

In our study, in line with the fact that SP is synthesized in DRG and mainly released from primary afferent neurons to the periphery in the post-surgery acute phases (Yaksh *et al.*, 1980; Adelson *et al.*, 2009; Chen and Marvizon, 2009a; Taylor *et al.*, 2014), spinal SP did not achieve statistical significance difference in WT mice. The absence of σ_1R did not alter significantly the expression of SP. However, spinal NPY expression was bilaterally (both dorsal horns) up-regulated in WT mice and attenuated

in σ_1R KO mice 4 h after plantar incision. It has been shown that NPY acts at neuropeptide Y1 receptors in the dorsal horn to decrease nociception by inhibiting SP release (Taylor *et al.*, 2014). Further molecular studies at different time points would be necessary to understand a possible involvement of σ_1R in the pattern expression of these neuropeptides. In fact, it is known that NPY-induced increases in hippocampal dopamine can be mediated by σ_1R (Meurs *et al.*, 2007), but no studies had been performed to link NPY with σ_1R at the spinal cord.

The pharmacological blockade of σ_1R with E-52862 attenuated the behavioural hypersensitivity to thermal and mechanical stimuli in the plantar incision model 4 hours after injury, although the absence of σ_1R (KO mice) failed to prevent the acquisition of pain-related behavioural hypersensitivity 4 h after surgery. To confirm that the efficacy of E-52862 in this particular model was due to the specific pharmacological blockade of σ_1R *in vivo*, we tested whether E-52862 shows activity in the absence of its putative target. No effects in σ_1R KO mice were found using E-52862 doses active in WT mice, thus suggesting the involvement of σ_1R in E-52862-mediated effects on mechanical allodynia and thermal hyperalgesia. This result is consistent with recent reports where E-52862 was devoid of efficacy when administered to σ_1R KO mice in other pain models (Gris *et al.*, 2014; Tejada *et al.*, 2014). These results bring out the difference between the effect of genetic blockade (i.e. the absence of the receptor and development of adaptive changes) and the pharmacological blockade of σ_1R (i.e. the modulatory effect of a ligand at the time of the test) (Zamanillo *et al.*, 2013). Similar results were also obtained with other pain-related behaviours such as inflammatory pain or PSNL-induced heat hyperalgesia (De la Puente *et al.*, 2009; Gris *et al.*, 2014), where the absence of σ_1R did not affect the development of behavioural hypersensitivity but pharmacological blockade was able to attenuate these pain behaviours.

In the present study, the analgesic efficacy of E-52862 was compared to morphine, ibuprofen and celecoxib in the postoperative pain model. Morphine produced a dose-dependent antinociceptive effect, whereas ibuprofen and celecoxib showed very low activity. Although the analgesic efficacy of opioids and NSAIDs (celecoxib and ibuprofen) have been validated in postoperative pain and are commonly used in the clinical setting (Derry and Moore, 2013), these compounds failed to be strongly effective in our study. A previous study using the same model but in rats found partial antihypersensitivity effects of celecoxib (Emax = 47%) and naproxen (Emax = 43%), whereas indomethacin and morphine reached >75% efficacy (Whiteside *et al.*, 2004). On the contrary, full antihypersensitivity effect of oral celecoxib has been reported in mice (Oliveira *et al.*, 2014). The reason for these discrepancies is unclear, but differences in the experimental protocols could account for them. The fact that mechanical hypersensitivity was resistent to the action of opioids and NSAIDs might suggest that these drugs are not fully effective for this type of pain. Opioid resistance is present in other painful conditions such as neuropathic pain (Benedetti *et al.*, 1998). In this sense, surveys have shown that approximately half of all patients experience moderate to severe pain after surgery, thus suggesting that postoperative pain is still poorly treated despite the use of available drugs (Pogatzki-Zahn *et al.*, 2007). Drugs that are clinically used to produce postoperative analgesia are NSAIDs, opioids, ketamine, peripheral local anesthetics, and gabapentin (Oliveira *et al.*, 2014). However, because these drugs are known to cause side effects that often limit their use (Dahl and Kehlet, 2006), the development of more effective drugs with fewer side effects for the treatment of postoperative pain is necessary.

In summary, while no differences between WT and σ_1R KO mice were found in the development of thermal hypersensitivity, σ_1R KO mice did show a faster recovery in

mechanical hypersensitivity and an attenuation of pERK, c-Fos, GFAP and NPY in the dorsal horn of the spinal cord 4 h after injury, i.e. at the maximal mechanical and thermal hypersensitivty. E-52862 exerted antinociceptive effects in both pain-related behaviours, comparing favourably with morphine and NSAIDs celecoxib and naproxen. The results of the present study suggest that σ_1R plays an important role in postoperative pain and support that σ_1R antagonists, particularly E-52862, could be effective for treating postoperative pain.

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3.2. Annex 2: *"Spinal modulation of pain-related molecular markers by genetic inactivation of σ_1R in postoperative models".*

The role of σ_1R in postoperative pain is investigated for the first time, using either genetic (σ_1R KO mice) or pharmacological (S1RA) tools, in Article 4. The immunohitochemistry results were expressed as percentages of change *versus* control animals for each hemicord (contralateral and ipsilateral side).

Annex 2 shows representative slices from immunohistochemistry studies and expresses the results of Article 4 as absolute values of immunopositive neurons (c-Fos, nNOS and NPY) or mean grey intensity values (GFAP and SP) of the ipsilateral dorsal hemicord.

The material and methods used are the same as those described in Article 4 (immunohistochemistry methods).

Table 1 shows a summary of the spinal expression changes in the pain-related molecular markers analysed in the postoperative model, and how the lack of σ_1R interferes in the expression pattern. The expression changes of pain-related markers observed in WT and KO mice are different when the two mice are compared: the lack of σ_1R attenuated ERK, c-Fos, GFAP and NPY up-regulation induced 4 hours after paw incision. These differences contrast with previous findings where the inflammatory injury is used (Annex 1). Acute inflammation failed to substantially alter the expression pattern while the chronic inflammatory injury tends to increase the expression in KO mice.

Therefore, changes in the spinal expression pattern (ERK, c-Fos, GFAP, nNOS, SP and NPY) in the absence of σ_1R depends on the nature of the injury and the

mechanism that triggers sensitization in the mouse (acute or chronic inflammation or acute postsurgery).

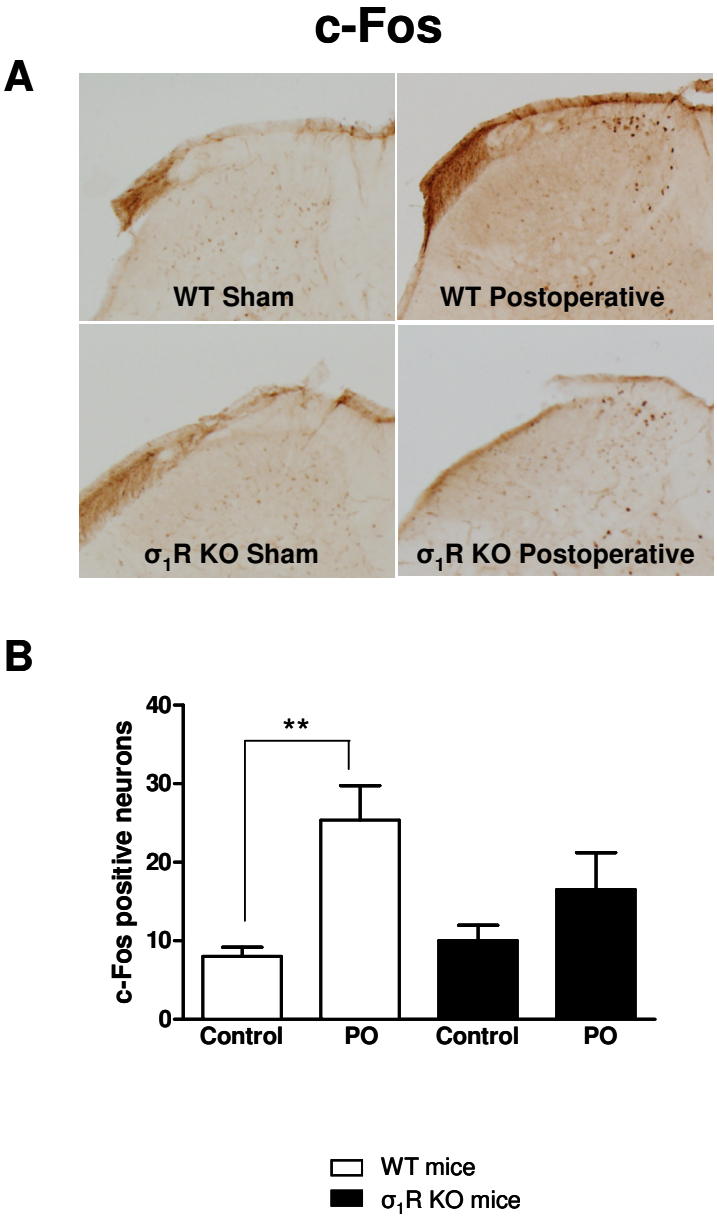

Fig. 1. c-Fos expression in the ipsilateral dorsal horn of the SC secondary to plantar incision in WT and σ_1R KO mice. Representative slices of c-Fos immunostaining in a dorsal horn of the SC of sham and operated WT and σ_1R KO mice 4h after surgery (A). Quantification of c-Fos as number of immunoreactive c-Fos neurons in the ipsilateral dorsal horn of the SC secondary to plantar incision in WT and σ_1R KO mice (B). Statistically significant differences comparing each control group in WT and σ_1R KO mice: ** $p < 0.01$ (two-way ANOVA followed by Bonferroni test). Each bar and vertical line represents the mean ± SEM of the values obtained in 4 animals.

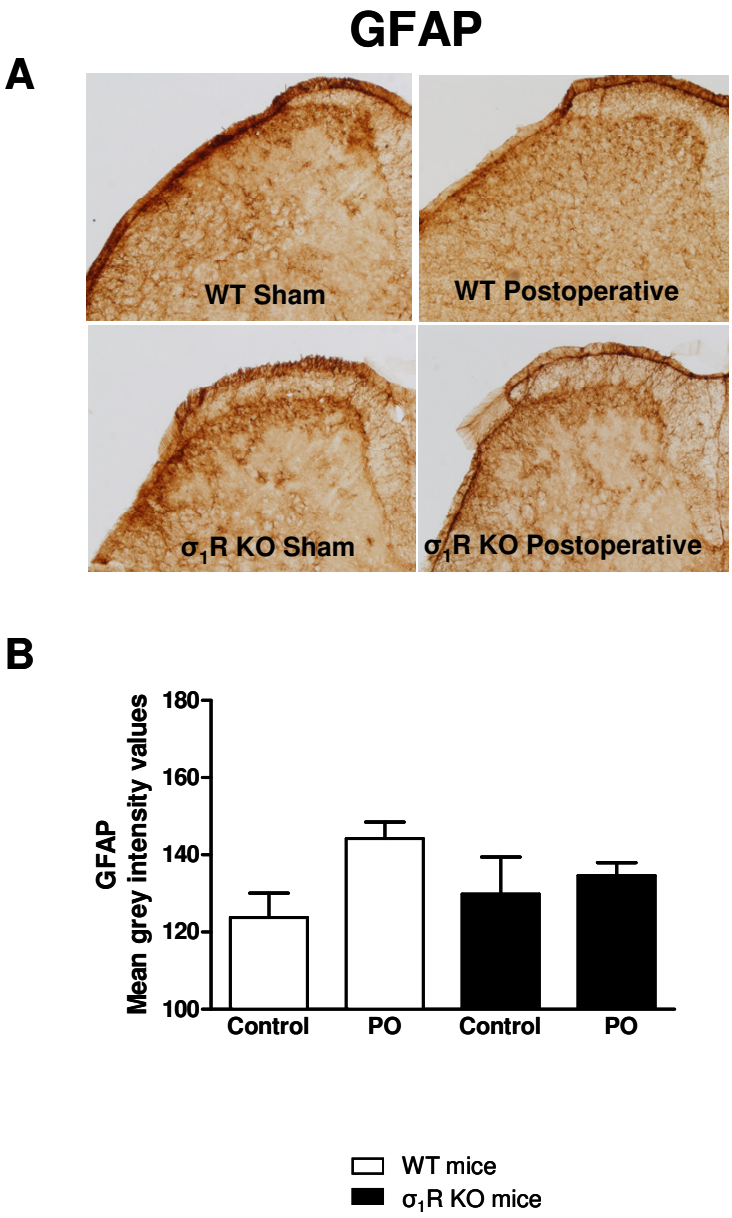

Fig. 2. GFAP expression in the ipsilateral dorsal horn of the SC secondary to plantar incision in WT and σ_1R KO mice. Representative slices of GFAP immunostaining in a dorsal horn of the SC of sham and operated WT and σ_1R KO mice 4h after surgery (A). Quantification of GFAP immunoreactivity (expressed in mean grey intensity values) in the ipsilateral dorsal horn of the SC secondary to plantar incision in WT and σ_1R KO mice (B). No significant differences *versus* corresponding control group (two-way ANOVA followed by Bonferroni test). Each bar and vertical line represents the mean ± SEM of the values obtained in 4 animals.

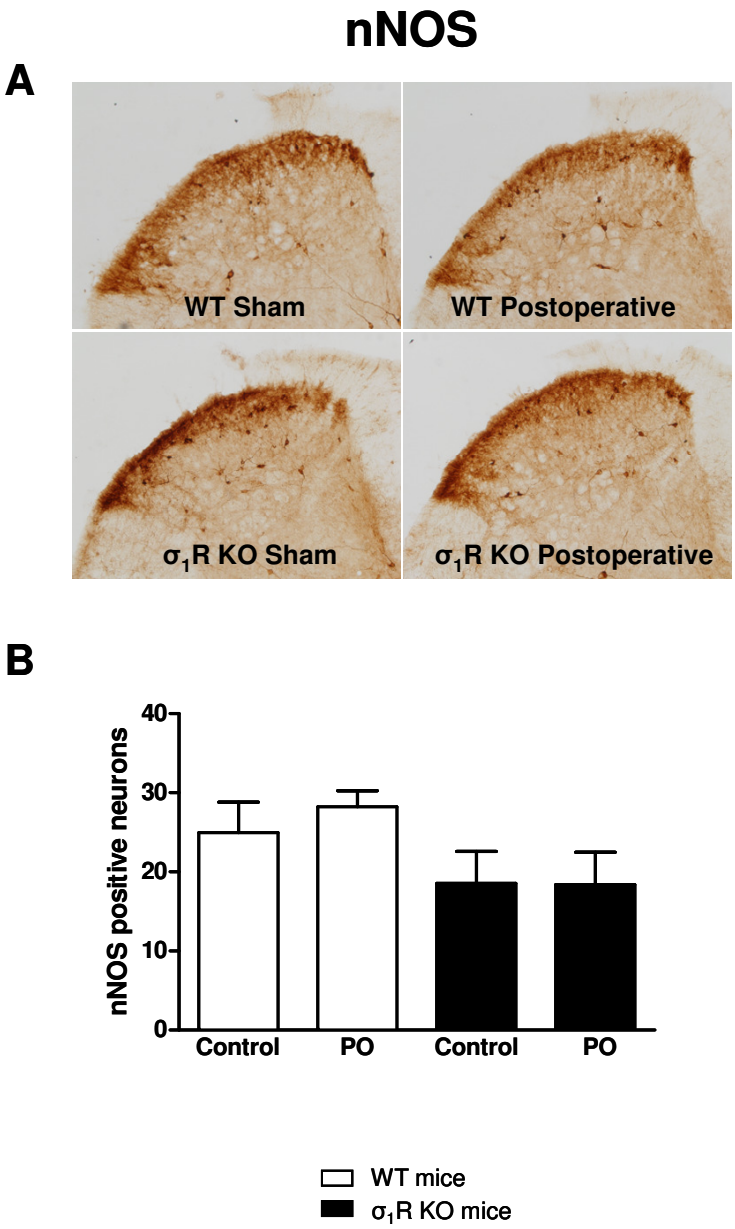

Fig. 3. nNOS expression in the ipsilateral dorsal horn of the SC secondary to plantar incision in WT and σ_1R KO mice. Representative slices of nNOS immunostaining in a dorsal horn of the SC of sham and operated WT and σ_1R KO mice 4h after surgery (A). Quantification of nNOS as number of immunoreactive nNOS neurons in the ipsilateral dorsal horn of the SC secondary to plantar incision in WT and σ_1R KO mice (B). No significant differences *versus* corresponding control group (two-way ANOVA followed by Bonferroni test). Each bar and vertical line represents the mean ± SEM of the values obtained in 4 animals.

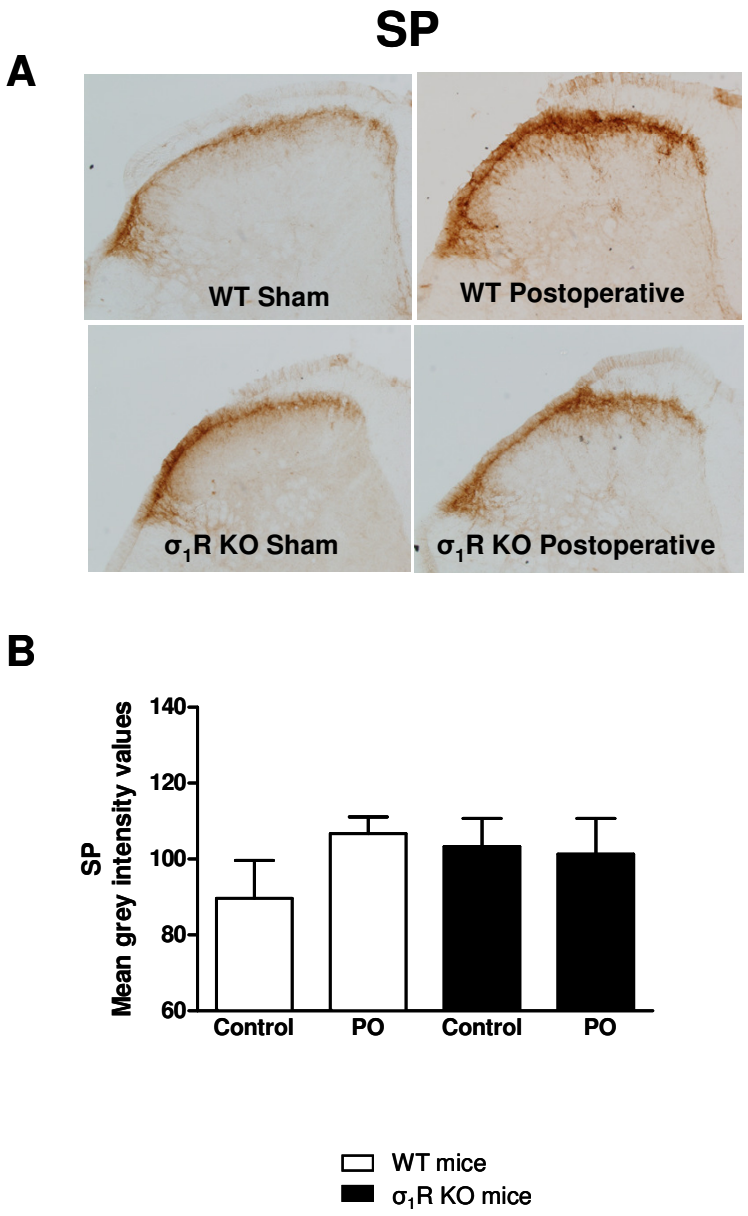

Fig. 4. SP expression in the ipsilateral dorsal horn of the SC secondary to plantar incision in WT and σ_1R KO mice. Representative slices of SP immunostaining in a dorsal horn of the SC of sham and operated WT and σ_1R KO mice 4h after surgery (A). Quantification of SP immunoreactivity (expressed in mean grey intensity values) in the ipsilateral dorsal horn of the SC secondary to plantar incision in WT and σ_1R KO mice (B). No significant differences *versus* corresponding control group (two-way ANOVA followed by Bonferroni test). Each bar and vertical line represents the mean ± SEM of the values obtained in 4 animals.

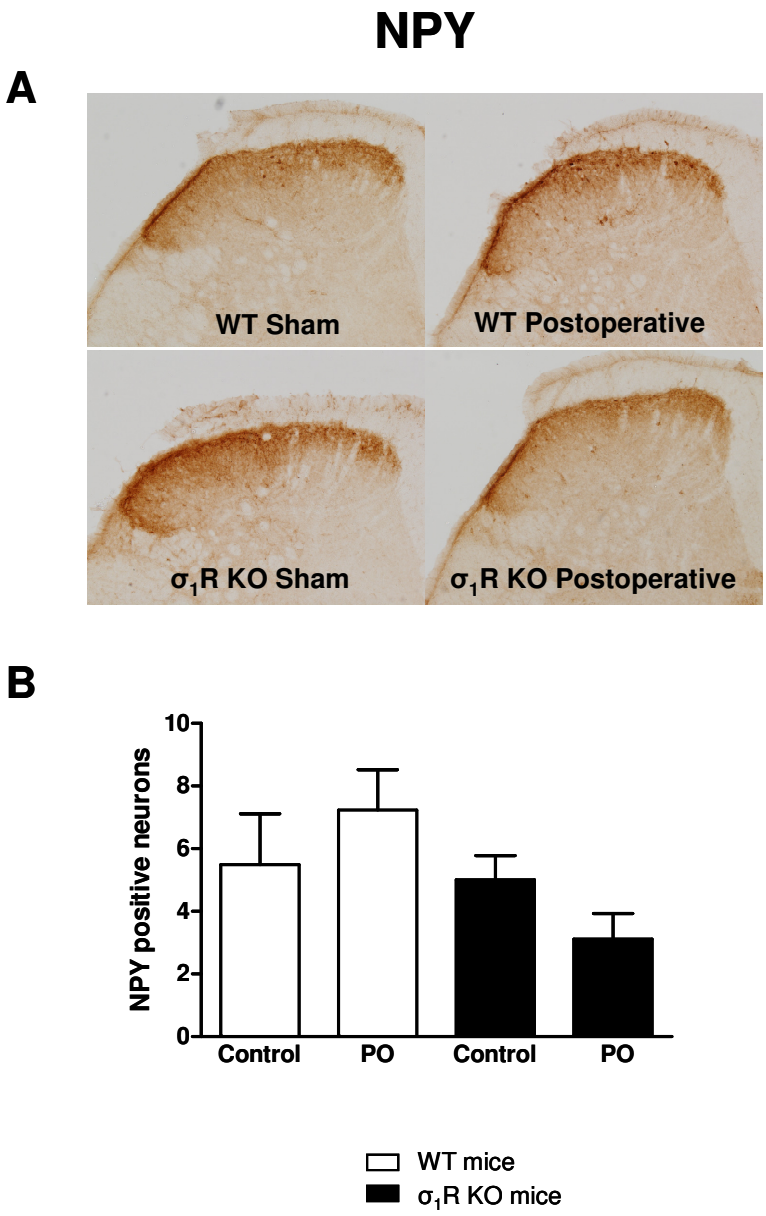

Fig. 5. NPY expression in the ipsilateral dorsal horn of the SC secondary to plantar incision in WT and σ_1R KO mice. Representative slices of NPY immunostaining in a dorsal horn of the SC of sham and operated WT and σ_1R KO mice 4h after surgery (A). Quantification of NPY as number of immunoreactive NPY neurons in the ipsilateral dorsal horn of the SC secondary to plantar incision in WT and σ_1R KO mice (B). No significant differences *versus* corresponding control group (two-way ANOVA followed by Bonferroni test). Each bar and vertical line represents the mean ± SEM of the values obtained in 4 animals.

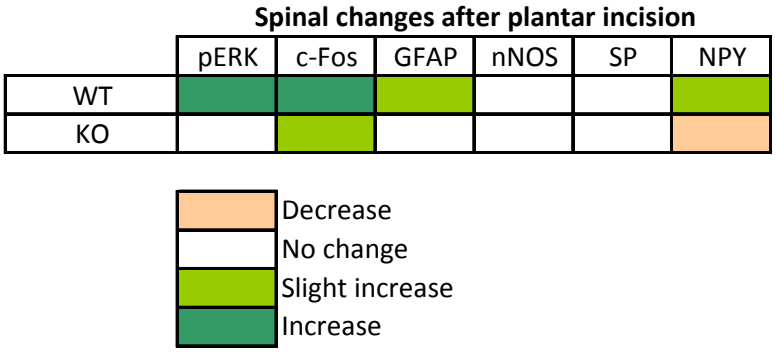

Table 1. Summary of the spinal expression of the pain-related molecular markers used in this study in WT and σ_1R KO mice in the plantar incision model of postoperative pain. Significant protein up-regulation is shown in dark green, slightly increased or tendency to protein up-regulation (no significant) is shown in light green, and significant protein down-regulation is shown in orange, according to previous discussion in Article 4. The lack of σ_1R seems to differentially affect the expression pattern in the postoperative model.

GENERAL DISCUSSION

The overall purpose of this Doctoral Thesis was to explore the role of σ_1R and its pharmacological blockade under important clinical pain conditions of different aetiology. To this end, two species, six pain-related animal models and different pharmacological, molecular and genetic approaches were used. The pharmacological antagonism of σ_1R was found to cause a reduction of the different pain behaviours in both species and in all pain models. Interestingly, a cumulative analgesic effect when σ_1R was chronically blocked, as well as a better analgesic profile as compared to some reference compounds in some of the pain models evaluated, were observed. Furthermore, the possible therapeutic effects of the pharmacological blockade of σ_1R for trigeminal-related and diabetic painful neuropathies, as well as in inflammatory and postoperative pain, has been demonstrated for the first time. Finally, we also found that σ_1R acts through different mechanisms depending on the type of pain, as evidenced by the differential expression patterns of several pain-related molecular markers. All these results strongly support the involvement of σ_1R in pain-related syndromes of different aetiology. Since most of our results have been obtained after the administration of E-52862 —a selective σ_1R compound developed by ESTEVE— the present Doctoral Thesis supports to move E-52862 forward into clinical development as a new therapeutic approach to pain management.

The potency and efficacy following acute treatment with E-52862 were different depending on the experimental model (Table 1). The highest antihypersensitivity effect obtained with E-52862 was found in inflammatory pain models and oxaliplatin-induced neuropathy. The inhibitory effects of E-52862 on pain-related hypersensitivity were not due to an antioedematous action because E-52862 was unable to reduce paw oedema in the carrageenan model. Moreover, these effects have shown to be specifically mediated by σ_1R given that E-52862 was devoid of any antinociceptive effect when administered

to σ_1R KO mice . The efficacy of σ_1R antagonism on oxaliplatin-induced neuropathy is consistent with previous reports demonstrating the involvement of σ_1R in chemotherapy (paclitaxel)-induced neuropathy in mice (Nieto *et al.*, 2012, 2014). Furthermore, our results obtained with a translational experimental model of facial pain support targeting σ_1R as a new strategy for the treatment of cephalic pain, including migraine. Accordingly, two previous reports with other σ_1R antagonist (BD1047) have shown some benefit in reducing acute facial pain in the formalin- and capsaicin-induced headache model (Kwon *et al.*, 2009; Roh and Yoon, 2014).

The antihypersensitivity (antiallodynic and antihyperalgesic) effects of E-52862 in neuropathic (diabetic and trigeminal) and postoperative pain models were superior than those obtained with some reference compounds (NSAIDs and pregabalin), which were unable to exert a significant effect.

Table 1. Antihypersensitivity effects of E-52862 administered by i.p. route. Abbreviations: n.a.: not applicable.

Pain type	Experimental pain model	Specie	Readout	Effect of E-52862 (Maximum efficacy reached; tested dose)	KO phenotype
Neuropathic pain	Chronic constriction of the infraorbital nerve (trigeminal)	Rats	Mechanical allodynia	33% (40mg/kg, acute) 62% (40mg/kg, repeated)	n.a.
	Streptozotocin-induced diabetic neuropathy		Mechanical hyperalgesia	43% (80mg/kg, acute) 46% (40mg/kg, repeated)	n.a.
	Oxaliplatin-induced chemotherapy neuropathic pain		Cold allodynia	100% (80mg/kg, acute) 93% (80mg/kg, repeated)	n.a.
Inflammatory pain	Carrageenan-induced acute inflammatory pain	Mice	Mechanical allodynia	88% (80 mg/kg, acute)	Similar to WT
			Thermal hyperalgesia	100% (80 mg/kg, acute)	Similar to WT
	CFA-induced chronic inflammatory pain	Mice	Mechanical allodynia	85% (80 mg/kg, acute)	Similar to WT
			Thermal hyperalgesia	n.a.	Similar to WT
Postoperative pain	Plantar incision-induced postoperative pain	Mice	Mechanical allodynia	70% (160 mg/kg, acute)	Attenuated
			Thermal hyperalgesia	58% (160 mg/kg, acute)	Similar to WT

No tolerance but increased activity respect to the acute treatment was found following repeated E-52862 administration in diabetic and oxaliplatin-treated rats and was not associated with drug accumulation (Romero *et al.*, 2012). While we cannot assume that E-52862 is a disease-modifying analgesic drug, we can assure that its effects last longer than expected based on its pharmacokinetic profile, which suggests a sustained pharmacodynamic effect (i.e., a modifying effect on the underlying baseline pain over time due to the continued action of the compound that results in a progressive attenuation of pain).

A genetic approach using σ_1R KO mice was used to study the involvement of this receptor in the development of inflammatory and postoperative pain, as well as to study the spinal expression of some key protein mediators involved in pain pathways (Table 2).

No differences in pain hypersensitivity were found in the carrageenan and CFA models when WT and σ_1R KO mice were compared. Moreover, whereas carrageenan did not elicit any significant difference in protein expression between mouse types, CFA did produce an increased pattern in σ_1R KO mice *versus* WT mice, probably due to the chronic nature of the CFA model. Interestingly, σ_1R KO mice recovered normal mechanical sensitivity faster than WT mice following plantar incision, thus suggesting that σ_1R plays a role in postoperative pain. The attenuated pattern in the expression of pain-related molecular markers (pERK, c-Fos, GFAP, SP and NPY) observed in σ_1R KO mice in postoperative pain also support the role played by σ_1R in this particular type of pain (Table 2 and 3).

Table 2. Summary of the spinal expression of the pain-related molecular markers in WT and σ_1R KO mice in the carrageenan and CFA inflammatory pain models and postoperative pain model of plantar incision.

Spinal changes after pain induction							
Pain model	**Genotype**	**pERK**	**c-fos**	**GFAP**	**nNOS**	**SP**	**NPY**
Carrageenan	WT						
	KO						
CFA	WT						
	KO						
Postoperative	WT						
	KO						

Decrease
No change
Slight increase
Increase

Table 3. Behavioural and molecular effects of the genetic blockade of σ_1R.

Pain type	Experimental pain model	Mechanical hypersensitivity	Protein expression pattern
Inflammatory pain	Carrageenan-induced acute inflammatory pain	Similar to WT	Similar to WT
	CFA-induced chronic inflammatory pain	Similar to WT	Increased pattern versus WT
Postoperative pain	Plantar incision-induced postoperative pain	Attenuation versus WT	Attenuation versus WT

Some limitations of the present study include the fact that behavioural studies have focused on reflex withdrawal responses that only rely on the sensory-discriminative component of pain. Also, the molecular studies have been performed at one time point. The investigation of molecular markers at different time points and in DRGs and additional somatosensory areas would be interesting, as it would be the investigation of the pharmacological modulation of pain-related changes of these molecular markers by σ_1R ligands.

In summary, the present Doctoral Thesis has demonstrated the potential therapeutic use of σ_1R antagonists for the clinical management of pain in a wide range of aetiologies, thus supporting progress to further studies in human populations.

CONCLUSIONS

Specific conclusions:

1.- Selective pharmacological σ_1R blockade with E-52862 was active in reducing mechanical and cold allodynia and mechanical hyperalgesia when administered acutely and after repeated administration in trigeminal neuralgia, chemotherapy-induced neuropathic pain and diabetic painful polyneuropathy, respectively. Preventive treatment with E-52862 inhibited the oxaliplatin-induced development of cold allodynia.

2.- Repeated daily treatment with E-52862 produced an increased antinociceptive effect and attenuated baseline pain behaviours, thus supporting not only a lack of tolerance but also a sustained inhibitory effect on underlying pain-generating mechanisms.

3.- Intraplantar injection of **carrageenan** caused both mechanical allodynia and thermal (heat) hyperalgesia that peaked at 3 h and reverted to baseline levels 3–4 days after injection in WT mice. The genetic inactivation of σ_1R failed to prevent the development of mechanical and heat hypersensitivity. Three hours after carrageenan injection c-Fos and GFAP expression was equally up-regulated in the dorsal horn of the spinal cord of inflamed WT and σ_1R KO mice. In contrast, nNOS expression was not modified in the dorsal horn of the spinal cord of WT mice, whereas it was clearly up-regulated in σ_1R KO mice. Finally, pERK, SP and NPY expressions were not modified in either WT or σ_1R KO mice.

4.- Acute systemic administration of E-52862 dose-dependently inhibited both mechanical and thermal (heat) hypersensitivity induced by carrageenan in WT mice. E-52862 failed to exert any antihypersensitivity effect in σ_1R KO mice suggesting that the

analgesic activity induced by E-52862 in this model was clearly mediated by the interaction with σ_1R. Unlike NSAIDs (ibuprofen and celecoxib), E-52862 did not modify the paw volume.

5.- Intraplantar injection of **CFA** produced a robust and long-lasting mechanical allodynia (not returning to baseline levels until day 11), but no or only mild thermal (heat) hyperalgesia in WT mice. The genetic inactivation of σ_1R failed to prevent the development of mechanical allodynia. Four days after CFA injection c-Fos expression was not modified in the dorsal horn of the spinal cord of WT mice, whereas it was ipsilaterally up-regulated in σ_1R KO mice. SP expression was reduced in the dorsal horn of the spinal cord of WT mice, and this change was attenuated in the σ_1R KO mice. Conversely, the NPY expression was not modified in WT mice, while it was decreased in σ_1R KO mice. Finally, pERK, GFAP and nNOS expressions were not modified in either WT or σ_1R KO mice.

6.- Acute systemic administration of E-52862 dose-dependently inhibited mechanical allodynia induced by CFA in WT mice. E-52862 failed to exert any antihypersensitivity effect in σ_1R KO mice, thus suggesting that the analgesic activity induced by E-52862 in this model was clearly mediated by the interaction with σ_1R.

7.- Plantar incision caused both mechanical and thermal (heat) hypersensitivity that peaked at 4 h and reverted to baseline levels 9 days after surgery in WT mice. σ_1R KO mice showed a faster recovery in mechanical hypersensitivity, whereas no differences were observed in WT and σ_1R KO mice in the acquisition of thermal hyperalgesia after plantar incision. Four hours after surgery the expression of pERK, c-Fos, GFAP and

NPY — but not nNOS and SP— were up-regulated in the dorsal horn of the spinal cord in WT mice, and these changes were attenuated in σ_1R KO mice.

8.- Unlike NSAIDs (ibuprofen and celecoxib), the acute systemic administration of E-52862 dose-dependently inhibited both mechanical and heat hypersensitivity induced by paw incision in WT mice. E-52862 was less potent but showed an efficacy similar to that of morphine. E-52862 failed to exert any antihypersensitivity effect in σ_1R KO mice, thus suggesting that the analgesic activity induced by E-52862 in this model was clearly mediated by the interaction with σ_1R.

General conclusion:

The pharmacological blockade of σ_1R represents a promising new therapy for the treatment of pain of different aetiologies such as neuropathic, inflammatory and postoperative pain. The *in vivo* efficacy observed in two species (mouse and rat), supports moving E-52862 forward into clinical development as a new therapeutic approach to pain management.

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APPENDIX

Other publications

1. ***"Sigma-1 receptors are essential for capsaicin-induced mechanical hypersensitivity: Studies with selective sigma-1 ligands and sigma-1 knockout mice".*** Pain. 2009 Jun;143(3):252-61. Entrena JM, Cobos EJ, Nieto FR, Cendán CM, **Gris G**, Del Pozo E, Zamanillo D, Baeyens, JM.

2. ***"Analgesic efficacy in rat models of experimental pain of a new selective sigma-1 receptor antagonist"***. Pain in Europe. Lisboa, 2009. European Journal of Pain, Volume 13, Supplement 1, September 2009, Page S102. **Gris G**, Vidal A, Fort M, Aubel B, Romero L, González A, Portillo-Salido E, Baeyens JM, Vela JM, Deseure K, Zamanillo D.

3. ***"Pharmacological characterization of a new selective sigma-1 receptor antagonist showing antihyperalgesic and antiallodynic activity in different pain models in mice".*** European Journal of Pain, Volume 13, Supplement 1, September 2009, Page S104. Zamanillo D, Burgueño J, Nadal X, Dordal A, **Gris G**, Vidal A, Romero L, Laloya M, Aubel B, Segalés C, Baeyens JM, López-García JA, Maldonado R, Vela JM.

4. ***"In vivo characterisation of the new selective sigma-1 receptor antagonist (S1RA): Potential use for the treatment of neuropathic pain".*** Neuropathic pain Congress. Athens, 2010. European Journal of Pain Supplements, Volume 4, Issue 1, April 2010, Page 52.Fort M, **Gris G**, Aubel B, Vidal A, Romero L, González A, Portillo-Salido E, Darbaky Y, Deseure K, Vela JM, Zamanillo D.

5. ***"A new selective sigma-1 receptor antagonist (S1RA) reduces neuropathic pain behaviors and activity-induced spinal sensitisation".*** Neuropathic pain Congress. Athens, 2010. European Journal of Pain Supplements, Volume 4, Issue 1, April 2010, Page 63. Zamanillo D, Romero L, Burgueño J, Nadal X, Dordal A, **Gris G**, Vidal A, Laloya M, Segalés C, Portillo-Salido E, Baeyens JM, López-García JA, Maldonado R, Vela JM.

6. ***"Analgesic efficacy in rat models of experimental neuropathic pain of a new selective sigma-1 receptor antagonist S1RA"***. 13th World congress on Pain. Montreal, 2010. Vela JM, Darbaky Y, **Gris G**, Fort M, Aubel B, Vidal A, Romero L, González A, Portillo-Salido E, Deseure K, Zamanillo D.

7. ***"Role of the sigma-1 receptor (σ_1R) in postoperative pain in mice".*** 15th World congress on Pain. Buenos Aries, 2014. **Gris G**, Portillo-Salido E, Vela JM, Merlos M, Zamanillo D.

8. ***"Comparison of antinociceptive effects of standard analgesics in attenuating carrageenan-induced mechanical allodynia and thermal hyperalgesia in rats".*** 9th congress of the European Pain Federation. Viena, 2015. Aubel B, Portillo-Salido E, **Gris G**, Merlos M, Zamanillo D.