Selective antagonism of sigma-1 receptors as a new strategy for pain treatment.

Behavioural and neurochemical studies

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ESTEVE

This thesis is gratefully dedicated to Àlex
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II. ABSTRACT

English abstract

In the present thesis, and in the context of the Sigma-1 receptor (σ_1R) research project running at the pharmaceutical company ESTEVE, we addressed, at the preclinical level, the role played by selective σ_1R antagonism/blockade in nociception in order to ascertain the therapeutic interest of σ_1R antagonists in pain treatment. Taking advantage of using selective σ_1R pharmacological tools and the *in vivo* microdialysis technique, the mechanism and the site of action of selective antagonism of σ_1R were investigated in pain conditions involving sensitization (formalin model) as well as in acute thermal pain (tail-flick test) to look at the modulation of opioid-induced efficacy and safety-related outcomes. The results of this thesis provide new knowledge about σ_1R as a pain target suitable for therapeutic intervention to get analgesia and support the use of σ_1R antagonists as opioid adjuvants to treat pain conditions.

Resum en català

En aquesta tesi, i en context amb el projecte de recerca sobre el receptor Sigma-1 (σ_1R) que té lloc a l'empresa farmacèutica ESTEVE, hem estudiat, a nivell preclínic, el paper de l'antagonisme/bloqueig selectiu del σ_1R en nocicepció, per tal de determinar l'interès terapèutic dels antagonistes del σ_1R per al tractament del dolor. Mitjançant l'ús d'eines farmacològiques selectives per al σ_1R i de la tècnica de microdiàlisi *in vivo*, el mecanisme i el lloc d'acció de l'antagonisme selectiu del σ_1R van ser estudiats en condicions de dolor que impliquen sensibilització (model de la formalina) així com en dolor agut (test del tail-flick) on vam analitzar la modulació de l'eficàcia produïda pels opioides i paràmetres de seguretat. Els resultats d'aquesta tesi aporten nou coneixement sobre el σ_1R com a diana terapèutica idònia per obtenir analgèsia i reafirmen l'ús d'antagonistes del σ_1R com a adjuvants als opioides per al tractament del dolor.

III. ABBREVIATIONS

5-HT: serotonin

ADME: absorption, distribution, metabolism, and excretion

AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

ANOVA: analysis of variance

AR: adrenoceptors

Asp: aspartate

AUC: area under the curve

BD-1047: *N*-[2-(3,4-Dichlorophenyl)ethyl]-*N*-methyl-2-(dimethylamino)ethylamine dihydrobromide

BD-1063: 1-[2-(3,4-Dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride

BiP: binding immunoglobulin protein

BMY14802: α-(4-Fluorophenyl)-4-(5-fluoro-2-pyrimidinyl)-1-piperazinebutanol hydrochloride

Ca^{2+}: calcium ion

CAMKs: calcium/calmodulin-dependent kinases

cAMP: cyclic adenosine monophosphate

CAR: carbetapentane

CB1 / CB2: cannabinoid receptor 1/2

cDNA: complementary deoxyribonucleic acid

CeA: central nucleus of the amygdala

CGRP: calcitonin gene-related peptide

CI: confidence intervals

CNS: central nervous system

COMT: catechol-O-methyl transferase

COX-2: cyclooxygenase-2

CPP: conditioned place preference

CSF: cerebrospinal fluid

CYP’s: cytochrome’s

D_1R / D_2R: dopamine D_1 / D_2 receptor

DA: dopamine

DAG: diacylglycerol

DH: dorsal horn

DHEA / DHEA-S: dehydroepiandrosterone / dehydroepiandrosterone sulphate ester

DMT: *N-N*-dimethyltryptamine

DRG: dorsal root ganglia

DRN: dorsal raphe nucleus

E-52862: see S1RA

E-5842: (4-(4-fluorophenil)-1,2,3,6- tetrahydro-1-[4-(1,2,4-triazol-1-il)butyl] pyridine citrate)

ED_{50}: effective dose 50

EPSPs: excitatory post-synaptic potentials

ER: endoplasmic reticulum

ERK: extracellular signal-regulated kinase

FDA: food and drug administration

Fig.: figure

FMO's: flavin monooxygenase's

GABA: gamma-aminobutyric acid

GFAP: glial fibrillary acidic protein

Glu: glutamate

GluR: glutamatergic receptors

GPCRs: G-protein-coupled receptors

GTPγS: guanosine 5'-O-[gamma-thio]triphosphate

HPLC: high-performance liquid chromatography

HPMC: hydroxypropyl methyl cellulose

IC_{50}: half maximal inhibitory concentration

i.c.v.: intracerebroventricular

i.p.: intraperitoneal

i.pl.: intraplantar

i.t.: intrathecal

IASP: international association for the study of pain

IP_3: inositol-1,4,5-trisphosphate

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

K^+: potassium ion

KAR: kainate receptor

KCl: potassium chloride

kDa: kilodalton

Ki: inhibition constant

LC: locus coeruleus

LT: low treshold

LTP: long-term potentiation

MAM: mitochondria-associated endoplasmic reticulum membrane

MAO: monoamine oxidase

Mg^{2+}: magnesium ion

mGluR: metabotropic glutamatergic receptors

(+)-MR200: [(+)-methyl (1R,2S)-2-{[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]methyl}-1-phenylcyclopropanecarboxylate]

NA: noradrenaline

Na^{+}: sodium ion

N.D.: not determined

NE100: 4-Methoxy-3-(2-phenylethoxy)-*N*,*N*-dipropylbenzeneethanamine hydrochloride

NET: noradrenaline transporter

NMDAR: *N*-methyl-D-aspartate receptor

NOS: nitric oxide synthase

nNOS: neuronal nitric oxide synthase

NPY: neuropeptide Y

NR1: N-methyl-D-aspartate receptor NR1 subunit

NRM: nucleus raphe magnus

NSAIDs: nonsteroidal anti-inflammatory drugs

PAG: periacueductal grey

PCP: phencyclidine

PG: prostaglandin

PGi: paragigantocelullaris

PGRMC1: progesterone receptor membrane component 1

PKA: protein kinase A

PKC: protein kinase C

PLC: phospholipase C

pNR1: phosphorylated form of N-methyl-D-aspartate receptor NR1 subunit

PNS: peripheral nervous system

(±)-PPC: (*S**,*R**)-2-[(4-Hydroxy-4-phenyl-1-piperidinyl)methyl]-1-(4-methylphenyl)-cyclopropanecarboxylic acid methyl ester

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

PREG / PREG-S: pregnenolone / pregnenolone sulphate ester

PrH: prepositus hypoglossi

PROG: progesterone

RNA: ribonucleic acid

RVM: rostroventromedial medulla

S1RA: 4-(2-(5-methyl-1-(naphthalen-2-yl)-1H- pyrazol-3-yloxy)ethyl) morpholine hydrochloride (E-52862)

s.c.: subcutaneous

SEM: standard error of the mean

SKF-10,047: *N*-allyl-normetazocine

SP: substance P

SR31747A: (Z)N-cyclohexyl-N-ethyl-3-(3-chloro4-cyclohexylphenyl)pro pen-2-ylamine hydrochloride

Th13: 13th thoracic vertebra

TKRs: tyrosine kinase receptors

TNC: trigeminal nucleus caudalis

TRPV: transient receptor potential vanilloid

(-)-U50,488H: *trans*-(-)-3,4-Dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide hydrochloride

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

WIPO: world intellectual property organization

WT: wild type

σR: sigma receptor

σ_1R: sigma-1 receptor

σ_1R-KO: sigma-1 receptor knockout

σ_2R: sigma-2 receptor

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1. INTRODUCTION

1.1 Pain overview

The actual definition of pain according to the International Association for the Study of Pain (IASP) is "An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage."

Based on the duration, pain can be classified as **acute** or **chronic** pain. Acute pain motivates the individual to withdraw from damaging situations, to protect a damaged body part while it heals, and to avoid similar experiences in the future. Most pain resolves promptly once the painful stimulus is removed and the body has healed. However, sometimes pain persists despite removal of the stimulus and apparent healing of the body, or as a result of a chronic condition (p.e. diabetes, nerve compression…) and gives rise to chronic pain, which does not seem to have any beneficial function.

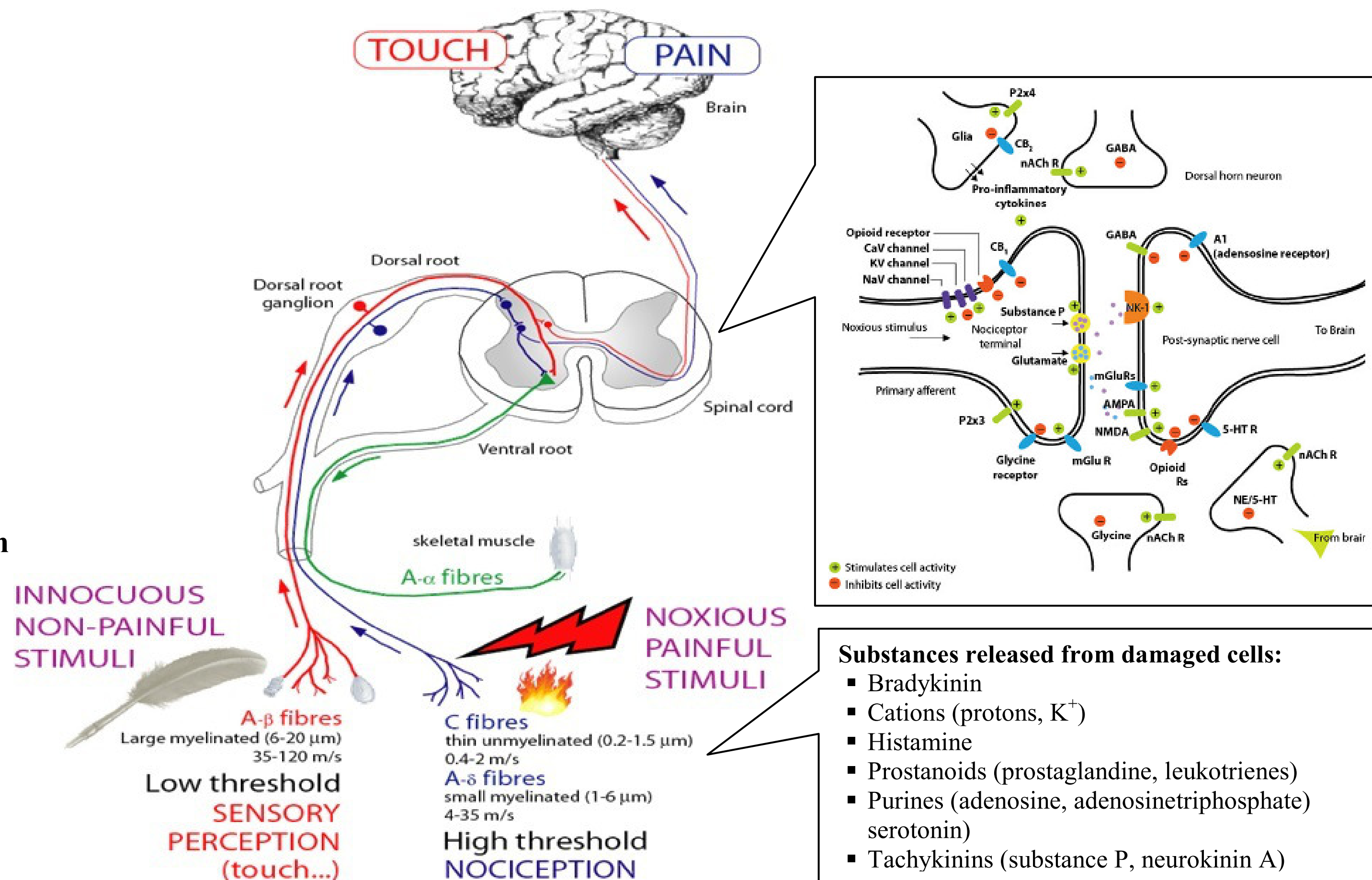
It is important to note that pain is the most common reason for physician consultation. It is a major symptom in many medical conditions, and can dramatically reduce the individual's quality of life and interfere general functioning. Nonsteroidal anti-inflammatory drugs (NSAIDs), opioids, triptans, anticonvulsants, and antidepressants are the most common analgesic drugs. However, they have a limited effectiveness in certain pain conditions (e.g., neuropathic pain) and important adverse effects. In this regard, investigation is essential in order to find new treatments or new drug combination approaches for pain treatment.

The signals of painful stimuli such as chemical, thermal and mechanical activate the primary sensory nociceptors (first order neurons) from the periphery and are transformed into electrical signals (**transduction**) that are propagated as nerve impulses. The first order neurons have their soma in the dorsal ganglia and make the synapses (synaptic **transmission**) with the dorsal horn (DH) neurons (second order neuron) of the spinal cord which project, after crossing to the contralateral side of the spinal cord, via the spinothalamic tract to supraspinal areas. Then, it synapses with third order neurons that transmit the impulse to the cortex were the information is processed (**perception**). The process is called ascending pain pathway (Fig. 1). However, the pain sensation is subject not only to modulation during its <u>ascending</u> transmission from the periphery to the cortex but also to segmental modulation and <u>descending</u> control from higher centres (Millan 2002). Thus, pain messages are two-way traffic.

Perception

Tranmission

Transduction



Substances released from damaged cells:

- Bradykinin
- Cations (protons, K^+)
- Histamine
- Prostanoids (prostaglandine, leukotrienes)
- Purines (adenosine, adenosinetriphosphate) serotonin)
- Tachykinins (substance P, neurokinin A)

Fig. 1. Scheme representing general aspects of the ascending pain pathway. Two main types of primary sensory afferent fibres transmit peripheral information to the brain: 1) Aβ fibres transmit innocuous non-painful stimuli (touch), and 2) C and Aδ fibres act as nociceptors and transmit noxious painful stimuli. After noxious painful stimuli, peripheral damaged cells release substances that activate the primary sensory nociceptors which transform the stimuli into electrical signals (**transduction**). Once the action potential from the periphery reaches the first synapse at the dorsal horn of the spinal cord with second order neurons, a number of neuroactive substances are released (synaptic **transmission**). After crossing to the contralateral side of the spinal cord, second order neurons project to supraspinal areas were the information is processed (**perception**).

There are different types of sensory fibres that transmit peripheral information to the brain: Aβ fibres transmit innocuous non-painful stimuli (touch), and C fibres and Aδ fibres transmit noxious painful stimuli (nociception). Aα fibres, in turn, participate in propioception and somatic control of skeletal muscles (Table 1).

Table 1. Summary of the different types of fibres and some of their characteristics.

Fibre Type	Information Carried	Myelin Sheath	Diameter (µm)	Conduction Velocity (m/s)
Aα	propioception	myelinated	13-20	80-120
Aβ	touch (and when stimulated they block pain impulses)	myelinated	6-12	35-90
Aδ	pain (mechanical and thermal)	myelinated	1-5	5-40
C	pain (mechanical, thermal, and chemical)	non-myelinated	0.2-1.5	0.5-2

Sensitization of the nociceptive system may result from enhanced membrane excitability, enhanced synaptic efficacy or reduced inhibition in pain pathways. Sensitization reduces pain threshold and drives pain perception against normal innocuous stimuli that can expand outside the primarily injured area (Eide, 2000). **Peripheral sensitization** is produced by inflammatory mediators such as bradykinin, prostaglandins (PG), neuropeptides, cytokines…, which activate primary afferent neurons. **Central sensitization** is produced by mediators at the spinal cord such as glutamate (Glu), substance P (SP), calcitonin gene-related peptide (CGRP)…. Altogether translates in spontaneous pain, allodynia, and/or hyperalgesia.

For many years, allodynia was defined as pain due to a stimulus that does not normally provoke pain while hyperalgesia was defined as an increased pain from a stimulus that normally provokes pain. However, according to actual definitions from IASP, all forms of pain amplification including lowering in thresholds are summarized under the umbrella term **hyperalgesia** and only when pain is clearly involving low-threshold fibres should the term **allodynia** be used (Fig. 2).

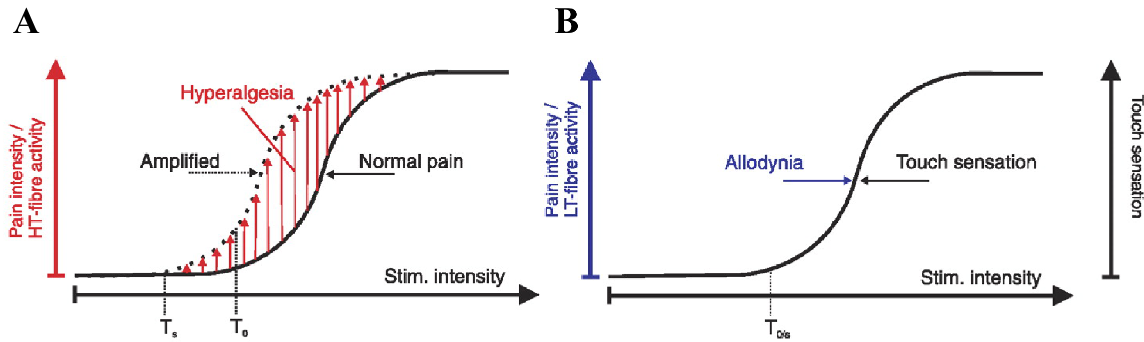


Fig. 2. In 2008 IASP redefined the terms "hyperalgesia" and "allodynia." All forms of pain amplification including lowering in thresholds are summarized under the umbrella term hyperalgesia (red area in A graph). Only if pain is clearly engaging low-threshold (LT) fibres should the term allodynia be used (blue ordinate in B graph). T_0 refers to the normal pain threshold, and T_S refers to the pain threshold after sensitization. (Sandkuhler 2009).

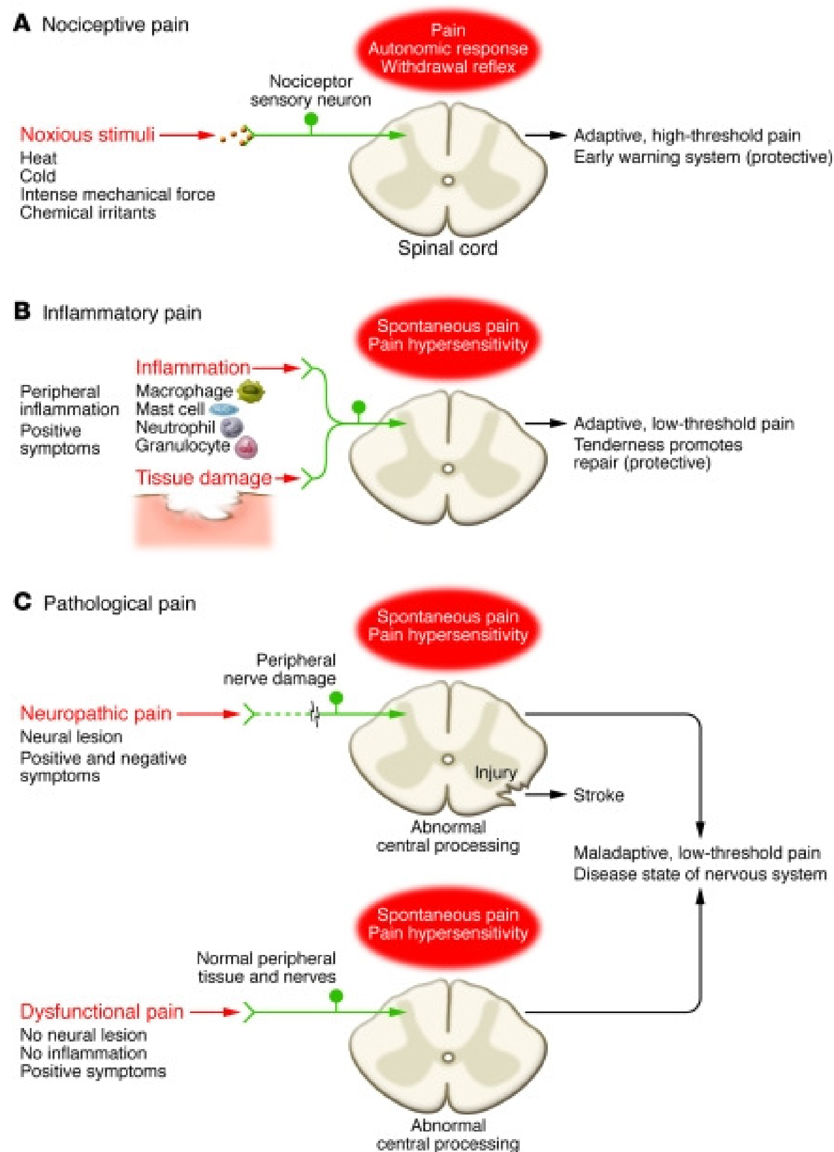


Fig. 3. Pain classification. Based on the aetiology pain can be divided in nociceptive (A), inflammatory (B), and pathological (C). (Woolf 2010).

1.2 Types of pain

Based on the aetiology, three types of pain can be distinguished: **Nociceptive, inflammatory and pathological** (Woolf 2010) (Fig. 3).

- **Nociceptive pain** is a high threshold pain only activated in the presence of intense stimuli. It is a vital physiologic sensation produced as a consequence of a somatic or visceral injury and it has a protective role for the survival of the organism. Examples of somatic pain include musculoskeletal (joint pain, myofascial pain) and cutaneous pain, which are often well localized. Pain produced in hollow organs and smooth muscle generates visceral pain, which is usually manifested as a referred pain.
- **Inflammatory pain** is adaptative and protective and it is produced as a result of activation and sensitization of the nociceptive pain pathway by a variety of mediators released at the site of tissue inflammation by the immune system. In this state, sensitivity is increased such that stimuli to the affected part that would normally not cause pain now do so. Examples include appendicitis, rheumatoid arthritis, inflammatory bowel disease, and herpes zoster pain.
- **Pathological pain** is not protective, but maladaptive, resulting from abnormal functioning of the nervous system and is a low-threshold pain. It is characterized by sensory abnormalities due to the hypersensitivity state that leads to hyperalgesia, allodynia, and spontaneous pain.
    - **Neuropathic pain** initiated or caused by a primary lesion or dysfunction in the somatosensory nervous system. Some examples include diabetic neuropathy, postherpetic neuralgia, spinal cord injury pain, phantom limb (post-amputation) pain, and post-stroke central pain.
    - **Dysfuntional pain** produced in conditions in which there is no apparent nerve damage or inflammation. Some examples include fibromyalgia, irritable bowel syndrome, tension type headache, temporomandibular joint disease, and interstitial cystitis.

1.3 Control of pain

As previous stated, pain messages are two-way traffic: pain sensation is modulated during the ascending transmission from the periphery to the cortex, but also by descending control from supraspinal areas.

1.3.1 Ascending pain pathways

Nociceptive transmission normally starts from peripheral endings of primary sensory afferents which are activated by noxious stimuli. Action potentials generated in these nociceptive afferents are propagated into the spinal cord DH. At the central terminals of primary afferents, the amino acid Glu is the major fast neurotransmitter which is released and mediates rapid excitatory post-synaptic potentials (EPSPs) in DH neurons. Other excitatory substances such Aspartate (Asp) and peptides including SP and CGRP are also released.

1.3.1.1 Glutamate

Glu plays a key role in central sensitisation and spinal pain transduction mechanisms (Hudspith 1997). The state of hyperalgesia is mimicked by spinal Glu application (Gerber and Randic 1989) and is blocked by antagonists of *N*-methyl-D-aspartate receptors (NMDARs) (Thompson *et al.* 1990). This sensitisation is marked by an increased responsiveness of the DH neurons to the excitatory amino acids (Dougherty and Willis 1992) and expansion in their receptive fields (Woolf 1983).
Glu and the glutamatergic receptors (GluR) are involved in the transmission of different types of stimuli in the spinal cord, ranging from noxious chemical, mechanical, and thermal stimuli relaying propioceptive information and the modulation of sensory information. In the nociceptive system, GluRs are expressed at the peripheral, spinal, and supraspinal levels. In the spinal cord, once Glu is released it acts on post-synaptic GluR localized on second-order neurons in the DH. There are three main classes of ligand-gated ionotropic GluRs: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), kainate receptor (KAR), and NMDARs; and three groups (I-III) of G-protein coupled metabotropic GluRs (mGluRs) (Woolf and Salter 2000; Larsson 2009) (Fig. 4).

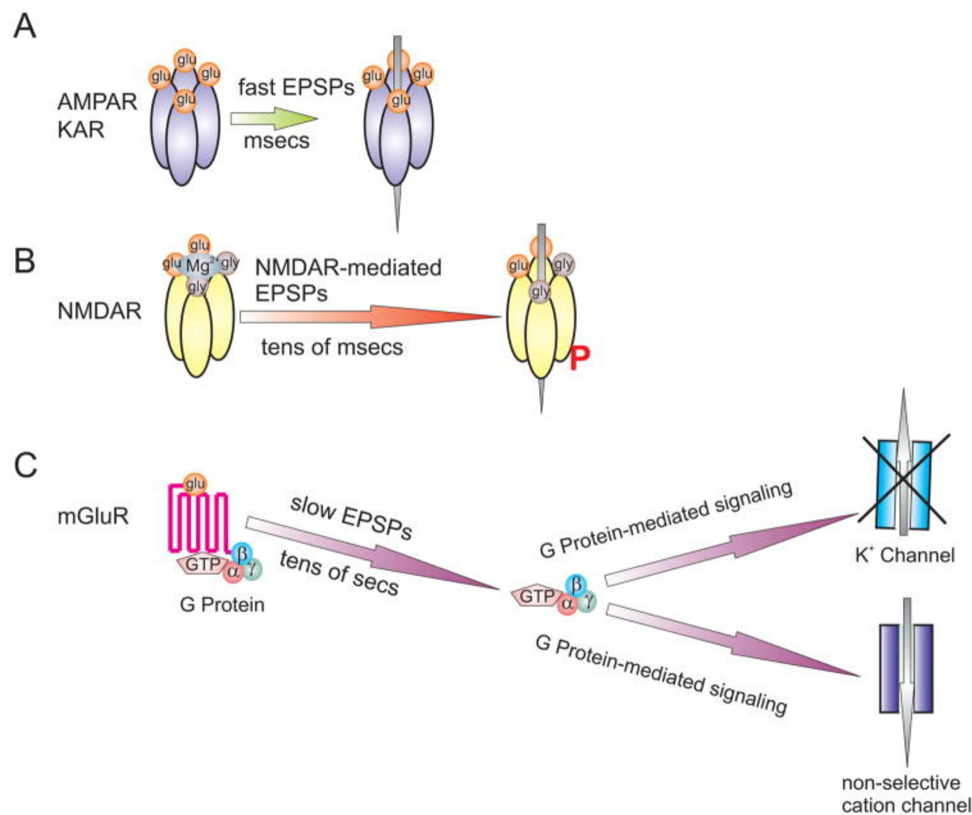


Fig. 4. Schematic illustration of the synaptic transmission from primary sensory afferents to second-order neurons in the DH. (**A**) Under normal circumstances, the fast EPSPs are mediated by AMPARs and KARs, on the scale of milliseconds. (**B**) NMDARs contribute little to fast excitatory transmission as NMDARs are down-regulated at resting potential by the Mg^{2+} block at the channel pore and receptor dephosphorylation. However, NMDARs are recruited into synaptic transmission under high frequency stimulation, producing EPSPs on the scale of tens of milliseconds. The recruitment of NMDARs involves disinhibition of Mg^{2+} block and receptor phosphorylation. **(C)** Sustained release of Glu leads to activation of post-synaptic group I mGluRs, resulting in G protein activation. The activated G protein subunits then associate with downstream effectors to affect membrane potential by inhibiting K^+ channels or facilitating non-selective cation channels, leading to slow EPSPs lasting up to tens of seconds. (Liu and Salter 2010).

GluRs are involved in initiating and maintaining neuroplasticity. AMPAR and KAR produce fast EPSPs (on the scale of **milliseconds**). NMDAR contributes little to the fast excitatory transmission, or to acute pain, as under normal conditions are down-regulated at resting potential by Mg^{2+} block at the channel pore and by phosphorylation/dephosphorylation systems. Under high-frequency stimulation, NMDARs are recruited (involving Mg^{2+} disinhibition and phosphorylation) producing post-synaptic potentials on the scale of **tens of milliseconds**. Upon sustained, intense noxious stimuli, primary sensory afferents release SP (Ikeda *et al.* 2006) and CGRP (Schaible *et al.* 1994) together with Glu leading to activation of post-synaptic G-protein-coupled receptors (GPCRs) including group I mGluR. The activated G protein subunits then associate with downstream effectors to affect membrane potential by

inhibiting K^+ channels or facilitating non-selective cation conductance, leading to slow post-synaptic potentials lasting up to **tens of seconds**. The sustained depolarization furthermore recruits voltage-gated Ca^{2+} currents (Morisset and Nagy 1999). Post-synaptic Ca^{2+} concentration increases substantially, resulting in subsequent activation of multiple intracellular signalling pathways, leading to increased neuronal excitability and enhanced synaptic transmission. The end result of these processes is central sensitization: the enhancement of the activity of central neurons and circuits in the nociceptive pathway. A large body of evidence indicates that persistent synaptic plasticity at glutamatergic synapses in the central nervous system (CNS) is critically dependent on post-translational modification of GluRs.

Because GluRs are critical for neuroplasticity in nociceptive networks, there have been considerable efforts to develop therapeutic approaches that suppress the function of these receptors. However, GluR-based therapies have had limited clinical success largely due to adverse-effects because the activities of GluR are essential for many important physiological functions in the CNS.

1.3.2 Descending pain pathways

The brainstem stimulation can be either **inhibitory** or **facilitatory**. Both systems seem to be activated simultaneously in conditions of acute nociception. Neuroplastic changes might occur to yield a sustained facilitatory influence that may contribute to the development and maintenance of hyperalgesia and thus contribute to chronic pain states in conditions of persistent nociceptive input.

The descending inhibitory influences engaged in the brainstem have clear evolutive physiological advantages. If an endogenous pain modulatory system is activated by a stimulus like stress, fear, intense exercise…, or the need to escape from a predator when injured, control of pain has relevant value (Gebhart 2004). On the other hand, the physiological advantages of a descending facilitatory system are not equally intuitive. When a tissue is injured, there is increased input and sensitivity to stimulation at the site of insult (primary hyperalgesia), and also an increased sensitivity from uninjured tissue adjacent to or at some distance from the site of injury (secondary hyperalgesia). The mechanisms that contribute to maintenance of secondary hyperalgesia, or “central sensitization”, are not restricted to the spinal cord as brainstem neurons in the rostral ventromedial medulla (RVM neurons) also undergo changes in excitability. But what

role does descending facilitatory influences play? In day-to-day life, sprains, cuts and bruises that lead to inflammation and pain are associated with both primary and secondary hyperalgesia, and descending facilitatory influences contributes to prevent further damage to already damaged tissue. In other cases, such as neuropathic pain, normally non-noxious stimuli are unbearably painful. There is permanent damage to the nervous system and likely anatomical reorganization of spinal terminations of surviving axons or ectopic activity from a neuroma that contributes to persistent input to the spinal cord. In addition to anatomical reorganization in the spinal cord, there could be some reorganization in the RVM, but more likely there is prolonged input to RVM that sustains facilitatory influences that descend to the spinal cord (Gebhart 2004).

1.3.2.1 Descending facilitation of pain

Stimulation in the RVM at relatively high current intensities (50-100 μA) was antinociceptive, and lower current intensities (5-25 μA) at the same sites were facilitatory (Zhuo and Gebhart 1990, 1992, 1997). Activation of this RVM facilitatory systems results from persistent noxious inputs that can enhance pain: pain begets pain (Porreca *et al.* 2002). In support of this idea, formalin injected into the tail increased responses of L_4-L_6 neurons to heating of the hind paw (Biella *et al.* 1999). Similarly, when injected into a hind paw, formalin facilitated reflexes of tail withdrawal from thermal and mechanical noxious stimuli (Calejesan *et al.* 1998).

Based on response characteristics to noxious thermal stimulation of the tail, Fields and colleagues described three types of neurons: ON, OFF and neutral cells. OFF-cells are tonically active and pause in firing immediately before tail (or hind limb) withdrawal from a noxious thermal stimulus. ON-cells accelerate firing immediately before the nociceptive reflex occurs. OFF-cells inhibit whereas ON-cells facilitate transmission of spinal nociceptive input and the subsequent responses. Neutral cells where initially characterized by the absence of a response to noxious thermal stimulation of the tail; however these cells respond to noxious stimuli applied elsewhere, and could represent a subtype of ON- or OFF-cells (Fields *et al.* 1983; Fields and Heinricher 1985).

1.3.2.2 Descending inhibition of pain

Electrical stimulation of the anterior hypothalamus, inferior septal areas, the anterior, mid or posterior periventricular grey, and the centrum medianum nucleus of the thalamus sites, all produce effective pain control in humans (Richardson 1990).

Microinjection of morphine in each of those regions also produces analgesia (Yeung *et al.* 1977). The stimulus-produced analgesia from these higher centres is mediated by activation of brainstem nuclei (Cross 1994), being most strongly implicated the periacueductal grey (PAG), the raphe nuclei and the locus coeruleus (LC) (Fig. 5).

- The PAG was the first region where electrical stimulation showed to evoke a degree of hypoalgesia adequate for surgical intervention in rats (Reynolds 1969), a finding reproduced in humans (Mayer 1984; Young and Brechner 1986). There is evidence that different subregions of the PAG may be involved in different forms of analgesia: opioid analgesia appears to be mediated via the ventrolateral PAG while the lateral portion of nucleus elicits non-opioid analgesia (Bandler and Shipley 1994). The rat PAG receives significant afferents from many nuclei in the diencephalon and brainstem. Weaker inputs come from the medial preoptic area, lateral septum and the anterior cingulated cortex (Marchand and Hagino 1983). There are three major projections from the PAG that are central to its role in descending inhibition of pain. First, there is a pathway from PAG to a rostroventromedial pericoerulear region that includes Barrington's nucleus but not the LC proper. Second, the PAG projects to the nucleus paragigantocelullaris (PGi), and third, and perhaps the most important, the PAG sends fibres to the nucleus raphe magnus (NRM).
- The raphe nuclei present several subdivisions. The two most widely implicated in descending control are the NRM and the dorsal raphe nucleus (DRN). The NRM receives an input from the PAG and it is thought to mediate at least some of the effects of PAG stimulation. Direct stimulation of NRM causes analgesia in behavioural algesiometric tests (Oliveras *et al.* 1975). There are high levels of serotonin (5-HT) in the raphe but there is also a significant amount of noradrenaline (NA).
- The nucleus paragigantocellularis (PGi) gives rise to a massive projection to the LC (Aston-Jones *et al.* 1986). It projects to the spinal cord and to the NRM, and it also receives projection from the PAG.
- The LC is considered the main noradrenergic nucleus involved in the descending control of pain. Its main inputs are from the nucleus prepositus hypoglossi (PrH) and the PGi. It does not receive a direct input from the PAG which instead innervates the pericoerulear region but may influence the LC via the PGi. LC stimulation causes antinociception (Margalit and Segal 1979) and also causes

elevation of spinal cord NA metabolites (Crawley *et al.* 1979). However, it cannot be assumed that the spinal effects of LC stimulation are wholly mediated by the release of NA in the cord.

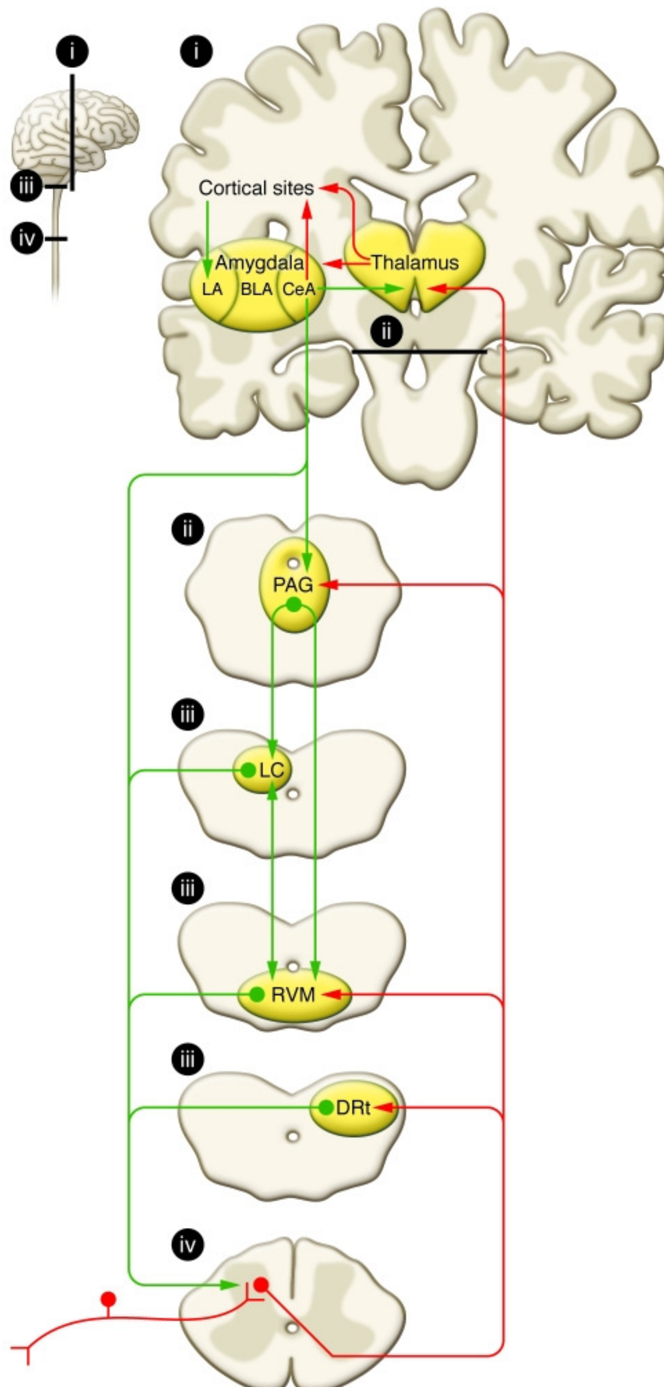


Fig. 5. Schematic representation of pain modulatory circuitry. Ascending projections (red) target the thalamus, and collateral projections target mesencephalic nuclei, including the dorsal raphe nucleus (DRt), the RVM, and the midbrain PAG. Descending projections (green) from the DRt are a critical component of the inhibitory pain pathway. Rostral projections from the thalamus target areas that include cortical sites and the amygdala. The lateral capsular part of the CeA ("nociceptive amygdala") sends outputs to cortical sites and the thalamus, in which cognitive and conscious perceptions of pain are integrated. Descending pain modulation is mediated through projections to the PAG, which also receives inputs from other sites, including the hypothalamus, and communicates with the RVM as well as other medullary nuclei that send descending projections to the spinal DH through the dorsal longitudinal fasciculus. The noradrenergic LC receives inputs from the PAG, communicates with the RVM, and sends descending noradrenergic inhibitory projections to the spinal cord. Antinociceptive and pronociceptive spinopetal projections from the RVM positively and negatively modulate nociceptive inputs and provide for an endogenous pain regulatory system. Areas labelled "i–iv" in the small diagram correspond with labeled details of the larger diagram (Ossipov *et al.* 2010).

The transmitters most clearly implicated in descending inhibition of pain are NA, 5-HT, endogenous opioids, and gamma-aminobutyric acid (GABA).

1.3.2.2.1 Noradrenaline

NA is biosynthesized from tyrosine, which is first converted to dopamine (DA), and in noradrenergic cells is further converted to NA. Noradrenergic cell groups are classified as A1-A7 (Dahlström and Fuxe 1964) and are present in neuronal circuits critical for pain modulation in the brain. Cell groups A5-A7, originating in the midbrain (PAG) and brainstem (mainly LC but also RVM), have significant descending noradrenergic projections to the spinal cord (Proudfit 1988; Kwiat and Basbaum 1992). NA receptors are classically divided into two main adrenoceptors (AR) categories (Ruffolo and Hieble 1994; Aantaa *et al.* 1995; Bylund 1995):

- α-$_{1A, 1B, 1D}$ and α-$_{2A, 2B, 2C}$.
- β_1, β_2, and β_3.

α_1-AR is coupled to phospholipase C (PLC) through G_q or it is coupled directly to Ca^{2+} influx; and α_2-AR decreases intracellular adenylcyclase activity through G_i or directly modifies activity of ion channels such as the NA^+/H^+ antiport, Ca^{2+} channels, or K^+ channels. On the other hand, β-AR increases adenylcyclase activity through G_s (Summers and McMartin 1993).

ARs located on the noradrenergic neurons are considered autoreceptors that either inhibit impulse discharge of neurons (those located in the somatodendritic area) or inhibit the release of NA (those located on noradrenergic axon terminals). ARs located on non-noradrenergic neurons are considered heteroreceptors which are activated by NA released from noradrenergic neurons.

Peripheral NA has only little influence on pain in physiological conditions, but in inflamed and neuropathic conditions it may aggravate pain (Davis *et al.* 1991; Drummond 1995; Torebjörk *et al.* 1995) or can be antinociceptive through α_1, α_2, or β AR activation (Binder *et al.* 2004). On the other hand, intracerebroventricular administration of noradrenergic compounds suppresses pain-related responses (Schmitt *et al.* 1974a,b). The sources of spinal NA are descending axons originating in the noradrenergic nuclei of the brainstem (Proudfit 1988; Jones 1991), particularly the noradrenergic cell groups A5, A6 and A7 in the pons (Westlund and Coulter 1980; Kwiat and Basbaum 1992). These noradrenergic cell groups are connected with other pain-related nuclei and receive projections from the PAG (Bajic and Proudfit 1999). In

the DH, NA terminals are mainly concentrated in the upper two laminae in the rat (Dahlström and Fuxe 1965). Intrathecal (i.t.) NA causes analgesia in the rat (Reddy and Yaksh 1980). Using selective drugs, it has been shown that the α_2-AR antagonist yohimbine is an order of magnitude more potent than the α_1-AR antagonist prazosin in the reversion of i.t. NA-induced analgesia. Consequently, it is considered that α_2-AR is the subtype responsible for NA spinal analgesic effects (Sagen and Proudfit 1984). Further studies with subtype-specific antagonists indicate that, in rodents, the α_{2D} subtype (human α_{2A}) mediates antinociception (Millan *et al.* 1994). Although α_2-AR exist both pre- and post-synaptically, the spinal α_2 sites more involved in antinociception are postsynaptically located. Accumulating evidence suggests that the mechanism of action of some analgesic drugs that inhibit pain sensitization, such as pregabalin and gabapentin, act supraspinally to activate the descending noradrenergic system resulting in spinal release of NA (Takeuchi *et al.* 2007; Hayashida *et al.* 2007) coupled with spinal α_2-AR activation to alleviate neuropathic pain (Takeuchi *et al.* 2007). In addition, there is evidence that NA uptake blockade at the spinal cord contributes clinically to the analgesic effect of some drugs such as tramadol and some antidepressants (Grond and Sablotzki 2004).

The intrinsic noradrenergic pain regulatory system in the spinal cord has only low tonic activity, since α_2-AR antagonists (Dennis *et al.* 1980; Sagen and Proudfit 1984) or the α_2-AR knockout (Malmberg *et al.* 2001) did not enhance spinal withdrawal responses to brief noxious stimulation in naïve animals. However, under persistent stimulation, α_2-AR antagonists or the α_2-AR knockout increased spinal nociceptive responses indicating an involvement of the noradrenergic feedback inhibition in the regulation of sustained pain (Green *et al.* 1998; Mansikka *et al.* 2004).

Spinal NA, at the cellular level, is inhibitory upon nociceptive transmission and α_2-AR mediates inhibition of spinal nociceptive cells with ascending axons. Particularly, NA reduces the release of Glu and SP from central afferent terminals (presynaptic inhibition) by the inhibition of N-type Ca^{2+} channels (Kuraishi *et al.* 1985b; Ueda *et al.* 1995) and hyperpolarizes DH neurons (postsynaptic inhibition) by an increase in K^+ conductance (North and Yoshimura 1984; Wolff *et al.* 2007). As previously stated the α_2-AR is coupled to a G_i protein which reduces the activity of adenylyl cyclase, simultaneously suppressing both the production of cyclic adenosine monophosphate (cAMP) and the activity of protein kinase A (PKA).

1.3.2.2.2 Serotonin

5-HT is biosynthesized from L-tryptophan. The current classification of 5-HT receptors is represented in Fig. 6.

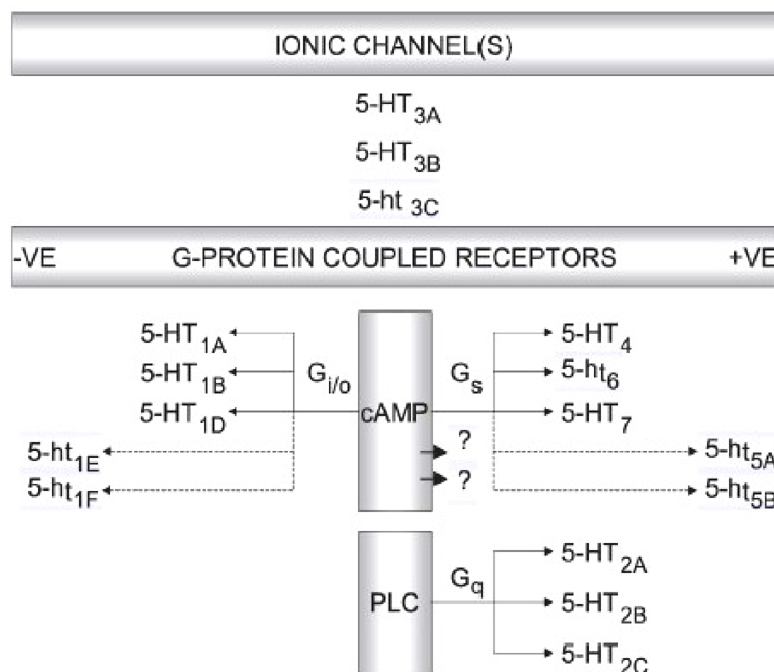


Fig. 6. Graphical representation of the current classification of 5-HT receptors. Abbreviations: negative (-ve); positive (+ve). (Hoyer *et al.* 2002).

The involvement of 5-HT in descending control of pain has long been recognized:

- A blocker of 5-HT synthesis abolishes central stimulation-induced analgesia (Basbaum 1981).
- Electrical stimulation of NRM has been shown to increase the release of 5-HT in the spinal cord (Rivot *et al.* 1982).
- Systemic morphine increases 5-HT synthesis in the spinal cord, mainly in the DH (Rivot *et al.* 1989).
- Antidepressants potentiate the action of morphine (Puig *et al.* 1993).

At the cellular level, 5-HT is thought to be an inhibitory transmitter in the DH. However, there is some evidence that 5-HT can be excitatory upon small cells (interneurons) in laminae I-III (Todd and Millar 1983). In contrast with the reasonably clear evidence that α_2-AR mediate the antinociceptive actions of NA, it is far from clear which 5-HT receptors mediate its spinal antinociceptive effects, although most of the recent reports point to 5-HT_{1B}, 5-HT_{1A}, and/or 5-HT_7 (Brenchat *et al.* 2012; Viguer *et al*. 2013).

1.3.2.2.3 Opioids

Opioids are involved in the ascending (μ, δ, and κ) and the descending (μ and κ) components of pain modulation. μ receptor binding is moderately found in PAG, DRN and NRM and highly found in LC; and moderate κ receptor binding is found in PAG, DRN and LC, while highly in DRN and LC (Mansour *et al.* 1995). Local microinjections of morphine at supraspinal sites have complex and variable actions on DH cell activity (Carstens *et al.* 1988). For instance, PAG administration of morphine may inhibit, enhance or not affect responses to noxious stimulation in the DH (Dickenson and Le Bars 1987; Jones and Gebhart 1988). Morphine administered in the NRM shows similar paradoxical properties: in some hands DH responses to noxious heat are inhibited (Jones and Gebhart 1988) while others find enhancement (Llewelyn *et al.* 1986). Morphine microinjected into the LC inhibits the response of most DH cells to noxious stimuli (Jones and Gebhart 1988).

1.3.2.2.4 Gamma-aminobutyric acid

GABA is biosynthesized from Glu. Two subtypes of GABA receptors are known:

- $GABA_A$ receptors are ligand-activated chloride channels (ionotropic receptors).
- $GABA_B$ receptors are GPCRs (metabotropic receptors).

Approximately 40% of terminals in the PAG are GABAergic. It has been shown that microinjection of the $GABA_A$ receptor agonist muscimol into either PAG or DRN causes hyperalgesia and also blocks the antinociceptive action of locally applied morphine (Moreau and Fields 1986). The role of $GABA_B$ is less clear: injection of the $GABA_B$ receptor agonist baclofen into the NRM can be either hypo or hyperalgesic in the tail-flick test depending on the dose used (Hammond *et al.* 1994).

In the spinal cord there are GABA receptors located in the DH on pre- and postsynaptic sites. Spinal $GABA_A$ and $GABA_B$ receptor activation results in an antinociceptive effect. Activation of presynaptic $GABA_B$ receptors enhance pain threshold by diminishing the release of SP and Glu (Enna and McCarson 2006).

1.4 Spinal cord microdialysis approaches

In the last years there has been little progress in developing new efficacious and safe analgesics probably because of the lack of understanding the pain itself and the lack of translational preclinical models of pain. The development of the microdialysis sampling technique can help to understand pain pathways and the mechanism of action of analgesics.

The microdialysis allows on-line estimation of neurotransmitters in the living animal (Fig. 7). It offers many benefits such as frequent data points, clean samples, no loss of body fluid and consumption of fewer experimental animals per study (Khan and Shuaib 2001). In microdialysis, a semipermeable dialysis membrane is introduced into the fluid or tissue compartment to be sampled and perfused with physiological fluid. As a result of passive diffusion, molecules migrate across the membrane along their concentration gradient. Molecules found in high concentrations within the tissue compartment migrate across the membrane into the dialysis tubing where they can be collected for subsequent quantification, whereas molecules found in high concentrations within the membrane diffuse outward into the surrounding tissue compartment. The recovery of substances from the compartment depends on the length and molecular weight cut-off of the membrane as well as the composition and rate of flow of the perfusate.

As introduced, the spinal cord represents an important synaptic relay in the pain pathways and a modulation of excitatory signals at the DH occurs in pain transmission states. Because of the increasing interest in transmitter pharmacology in this region, the spinal cord microdialysis in rat was introduced in pain research (Sluka and Westlund 1992; Malmberg and Yaksh 1995a; Scheuren *et al.* 1997). Skilling and coworkers (Skilling *et al.* 1988) successfully demonstrated for the first time the release of Glu and Asp in response to formalin-induced acute nociceptive stimulation. The results from Vetter and Okuda (Vetter *et al.* 2001; Okuda *et al.* 2001) also provided evidence to support the hypothesis that Glu and Asp are DH neurotransmitters involved in nociception. Nevertheless, measurement of neurotransmitter release in the spinal DH in conscious animals remains a technical challenge (for review see Stiller *et al.* 2003).

Three different methods for spinal microdialysis have been described based on the location of the fibre: transversal, intrathecal and concentric (Fig. 8).

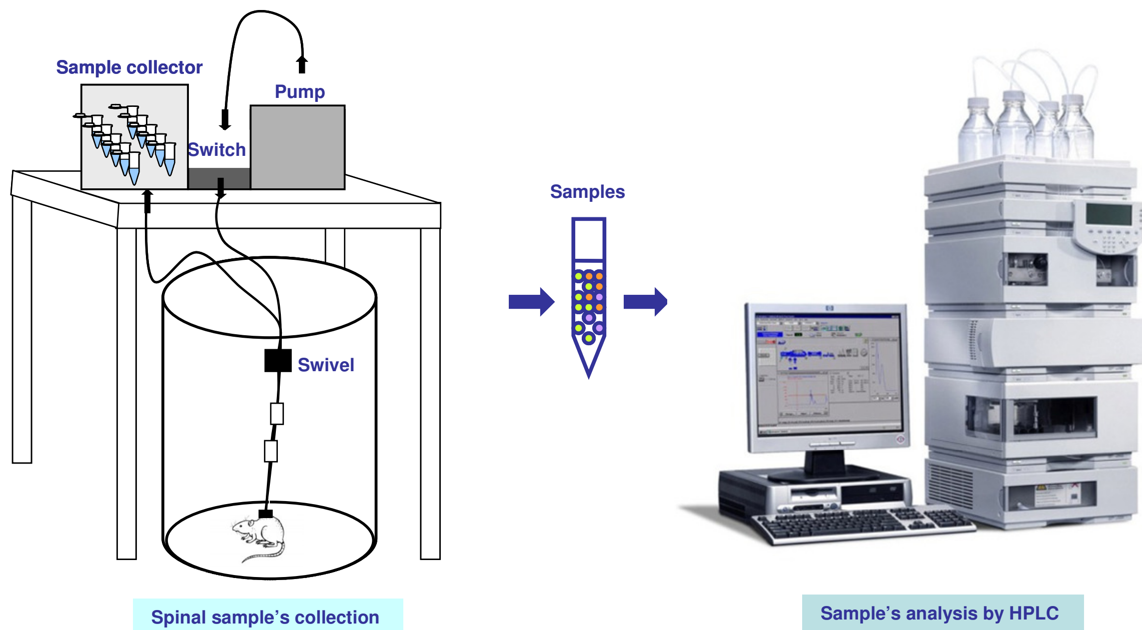


Fig. 7. Sequence of steps in performing spinal microdialysis in the rat. A semipermeable dialysis membrane is introduced into the spinal fluid or into the spinal cord to be sampled. Artificial cerebrospinal fluid is pushed through the probe and as a result of passive diffusion molecules migrate across the dialysis membrane along their concentration gradient. Analytes are collected into the perfusate and then pushed through the outflow tubing into a collection vial. The recovery of substances from the compartment depends on the length and molecular weight cut-off of the membrane as well as the composition and rate of flow of the perfusate. The collected sample is analysed by whatever method is most appropriate for detection of the particular analyte(s) under study (e.g., HPLC, RIA…).

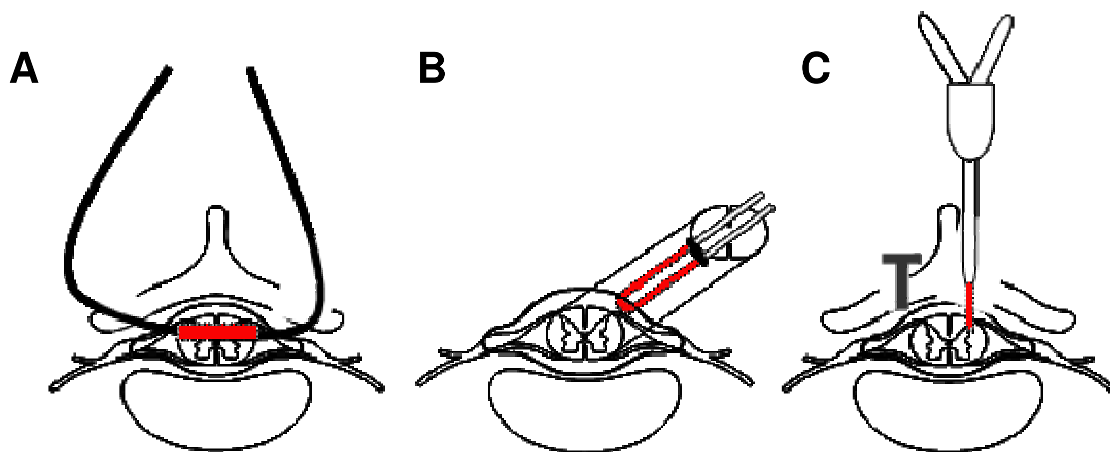


Fig. 8. Drawings showing three different methods for spinal microdialysis based on the location of the fibre: a) transversal, b) intrathecal, and c) concentric.

1.4.1 Transversal dialysis

In this microdialysis approach, the transverse probes are passed transversally through the DH through two opposing holes drilled into the overlaying vertebrae after removing portions of the paravertebral musculature (Skilling *et al.* 1988; Sluka and Westlund 1992; Sorkin and McAdoo 1993). The transverse system provides the ability to locate precisely the spinal terminals from which the neurotransmitter release occurs. However, its utility is limited basically by the difficulty of surgical preparation and for the consequences of the extensive surgery (incisions and dissections) and trauma associated with the implant. In addition, the complicated construction of the probe limits its routine use (for details on preparation of probes see Zapata *et al.* 2009). Each laboratory manufactures its own probes which may contribute to larger inter-laboratory variation.

1.4.2 Intrathecal dialysis

In this case, the spinal loop catheters (intrathecal) are inserted through an incision in the atlanto-occipital membrane, passed to the lumbar enlargement and placed within the cerebrospinal fluid (CSF) (Marsala *et al.* 1995; Malmberg and Yaksh 1995a). The surgical procedures are essentially identical to the insertion of an intrathecal catheter for drug administration as originally described by Yaksh and Rudy (Yaksh and Rudy 1978). Major advantages of this technique include:

- Preservation of spinal tissue integrity.
- Minimal surgical preparation and dissection.
- Quick recovery of the animal because of a less invasive procedure than the transversal one.
- The 4 cm active membrane of the loop probe offers much larger volume recovery than the 3 mm of the linear probe. Thus, in contrast to the transversal one is more reproducible for routine investigation of drug effects.
- The placement via the cisternal route allows the catheters to exit skin at a site which is relatively protected from attention by the animal.

However, the loop probe technique samples the neurotransmitters from the CSF and consequently, it is unable to locate precisely the origin of the release.

1.4.3 Concentric dialysis

Finally, the third described approach consists in concentric probes which are placed unilaterally into a specific region of the spinal cord (Walwyn *et al.* 1999; Gerin *et al.* 2008). In contrast to the transversal or the intrathecal approaches, it is the only one that allows ipsilateral intra-DH microdialysis, which is relevant in most animal models of pain to focus on ipsilateral pain-related changes compared to control contralateral ones. Noteworthy, the concentric microdialysis approach has never been used in conscious animals in pain research. Only some studies are available in anaesthetized animals (Tzschentke *et al.* 2012). It is well known that anaesthesia modifies pain perception, and it is probably that the neurochemical modulation of pain as well as the effect on analgesics could be in part interfered in studies under anaesthesia. It is obvious that anaesthesia is a potential problem and it is difficult to study the normal physiological mechanisms of pain or pain killers under such circumstances. Indeed, differences in the depth of anaesthesia and type of anaesthetic used have often been touted as explanations for variable or inconsistent results between laboratories. In this way, certain anaesthetic agents modulate Glu central release (Wang *et al.* 2008). In addition, components of the pharmacology of descending inhibition may be differentially modified by anaesthesia. For instance, morphine may increase LC neuron firing in conscious cats while decreasing activity under chloral hydrate anaesthesia (Rasmussen and Jacobs 1985).

Only a few microdialysis studies on locomotor activity have employed concentric probes in awake animals (Gerin and Privat 1998; Walwyn *et al.* 1999; Gerin *et al.* 2008) and have demonstrated that lumbar DH, site of placement of the microdialysis probe, is compatible with exercise and with local sampling (Gerin *et al.* 2011).

1.5 Drugs for pain treatment

Although there are an important number of drugs for pain treatment and a huge diversity of potential targets involved in pain, the majority of analgesics available to prescribers and patients are compounds based on mechanisms of action that are known for many years.

The most common drugs approved for the treatment of pain are NSAIDs, opioids, and antimigraine triptans. In addition, drugs approved for other medical conditions such as depression and epilepsy (anticonvulsants) are increasingly being used since their effectiveness in pain was later confirmed by a meta-analysis or by an FDA review (Kissin 2010).

However, the actual pain killers show limited effectiveness in many pain conditions (e.g., neuropathic pain) and multiple serious adverse effects. Thus, there is a high interest in developing new analgesics. Table 2 summarizes the drugs developed during the period 1960 to 2009 and presently in use for the treatment of pain.

Unfortunately, real innovation is poor as most new treatment approaches are based on the same previously described mechanisms, involve novel reformulations or routes of administration, or are combination products (Burgess and Williams 2010). Only few drugs (although some of them were identified some time ago) act on novel molecular targets such as TRPV1 receptor agonists (topical capsaicin), N-type Ca^{2+} channel blockers (Ziconotide), NMDAR antagonists (Ketamine), and CB1/CB2 agonists (Dronabinol).

Emerging approaches are nerve growth factor monoclonal antibodies and mechanisms based on activated microglia (Burgess and Williams 2010). In addition, the fields of genetics and epigenetics are an exciting new hope for pain research (Crow *et al.* 2013).

Table 2. Analgesics developed during 1960-2009 and currently in use. (Adapted 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]	Valproate 1979–2000 (FDA)[c]
	Phenytoin 1964–1995	Gabapentin 1996–2002 (FDA)
	Clonazepam 1975–1995	Topiramate 2001–2003 (FDA)
Antidepressants	Amitriptyline 1964–1992	Desipramine 1984–1996
	Doxepin 1973–1992	Venlafaxine 1996–2005
	Imipramine 1962–1996	Duloxetine 2004 (FDA)
Other drugs	Propranolol 1968–1991	Mexiletine 1986–2005
	Capsaicin (topical) 1987–1994	Ketamine 1974–2006
	Cyclobenzaprine 1989–2004	Dronabinol 1975–2007
	Lidocaine (systemic, topical) 1982–2005	Dexamethasone 1967–2008

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 FDA approval for the treatment of pain as an additional indication.

1.6 Sigma-1 receptors

1.6.1 A little bit of history

Sigma receptors (σRs) were first proposed in 1976 by Martin and coworkers based on the psychomimetic actions of *N*-allyl-normetazocine (SKF-10,047) and related benzomorphans (Martin *et al.* 1976). These effects could not be explained by the actions on μ-opioid or κ-opioid receptors, and contributed to the proposal of a σ-opioid receptor, derived from the first letter "S" of SKF-10,047. The enantiomeric selectivity of the σR for the (+)-isoforms of the benzomorphans rather than for their (−)-isoforms, and the fact that the effects of sigma ligands neither *in vivo* nor *in vitro* were blocked by classical opioid antagonists (Iwamoto 1981; Vaupel 1983; Young and Khazan 1984) led to some confusion regarding σR binding sites. Later, it was determined that (+)SKF-10,047 interacted with the phencyclidine (PCP) binding site, within the ionophore of the NMDAR (Quirion *et al.* 1981; Zukin *et al.* 1984; Mendelsohn *et al.* 1985; Sircar *et al.* 1986; Wong *et al.* 1988). However, further research conclusively became apparent that (+)SKF-10,047 binding was partially displaced using selective NMDAR ligands (Wong *et al.* 1988) evidencing another binding site, identified as the σR. In the early 1990´s, and based on the selectivity profile of some ligands and the molecular mass of binding sites, two σR subtypes were designated: σ_1 and σ_2 (Hellewell and Bowen 1990), but only the σ_1R has been unequivocally cloned so far.

1.6.2 Cloning and putative structure

The σ_1R has been cloned from guinea pig liver (Hanner *et al.* 1996), human placental cells and brain (Kekuda *et al.* 1996; Prasad *et al.* 1998), rat brain (Seth *et al.* 1997; Mei and Pasternak 2001), and mouse brain (Pan *et al.* 1998). These four different mammalian σ_1R proteins thus far cloned all consist of 223 amino acids with a sequence identity of >90%, indicating its importance in cellular functions. The human gene encoding the σ_1R is located on chromosome 9 and consists of four exons interrupted by three introns (Prasad *et al.* 1998). The σ_1R cDNA is 1.7 kb long (23.25 kDa).

The σ_1R exhibits no significant sequence homology to any of the mammalian proteins thus far cloned. However, it shares 30% identity with a yeast gene that codes a sterol C8-C7 isomerase that is necessary for cholesterol synthesis, but the σ_1R showed no

sterol isomerase activity. In addition, mammals express a totally different protein with C8-C7 isomerase activity with no structural similarity to either the yeast C8-C7 sterol isomerase or mammalian σ_1R (Hanner *et al.* 1995; Silve *et al.* 1996).

The σ_1R is an integral membrane protein. Hydropathy analyses have led to the prediction of two topology models consisting of one (Hanner *et al.* 1995; Kekuda *et al.* 1996) or two (Jbilo *et al.* 1997; Pan *et al.* 1998) transmembrane domains. Aydar studies (Aydar *et al.* 2002) provided evidence in support to the second model demonstrating that the N-terminus as well as the C-terminus are located on the cytoplasmic surface, and predicted two putative transmembrane domains (amino acid positions 13-34 and 86-108), an extracellular loop of approximately 50 amino acids and an intracellular C terminus of 125 amino acids (Fig. 9).

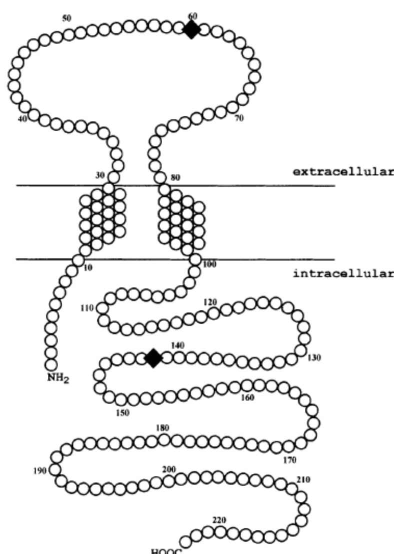


Fig. 9. A structural model of the σ_1R. This model contains two transmembrane segments. The N and C termini are shown on the intracellular side of the membrane. The two lysines in the guinea pig σ_1R are indicated by closed diamonds. In the rat σ_1R, residue 60 is arginine, so the only primary amino groups are lysine 142 and the terminal amino group. Their intracellular location in this model is consistent with poor biotin labelling prior to permeabilization and efficient labelling thereafter (Aydar *et al.* 2002).

Sequence-specific antibodies (Alonso *et al.* 2000; Palacios *et al.* 2003), antisense oligodeoxynucleotides (Matsumoto *et al.* 2001; Maurice *et al.* 2001a; Hiramatsu and Hoshino 2004) and a σ_1R knockout mouse (σ_1R-KO) (Langa *et al.* 2003) have been developed as a tools to study this protein.

However, although the σ_2 subtype is a different physical entity from the σ_1R (18-21 kDa versus 25 kDa) (Hellewell and Bowen 1990), cloning of the σ_2R has not yet been reported. It was recently identified the putative σ_2R binding site as progesterone receptor membrane component 1 (PGRMC1) (Xu *et al.* 2011). In contrast to σ_1R that translocates to different membrane compartments, σ_2R appears to be a lipid raft protein related to Ca^{2+} signalling via sphingolipid products. The σ_2R has been implicated in the

regulation of cell proliferation and cell viability (Bowen 2000; Hashimoto and Ishiwata 2006).

There is evidence for splice variants for the σ_1R subtype that lead to at least two truncated versions of the σ_1R (Ganapathy *et al.* 1999; Zamanillo *et al.* 2002; Guitart *et al.* 2004; Shioda *et al.* 2012). In addition, nascent data describe σ_1R polymorphisms that have functional consequences in disease states such as schizophrenia or alcoholism (Ishiguro *et al.* 1998; Miyatake *et al.* 2004).

1.6.3 Anatomical distribution of σ_1R

The σ_1R is widely distributed in peripheral organs. In the digestive tract (Samovilova and Vinogradov 1992), the σ_1R is enriched in the mucosal and submucosal regions, with fewer amounts in the muscular regions. The σ_1R is also present in the liver (Dumont and Lemaire 1991; Hellewell *et al.* 1994; Maurice *et al.* 1996), kidney (Hellewell *et al.* 1994), heart (Jansen *et al.* 1992; Ela *et al.* 1994), sexual organs (Jansen *et al.* 1992), and skin (Sánchez-Fernández *et al.* 2013a).

In the **CNS**, the σ_1R seems to be concentrated in brain areas involved in motor functions, in limbic areas, sensory areas, and areas associated with endocrine functions. It is highly expressed in neuron cell bodies (perikarya) and dendrites but its expression has not been described in axonal fibres or terminals. Studies in rats showed that the σ_1R is concentrated in the granular layer of the olfactory bulb, hypothalamic nuclei, septum, central gray, motor nuclei of the hindbrain, DH of the spinal cord and dentate gyrus of hippocampus (McCann *et al.* 1994; Alonso *et al.* 2000). In contrast, studies in mice showed low immunoreative signal in those areas reported by Alonso's group (Alonso *et al.* 2000) but enriched labelling in motoneurons of the brainstem and spinal cord (Mavlyutov *et al.* 2010). These discrepancies could be explained by a different species distribution of the σ_1R or the different specificity of the antibodies used in the studies. At the level of synaptic contacts, intense immunostaining was associated with postsynaptic structures including postsynaptic thickening and some polymorphous vesicles, whereas the presynaptic axons were devoid of immunostaining (Alonso *et al.* 2000). The σ_1R immunostaining was never found to be associated with structures immunostained for the astrocyte marker glial fibrillary acidic protein (GFAP) but was associated with the ependymocytes, which border the ventricular compartments extending from the olfactory bulb to the spinal cord (Alonso *et al.* 2000). The σ_1R is

also present in oligodendrocytes in rat brains (Palacios *et al.* 2003; Hayashi and Su 2004) and knockdown of σ_1Rs by small interfering RNA is shown to inhibit myelination of oligodendrocytes (Hayashi and Su 2004), suggesting a role in formation/maintenance of myelin.

In the peripheral nervous system (**PNS**), double immunofluorescence studies showed that the σ_1R co-localized with S100 protein, a specific marker of Schwann cells (Palacios *et al.* 2004). Although oligodendrocytes and Schwann cells have different origin and location, both share a common role in the myelination process. In this way, progesterone (PROG) (shows moderate σ_1R affinity) is known to promote the formation of new myelin sheaths by Schwann cells in rodent sciatic nerve lesions (Baulieu and Schumacher 1997). Therefore, the interaction of neurosteroids with σ_1Rs has been suggested to play a role in myelination during development, in remyelination during recovery after demyelinating lesions and/or maintenance of the myelin sheath in normal conditions. Recently, an important amount of σ_1R protein has also been described in both sensory neurons and satellite cells in the dorsal root ganglia (Sánchez-Fernández *et al.* 2013b; Bangaru *et al*. 2013).

Subcellular localization studies by electron microscopy in neurons have shown that the σ_1R distribution is primarily associated with the membrane of mitochondria, some cisternae of the endoplasmic reticulum (ER) and the plasma membrane (McLean and Weber 1988; Alonso *et al.* 2000). Given this unusual distribution, it has been proposed that, on activation, the σ_1R is translocated from the ER to the plasma membrane or to the nuclear membrane (Morin-Surun *et al.* 1999; Hayashi and Su 2001).

1.6.4 σ_1R as a chaperone

The σ_1R is structurally different from GPCRs and ion channels. The σ_1R is apparently devoid of its own specific signalling machinery but it can modulate (amplify or reduce) the signalling initiated when the target protein (receptor, enzyme or ion channel) it is interacting with becomes activated (Lupardus *et al.* 2000; Su and Hayashi 2003; Mavlyutov and Ruoho 2007; Tsai *et al.* 2009; Hayashi *et al.* 2010, 2011; Su *et al.* 2010). Under normal physiological conditions, most target proteins are not affected by the σ_1R, and only when disturbed or stressed, σ_1R chaperones can assist them and exert its modulatory effects. As a ligand-regulated chaperone, the modulatory activity on the target protein can be enhanced or inhibited by agonists or antagonists acting on the σ_1R.

One well characterized example of this σ_1R-chaperone activity occurs at the **ER**. The σ_1R is located at the interface with mitochondria at the mitochondria-associated endoplasmic reticulum membrane (MAM) where it regulates the stability of inositol-1,4,5-trisphosphate (IP_3) receptor (IP_3R) to ensure proper Ca^{2+} signalling between ER and mitochondria. The σ_1R forms a complex with another chaperone (BiP) at resting conditions, but in a pathological/stressful scenario, in the presence of high concentrations of cytosolic IP_3, the Ca^{2+} concentration at the ER dramatically drops and the σ_1R dissociates from BiP and binds to unstable IP_3R, preventing from being degraded and ensuring the proper Ca^{2+} influx into the mitochondria (Hayashi and Su 2007; Hayashi *et al.* 2009; Tsai *et al.* 2009). The σ_1R translocates from ER to the plasma membrane when cells are stimulated or undergo prolonged stress (Fig. 10).

At the **plasma membrane**, the σ_1R modulates the activity of Ca^{2+} (Zhang and Cuevas 2002; Tchedre *et al.* 2008), K^+ (Aydar *et al.* 2002), acid-sensing (Herrera *et al.* 2008), and Na^+ (Cheng *et al.* 2008; Zhang *et al.* 2009) channels, and regulates the activity of PLC and PKC. The σ_1R also modulates NMDARs (Monnet *et al.* 1990; Bergeron *et al.* 1996), probably by regulating the opening of a small conductance Ca^{2+}-activated K^+ current (SK channels) that shunts NMDAR-mediated responses (Martina *et al.* 2007). GPCRs such as DA (Peeters *et al.* 2004; Navarro *et al.* 2010) GABA (Mtchedlishvili and Kapur 2003) and μ (Kim *et al.* 2010) receptors have also been described to be susceptible to regulation by σ_1R ligands. A direct physical interaction with the σ_1R has been demonstrated for K^+ channels (Aydar *et al.* 2002), dopamine D1 receptor (Navarro *et al.* 2010) and μ-opioid receptor (Kim *et al.* 2010).

At the **nucleus**, the σ_1R located at the MAM neither associate physically nor regulate stability of the antiapoptotic protein Bcl-2, but controls its gene expression by activating nuclear factor κB (Meunier and Hayashi 2010).

Fig. 10 summarizes the involvement of the σ_1R in signal transduction pathways.

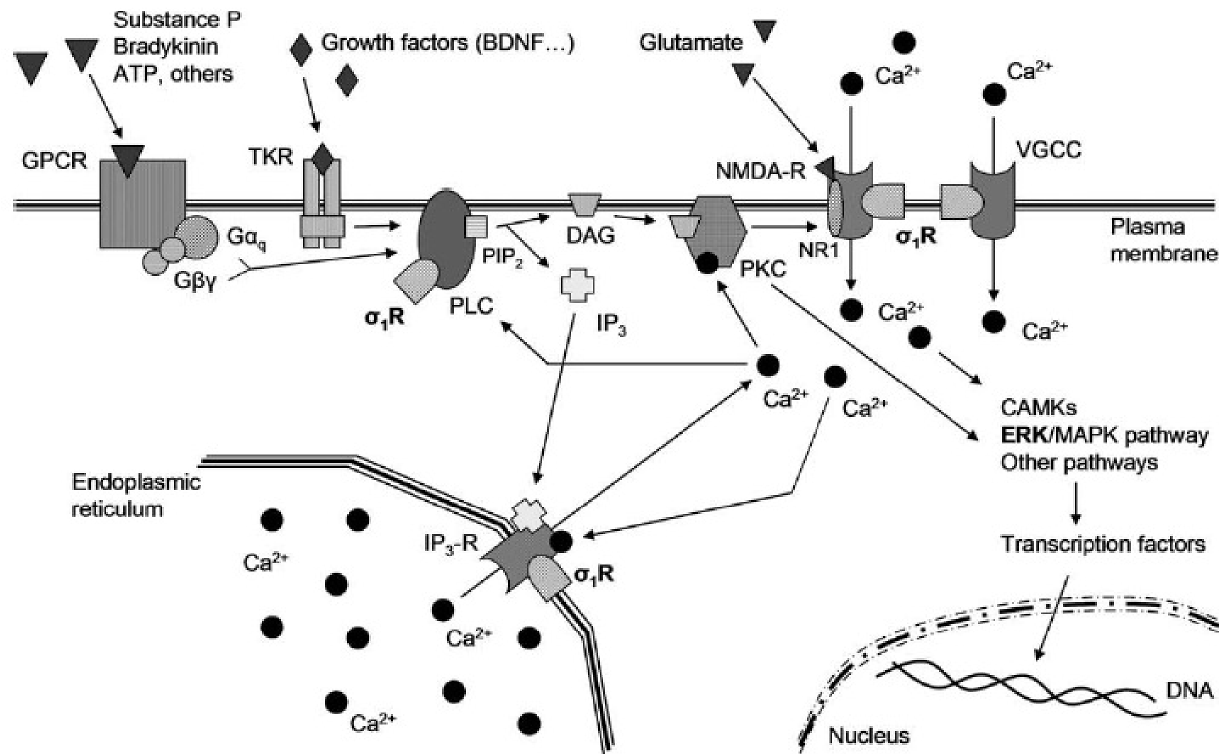


Fig. 10. Involvement of the σ$_1$R in signal transduction pathways. Activation by nociceptive mediators released into the DH of GPCRs involving Gα$_q$-coupling and other PLC-activating G proteins, and/or tyrosine kinase receptors (TKRs), stimulates PLC enzymes to hydrolyze phosphorylated phosphatidylinositols (e.g., PIP$_2$) to produce diacylglycerol (DAG) and IP$_3$. IP$_3$ binds then to IP$_3$ receptors (IP$_3$R) in the ER to promote the efflux of Ca^{2+} to the cytoplasm. Raises of cytosolic Ca^{2+} are also produced by Ca^{2+} influx through ionotropic NMDARs and voltage-gated Ca^{2+} channels (VGCCs). In turn, some PLC isoforms are activated by Ca^{2+} and low increases in intracellular Ca^{2+} concentrations activate IP$_3$R (Ca^{2+}-induced Ca^{2+} release) to promote more Ca^{2+} to be released from the ER. Interestingly, PKC as well as calcium/calmodulin-dependent kinases (CAMKs) is activated by Ca^{2+}. These enzymes phosphorylate diverse plasma membrane receptors and ion channels (e.g., NR1 subunit of NMDAR), being responsible for their rapid sensitization, and activate different signalling pathways, including the ERK pathway, leading to both rapid kinase-dependent posttranslational regulation and long-term changes via transcriptional regulation in DH neurons. σ$_1$R plays a key role in the control of intracellular Ca^{2+} levels. Activation of σ$_1$Rs increases intracellular Ca^{2+} concentrations by potentiating both Ca^{2+} entry at the plasma membrane level (via NMDAR and VGCC) and Ca^{2+} mobilization from endoplasmic stores (via PLC and IP$_3$R), which is followed by increased kinase sensitization (rapid) and transcriptional activation (long-term) of key gene products underlying pain hypersensitivity. Absence/blocking of σ$_1$Rs would avoid upregulation of Ca^{2+}-dependent sensitizing intracellular cascades (de la Puente *et al.* 2009).

1.6.5 σ_1R endogenous ligands

Steroids, neurosteroids, neuropeptide Y (NPY), CGRP, sphingolipids, *N*-*N*-dimethyltryptamine (DMT), are different potential endogenous ligands for the σ_1R.

Su and coworkers were the first to suggest that some neurosteroids (steroids that are locally synthesized in the CNS and PNS) serve as endogenous ligands for σR (Su *et al.* 1986), which they were collectively named as sigmaphins. There is evidence supporting that pregnenolone (PREG), dehydroepiandrosterone (DHEA), and their sulphate esters act as agonists at the σ_1R; and PROG act as a potent antagonist (Monnet *et al.* 1995; Bergeron *et al.* 1996, 1999; Maurice *et al.* 1999, 2001b). *In vitro* pharmacological tests have shown that PREG-S and DHEA enhanced Glu release in hippocampus and prelimbic cortex, respectively (Meyer *et al.* 2002; Dong *et al.* 2007); and that DHEA-S and PREG-S enhanced, while progesterone blocked, the NMDA-stimulated NA release in hippocampus (Monnet *et al.* 1995). *In vivo* pharmacological studies point to effects on stress, drug addiction, depression, amnesia, anxiety, pain and schizophrenia (Maurice *et al.* 2001a,b, 2006; Romieu *et al.* 2003; Maurice 2004; Díaz *et al.* 2009; Yadid *et al.* 2010; Ritsner 2010; Ritsner *et al.* 2010).

Endogenous ligands known to interact with the σ_1R are summarized in Table 3.

Table 3. Summary of endogenous ligands known to interact with the σ_1R (Adapted from Zamanillo *et al.* 2012).

Compounds	σ_1R Affinity [Ki nM]	σ_2R Affinity [Ki nM]	Funtion on σ_1R	Pharmacological action/s
D-erythro-sphingosine	140	13000	Agonist?	Endogenous amine involved in lipid signalling
DHEA-S	5200	?	Agonist	$GABA_A$ negative modulator
L-threo-sphingosine	20	8300	Agonist?	Endogenous amine involved in lipid signalling
N,N-dimethylsphingosine	120	2800	Agonist?	Endogenous amine involved in lipid signalling
DMT	14750	21710	Agonist	$5\text{-}HT_{2A}$ receptors agonist, psychedelic drug
NPY	~10	?	Agonist?	Anti-amnesic, anticonvulsant
PREG-S	980	?	Agonist	NMDA positive/$GABA_A$ negative modulator
PROG	130	?	Antagonist	NMDA negative/$GABA_A$ positive modulator
Sphinganine	70	35000	Agonist?	Endogenous amine involved in lipid signalling

1.6.6 σ_1R exogenous ligands

The discovery of selective σ_1R ligands with a defined functionality presents some inconveniences:

- Most σ_1R ligands show modest σ_1/σ_2 selectivity and/or a modest selectivity in comparison to other targets. The stereospecificity toward dextrorotatory isomers of benzomorphans is a characteristic that distinguishes σ_1 from σ_2 binding sites. Another distinguishing property is that the σ_1R is allosterically modulated by rozipine and phenytoin (Musacchio et al., 1989; DeHaven-Hudkins et al., 1993) whereas σ_2R is not affected. Phenytoin is able to shift agonists to significant higher affinity values while in the case of antagonists it does not shift or produces a very little shift to lower affinities (Cobos *et al.* 2005, 2006).
- Some σ_1R ligands do not show the classical linear dose-response curve in behavioural, biochemical, and electrophysiological studies (the effects disappear at high doses producing a biphasic bell-shaped curve).
- σ_1R seems to be quite promiscuous as many different ligands with very diverse structure display affinity for this receptor: antipsychotics (haloperidol), antidepressants (fluvoxamine, sertraline), antitussives (dextromethorphan, carbetapentane), drugs for the treatment of Parkinson's disease (amantadine) or Alzheimer's disease (memantine), and drugs of abuse (cocaine, methamphetamine).
- σ_1R is not a typical GPCR. It is a chaperone, and consequently, there is a lack of appropriate functional assays that makes it difficult to determine an agonist/antagonist at σ_1R.
- σ_1R is mainly an intracellular target and consequently hydrophobicity may be determinant for the potency *in vivo* (a high *in vitro* affinity for the σ_1R may not be correlated to high efficacy *in vivo*).

Exogenous σ_1R ligands are summarized in Table 4.

Table 4. Summary of exogenous ligands that interact with the σ_1R. Abbreviations: N.m.: not measurable; L: launched; R: registered; I/II/III: phase I/II/III; ADHD: attention deficit/hyperactivity disorder; OCD: Obsessive-compulsive disorder; PTSD: posttraumatic stress disorder; TCA: tricyclic antidepressant; *: under active development. Other abbreviations see the abbreviations list. (Adapted from Zamanillo *et al.* 2012).

Ligands		Pharmacological Activity				Clinical Development, if it has been reached
		σ_1 Affinity [Ki nM]	σ_2 Affinity [Ki nM]	Function on σ_1R	Pharmacological action/s	Indication (Highest Phase)
Benzomorphans	(+)-Pentazocine	16.7	6611	Agonist		
	(–)-Pentazocine	807	2324	Agonist	k_1 agonist, μ_1 μ_2 ligand, low affinity δ and k_3 opioid ligand	
	(±)Pentazocine	12.1	92	Agonist	k_1 agonist, μ_1 μ_2 ligand, low affinity δ and k_3 opioid ligand	Pain (L)
	(+)-SKF-10.047	597	39740	Agonist	NMDAR ligand (PCP site)	
	(-)-SKF-10.047	50399	41461	Agonist		
Antipsychotics	BMY-14802	66	51	Antagonist	5-HT_{1A} agonist	Psychosis (II); Schizophrenia (II)
	Dup734	2.6	23	Antagonist	5-HT_2 antagonist	Psychosis (I); Tardive dyskinesia (I)
	E-5842	4	220	Antagonist	High affinity for α_{2B}, α_{1A} and α_{1B} AR, low to moderate affinity for dopamine, 5-HT and Glu receptors	Schizophrenia (II)
	Eliprodil (SL-82.0715)	132	634	Antagonist	NMDAR antagonist, α_1-AR ligand	Schizophrenia (II); Head injury (II/III); Cerebrovascular ischemia (II/III); Parkinson's disease (II)
	Chlorpromazine	453	1628		Dopamine D_2 antagonist	Psychosis (L)
	CYR-101; MT-210*	47	56		5-HT_{2A} antagonist; σ_2R antagonist	Schizophrenia (II)
	Haloperidol	6.44	221	Antagonist	Dopamine D_2 and D_3 antagonist; σ_2 agonist	Psychosis (L); Schizophrenia (L); Tourette's disease (R)
	NE-100	1.5	84.6	Antagonist		Schizophrenia (II)

	Compound	σ1	σ2	Activity	Other mechanism	Indications
	Panamesine (EMD-57445)	6 (IC50 nM)		Antagonist		Schizophrenia (II)
	Rimcazole (BW-234U. SH 1/76)	2380	1162	Antagonist	Dopamine transporter inhibitor	Psychosis (II); breast, lung and prostate cancer (I)
	SR-31742A	0.4 (IC50 nM)	25 (IC50 nM)	Antagonist	High affinity for C8-C7 sterol isomerase	Psychosis (II); Schizophrenia (II)
	SSR-125047				σ ligand	Schizophrenia (I)
Antidepressants	(±)Fluoxetine*	240	16100	Agonist	Selective 5-HT reuptake inhibitor	Depression (L); OCD (L); Panic disorder (L); Bulimia nervosa (L); Obesity (L); Premenstrual syndrome (L); Fibromyalgia (II)
	(±)Norfluoxetine	2377	34630			
	Citalopram*	292	5410		Selective 5-HT reuptake inhibitor	Depression (L); Panic disorder (L); Mood disorder (II); Huntington's disease (II); Bipolar disorder (II/III)
	Clorgyline	2.9	505	Agonist?	Irreversible monoamine oxidase A inhibitor	
	Cutamesine (Msc1; SA-4503; AGY-94806) *	4.6	63.1	Agonist	Acetylcholine release enhancer	Depression (II); Stroke (II)
	Desipramine*	1987	11430		Monoamine reuptake inhibitor (TCA)	Depression (L); Gastroesophageal reflux disease (III)
	Fluvoxamine	36	8439	Agonist	Selective 5-HT reuptake inhibitor	Depression (L); OCD (L); Social phobia (L)
	Igmesine (JO-1784. CI-1019)	75	>1000	Agonist		Depression (III); Functional diarrhea (I); Alzheimer's disease (II)
	Imipramine*	343	2107	Agonist	Monoamine reuptake inhibitor (TCA)	Depression (L); Enuresis (L); Dyspepsia (III)
	Opipramol	0.2				Depression (L); Anxiety disorder (II); Somatoform disorders (II); Premedication prior surgery (II)
	Paroxetine*	1893	22870	No effect	Selective 5-HT reuptake inhibitor	Depression (L); Panic disorder (L); Anxiety (L); Post-traumatic stress (L); Depression (L); Social phobia (L); Premenstrual syndrome (L); OCD (L); Dementia AIDS related (I/II)

	R(-)Fluoxetine	2180	24100			
	S(+)Fluoxetine	120	5480			
	Sertraline	57	5297	Agonist	Selective 5-HT reuptake inhibitor	Depression (L); OCD (L); Post-traumatic stress (L); Panic disorder (L); Social phobia (L); Premenstrual syndrome (L)
	VPI-013; OPC-14523	47	56	Agonist	Agonist of pre- and postsynaptic 5-HT_{1A} receptors, SERT inhibitor	Depression (II); Neuropathic pain (II); Sexual dysfunction (II)
Antitussives	Carbetapentane	128	1953	Agonist	Muscarinic antagonist, σ_2 agonist	
	Dextromethorphan*	205	11060	Agonist	NMDAR allosteric antagonist	Cough (L); Rett's syndrome (II); Diabetic macular edema (I/II)
	Dimemorfan	151	4421	Agonist		Cough (L); Epilepsy (L)
Parkinson's and / or Alzheimer's disease	Amantadine	7440		Agonist?	NMDAR antagonist, antiviral properties	Influenza A (L); Parkinson's disease (L)
	ANAVEX 2-73; AE-37*			Agonist	NMDARs; Na^+ channels (voltage-gated); Lipid peroxidation inhibitor	Alzheimer's type dementia (UAD)
	Donepezil*	14.6		Agonist	Cholinesterase inhibitor	Dementia Alzheimer's type (L); ADHD (III); Dementia Lewy's bodies (III); Ischemic stroke (II); Cocaine dependency (II); Autism (II); Down's syndrome (II); Neurological disorders (II); Fragile X syndrome (II); Amblyopia (I)
	Memantine*	2600		Agonist?	NMDAR antagonist, antiviral properties	Spasticity (L); Dementia (L); Dementia Alzheimer's type (L); Cancer therapy associated disorders (III); Cognitive disorders (III); Depression (III); Heroin dependence (II/III); Alcoholism (II); Autism (II); Glioblastoma multiforme (II)
	T-82	24			5-HT_3 receptor antagonist; Acetylcholinesterase inhibitor	Alzheimer's type dementia (II)
Drugs of abuse	Cocaine	2000	31000	Agonist	Monoamine transporters inhibitor, psychostimulant	
	MDMA*	3057	8889		Preferential SERT inhibitor, psychostimulant	Post-traumatic stress (II)
	Metamphetamine	2160	46670		Preferential dopamine transporter inhibitor, psychostimulant	

Anti-convulsants	Phenytoin (DPH)	N.m.	N.m.	Allosteric modulator σ_1	Delayed rectifier K^+ channel blocker, T-type Ca^{2+} current inhibitor, Na^+ current inhibitor	Arrhythmia (L); Epilepsy (L); Neuropathic pain (L)
	Ropizine	N.m.	N.m.	Allosteric modulator σ_1		
Other σ_1R ligands	(-)-3-PPP	~310	~1200			
	(+)-3-PPP	79	120	Agonist	σ_2 agonist, NMDAR ligand, dopaminergic agonist	
	AC-927	30	138	Antagonist	σ_2 antagonist	
	BD-1008	2	8	Antagonist	σ_2 agonist	
	BD-1047	0.9	47	Antagonist	α AR ligand	
	BD-1063	9	449	Antagonist		
	BD-737	8.78	68.3	Agonist		
	CM-31747; SR-31747A				σ ligand	Autoimmune disease (II); Rheumatoid arthritis (II); Prostate cancer (II)
	DTG	77	43		σ_2 agonist	
	MR-200	1.5	21.9	Agonist		
	PRE-084	44		Agonist		
	S1RA* (E-52862)	17	6300	Antagonist		Pain (II); Neuropathic pain (II)
	Siramesine (LU-28-179)	17 (IC50 nM)	0.12 (IC50 nM)	Antagonist	σ_2 agonist, α_1-AR ligand	Anxiety disorder (II); Cancer
	SM-21	>1000	67.5		σ_2 agonist, cholinergic muscarinic receptor affinity	
	SSR-125329A; SR-125329; SSR-125329	0.4 (IC50 nM)	25 (IC50 nM)		Delta8-Delta7-sterol isomerase ligand	Prostate cancer (I); Immunological disorders (I); Rheumatoid arthritis (I)

1.6.7 σ_1R pharmacophore models

As mentioned, the σ_1R protein seems to be promiscuous as a long variety of drug classes with very diverse structure, including antipsychotic agents, anxiolytics, antidepressants, monoamine oxidase inhibitors, antineoplastic agents or anticholinergics, display affinity for this receptor. This led to several laboratories to try to find a universal pharmacophore model to explain the diversity of binding ligands.

The first pharmacophore models did not differentiate between σ_1 and σ_2 ligands (Largent *et al.* 1987; Gilligan *et al.* 1992). The first selective model for the σ_1R binding site (Glennon *et al.* 1994) defined an "Amine Site" flanked by one "Primary Hydrophobic Region" bigger than the "Secondary Hydrophobic Region" which optimally accommodates a three-carbon chain.

Based on 23 structurally diverse σ_1 ligands Langer developed the first 3D computer generated σ_1R pharmacophore model, consisting in four hydrophobic groups and one positive ionizable group (Laggner *et al.* 2005). Another computer based model (Zampieri *et al.* 2009) defined one basic amine, two hydrophobic aromatic groups, one hydrophobic group and one H-bond acceptor. A completely different approach is described by Oberdorf (Oberdorf *et al.* 2010). According to this model, the central protonated amino group of σ_1 ligands forms an H-bond with and H-bond acceptor group on the receptor surface instead of an ionic interaction with a negatively charged group. The receptor surface consists of many hydrophobic particles leading to many hydrophobic interactions during ligand binding.

1.6.8 σ_1R therapeutic interest

A search on the World Intellectual Property Organization (WIPO) gave a result of about 100 Patent Cooperation Treaty applications for various agents that bind to the σR (Zamanillo *et al.* 2012). The analysis indicates that neurologic and neuropsychiatric disorders, pain, and cancer are the main focuses of interest (Fig. 11). Some σ_1R ligands are being developed for depression (igmesine, cutamesine, OPC14523, opipramol), anxiety (opipramol, siramesine), schizophrenia (panamesine, rimcazole, eliprodil, BMY14802, SR31742A, NE100, MT210, E-5842), Alzheimer's disease (igmesine, T82), Parkinson's disease (eliprodil), autoimmune disease (SR31747A), prostate cancer (SR31747A), rheumatoid arthritis (SR31747A), stroke (cutamesine, eliprodil), sexual dysfunction (OPC14523), neuropathic pain (OPC14523), somatoform disorders

(opipramol), and premedication prior surgery (opipramol). Other therapeutic indications are being actively investigated: neuroprotection, drug dependence, cancer, cardioprotection, and pain (Table 4).

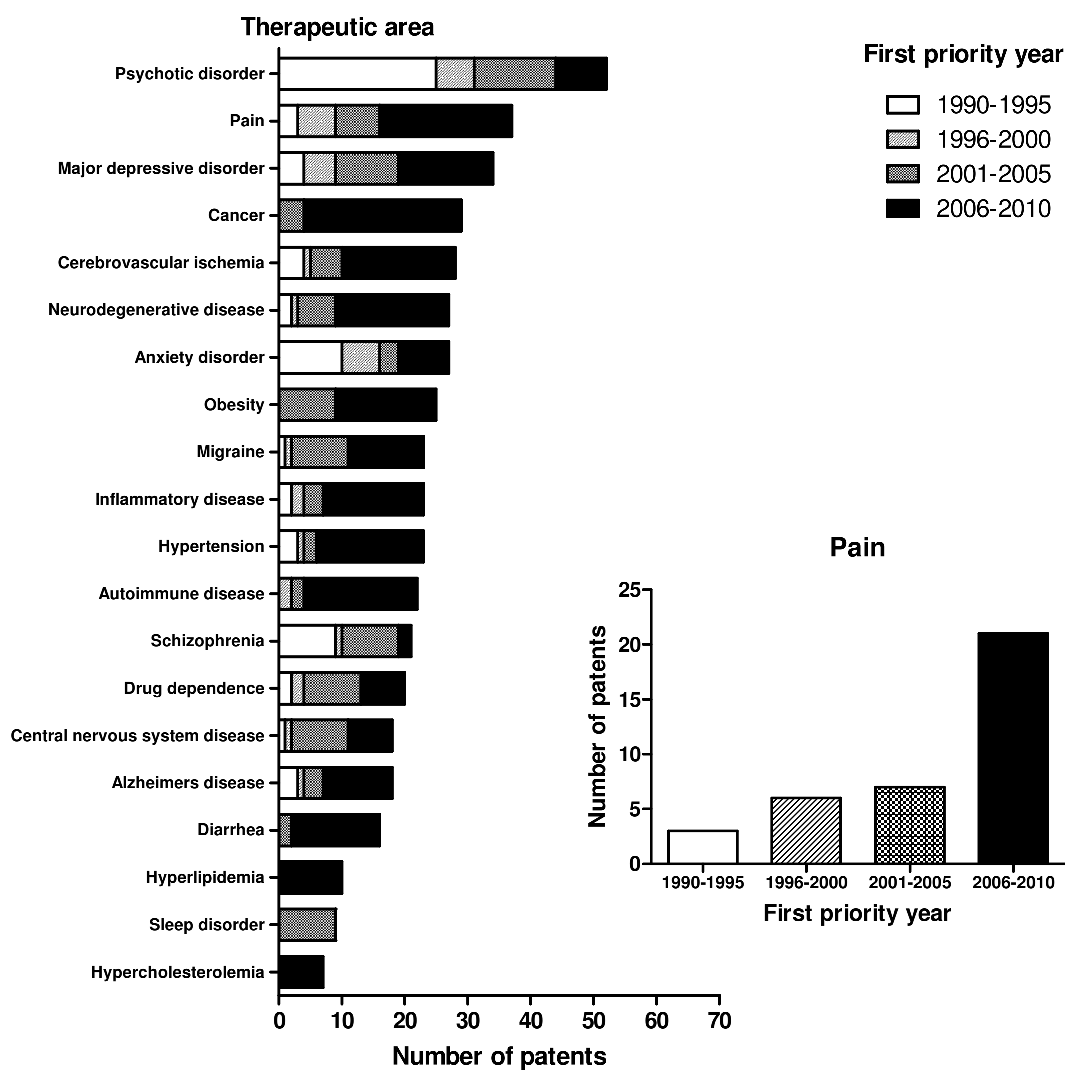


Fig. 11. Patent activity surrounding σ_1R ligands by indication (therapeutic area). Source: WIPO-World Intellectual Property Organization (January, 2011). (Zamanillo *et al.* 2012).

1.6.9 σ_1R and pain

As previously stated, the σ_1R is distributed in important CNS areas for pain processing such as the superficial layers of the spinal cord DH, PAG, LC, and RVM (Walker et al, 1992).

Interestingly, the σ_1R is involved in the modulation of opioid analgesia and in the modulation of pain behaviour in the absence of opioids (Zamanillo *et al.* 2013).

1.6.9.1 σ_1R modulation of opioid-induced antinociception

Opioids are powerful analgesics with basically no ceiling effects, but their use is limited by adverse effects at doses necessary to elicit antinociception, which reduce quality of life and medication compliance (Hjalte *et al.* 2010). Thus, in order to minimize opioid-related adverse effects, several approaches combining other drugs with opioids to increase their potency (and thus reduce the opioid doses) have been proposed. It is known that a number of transmitters modulate (decrease) the sensitivity of animals to opioid analgesics, including orphanin FQ/nociceptin (Grisel *et al.* 1996; Mogil *et al.* 1996; Tian *et al.* 1997; King *et al.* 1998), cholecystokinin (Faris *et al.* 1983; Cesselin 1995; Nichols *et al.* 1995; Xu *et al.* 1996), neuropeptide FF (Cesselin 1995; Roumy and Zajac 1998), and DA (Rooney and Sewell 1989). On the other hand, pain drugs such as NSAIDs and α_2-AR agonists (Ossipov *et al.* 1989; Yesilyurt and Uzbay 2001; Déciga-Campos *et al.* 2003; Zelcer *et al.* 2005), selective cyclooxygenase-2 (COX-2) inhibitors, cannabinoid receptor agonists, and GluR antagonists have also shown to modulate (enhance) opioid antinociception (Fischer 2011). Involvement of the σ_1R in the modulation of opioid analgesia is also known time ago (Chien and Pasternak 1993) and the site of action for this modulation points to supraspinal structures (Mei and Pasternak 2002).

Unlike opioids, σ_1R antagonists do not affect acute pain perception (seen in the animal model of tail-flick or the hot plate tests) but some studies suggest a potential use as opioid adjuvants. Investigations on the role of σ_1R on opioid antinociception began in Pasternak's laboratory in 1993. They found that haloperidol (σ_1R antagonist) enhanced and (+)-pentazocine (σ_1R agonist) lowered morphine antinociception (Chien and Pasternak 1993), pointing to the presence of an anti-opioid sigma system where the σ_1R exerts a tonic inhibitory control on the opioid receptor-mediated signalling pathways. Then, the concept of modulation by the σ_1R was expanded to other opioid analgesic systems (δ- and κ- in addition to μ-opioid receptors) (Chien and Pasternak 1994). Patients show a wide range of sensitivity to opiate drugs. Similarly, the activity of the σ_1 anti-opioid systems varies among strains, possibly explaining some of their sensitivity differences to opioids (Chien and Pasternak 1994). Thus, these sensitivity differences might reflect varying levels of tonic activity of the σ_1 system. For example, κ drugs are far less effective analgesics in BALB-c mice than in CD-1 mice (the κ_1 drug (-)-U50,488H is 6-fold more potent in CD-1 mice). Thus, the anti-opioid σ_1 system is more tonically active against κ than μ antinociception.

As commented, the first report on σ modulation of the opioid system used (+)-pentazocine, haloperidol, and morphine as pharmacological tools. Pentazocine is widely used clinically as an opiate analgesic and is provided as a racemate of both the (+)- and (-)- isomers. Although (-)-pentazocine is an effective opioid with activity at both μ and $κ_1$ receptors, (+)-pentazocine, in contrast, has no opioid activity but is a potent $σ_1$R agonist. Systemic (+)-pentazocine reduced systemic opioid antinociception mediated by μ, δ, $κ_1$, and $κ_3$ receptors (Chien and Pasternak 1994) in rodents without affecting morphine's inhibition of gastrointestinal transit and lethality. On the other hand, haloperidol is an old antipsychotic drug used in the treatment of schizophrenia and in the treatment of acute psychotic states and delirium. In addition to its high affinity for the dopamine D_2R, haloperidol also blocks the $σ_1$R and the $σ_2$R quite effectively (2.1, 2.2 and 16 nM, respectively) (Moison *et al.* 2003). Haloperidol potentiates opioid analgesia both in patients (Maltbie *et al.* 1979) and rodents (Chien and Pasternak 1994). However, haloperidol is not selective and this makes the interpretation of some of the studies difficult. Thus, the actions of haloperidol in opioid modulation might also be due to activity at other receptors different from $σ_1$R as D_2R. Indeed, D_2R deficient mice show an enhancement in opioid antinociceptive effects and a similar potentiation was obtained when sulpiride (a D_2R antagonist) was administered in wild-type (WT) but not in D_2R-KO mice (King *et al.* 2001). Interestingly, (+)-pentazocine and haloperidol retained its ability to reduce or potentiate opioid antinociceptive effect in these D_2R-KO mice, respectively. Because these mice have no D_2R, the actions are most probably mediated through $σ_1$ sites. These observations argue strongly for independent $σ_1$R and D_2R modulation of opioid antinociception.

Later on, the observations on opioid modulation were confirmed using other $σ_1$R ligands. (+)-MR200 and (-)-(1S,2R)-methyl 2-(bromomethyl)-1-phenylcyclopropanecarboxylate, structurally related to haloperidol, have shown to potentiate opioid antinociception suggesting an antagonist profile (Marrazzo *et al.* 2006, 2011). Although (+)-MR200 is selective for σRs, the $σ_1/σ_2$ selectivity is not very high (3.9 *vs*. 21.9 nM). (-)-(1S,2R)-methyl 2-(bromomethyl)-1-phenylcyclopropanecarboxylate shows a better $σ_1/σ_2$ selectivity ratio (7.0 *vs*. 571 nM), but the selectivity against other different receptor systems and transporters is not published. On the other hand, (±)-PPC, which displays high affinity for $σ_1$ and $σ_2$ sites (1.5 and 50.8 nM, respectively) and shows a marked σ selectivity over other tested receptors (Prezzavento *et al.* 2007), antagonized the analgesic response induced by the

κ-opioid selective agonist (-)-U-50,488H in a similar way than (+)-pentazocine (Prezzavento *et al.* 2008) ruling out an agonist profile. In addition, the σ_1R antagonist BD-1047 reversed the attenuation of (-)-morphine-produced tail-flick antinociception exerted by the administration of the non-selective σ_1R agonist (+)-morphine (Tseng *et al.* 2011).

In summary, all the σ_1R ligands exposed above have shown to modulate opioid antinociception but morphine is the only opioid used as a μ opioid agonist. In addition, the antagonists used as pharmacological tools in these studies have compromised selectivity as they also bind to σ_2R and/or other receptors, at the nanomolar range. Although it has also been shown that σ_1R antisense treatment also enhances opioid antinociception (Mei and Pasternak 2007), there is a lack of studies demonstrating that blockade of σ_1Rs by selective compounds results in an enhancement of opioid antinociception and there is still unclear if this potentiation affects the undesirable opioid-induced effects. In addition, the site of action and the possible mechanisms that may underlie the effect of systemic σ_1R antagonism treatment on opioid modulation have been poorly addressed. Thus, part of the experiments of this thesis aimed to study these questions by using the selective σ_1R antagonist S1RA (E-52862) developed by ESTEVE and σ_1R-KO mice.

1.6.9.2 σ_1R modulation of pain behaviour in the absence of opioids

σ_1R ligands by themselves also play a role in modulating pain in the absence of opioids, particularly in sensitizing and chronic pain conditions (Table 5), and the site of action for this modulation points to the spinal cord. These issues have been studied using σ_1R-KO mice and selective σ_1R ligands.

The first indication that the σ_1R plays an important role in pain in absence of opioids came from studies using σ_1R-KO mice. Such studies with σ_1R-KO mice revealed that the absence of σ_1Rs reduced >50% pain responses (licking) in both phases of the formalin test in comparison to WT animals (Cendán *et al.* 2005b). These results indicate that the σ_1R seems necessary for the full expression of formalin-induced pain. σ_1R-KO mice neither developed mechanical hypersensitivity following intraplantar administration of capsaicin (Entrena *et al.* 2009b). In addition, σ_1R-KO mice were unable to normally develop neuropathic pain behaviours after partial sciatic nerve ligation (reduction of mechanical and cold allodynia) and nerve injury-induced ERK phosphorylation in the spinal cord was inhibited (de la Puente *et al.* 2009). Recently,

pain-related behaviours induced by intracolonic capsaicin (visceral pain model) were found to be reduced in σ_1R-KO mice and following paclitaxel administration (antineoplastic drug-induced neuropathic pain model) (Nieto *et al.* 2012; González-Cano *et al.* 2013). At a mechanistic level, *in vitro* spinal cord electrophysiological recordings revealed that the wind-up amplification response that normally appears following repeated C-fibre stimulation of the dorsal root is inhibited in σ_1R-KO mice (de la Puente *et al.* 2009). Wind-up represents an amplification in the spinal cord of the nociceptive message coming from the periphery, and together with long-term potentiation (LTP), both are important mechanisms underlying central sensitization and synaptic plasticity.

Interestingly, the effects seen in σ_1R-KO mice were mimicked in WT using some pharmacological tools. Systemic treatment with haloperidol and its metabolites I and II, which act as antagonists at σ_1R (Cobos *et al.* 2007), produced similar results: inhibition of formalin-induced pain (licking) (Cendán *et al.* 2005a) and capsaicin-induced mechanical hypersensitivity (Entrena *et al.* 2009a). Intrathecal BD-1047 (σ_1R antagonist) reduced formalin-induced nociception in the second phase of formalin (Kim *et al.* 2006) and prevented the upregulation of both the NMDA receptor NR1 subunit and its phosphorylated form (pNR1) induced by nerve injury in rats (Roh *et al.* 2008). Systemic administration of BD-1047 produced an antinociceptive effect on the allodynia and reduced spinal ERK phosphorylation induced by chronic compression of the dorsal root ganglion (Son and Kwon 2010).

In the same way, the new selective σ_1R antagonist S1RA developed by ESTEVE dose-dependently inhibited formalin-induced nociception (licking), capsaicin-induced mechanical hypersensitivity and nerve injury-induced mechanical and thermal hypersensitivity (Romero *et al.* 2012; Bura *et al.* 2013), paclitaxel-induced cold and mechanical allodynia (Nieto *et al.* 2012), and exerted antinociceptive effect on capsaicin-induced visceral pain (González-Cano *et al.* 2013).

Consequently, all the above exposed evidences have lead to consider σ_1R antagonists as a potential new pharmacological approach for the treatment of neuropathic pain. σ_1R modulation points to spinal Ca^{2+}-dependent second messenger cascades and enhanced NMDA responses as key mechanisms underlying its modulatory effects. However, the site of action and the possible mechanisms that may underlie the effect of systemic σ_1R antagonism treatment have been poorly addressed and are still unclear. Some of the experiments of this thesis aimed to address these issues.

Table 5. Summary of the involvement of the σ_1R in pain models: results in σ_1R-KO mice and effect of σ_1R antagonists. Other abbreviations see the abbreviations list.

Experimental pain model	Outcome in σ_1R-KO mice	σ_1R antagonist effect	References
Tail-flick test	No difference respect to WT	No effect	Chien and Pasternak 1995a,b; Ronsisvalle *et al*. 2001; Marrazzo *et al*. 2006; Cendán *et al*. 2005a; de la Puente *et al*. 2009; Tseng *et al*. 2011
Hot plate test (50ºC)	No difference respect to WT		de la Puente *et al*. 2009
Formalin test	Antinociception (phase I and II)	Antinociception (phase I and II) Decreased Fos expression and phosphorylation of NR1 in the spinal cord (phase II)	Cendán *et al*. 2005a,b; Romero *et al*. 2012; Díaz *et al*. 2009; Kim *et al*. 2006
Capsaicin-induced mechanical allodynia	No development of mechanical allodynia	Antiallodynic effect (mechanical allodynia)	Entrena *et al*. 2009a,b; Romero *et al*. 2012
Capsaicin-induced headache model		Reduced pain-related behaviours Decreased Fos-like immunoreactive neurons and pNR1 immunoreactivity in TNC.	Kwon *et al*. 2009
Intracolonic capsaicin-induced visceral pain	Reduced pain-related behaviours Development of referred mechanical hyperalgesia	Inhibition of pain-related behaviours and mechanical hyperalgesia	González-Cano *et al*. 2013
Neuropathic pain-chronic constriction injury		Antiallodynic effect (mechanical allodynia) but not antihyperalgesic effect (thermal hyperalgesia) Blockade of the increase in NR1 expression and phosphorylation	Roh *et al*. 2008
Neuropathic pain-chronic compression of the spinal root ganglion		Antiallodynic (mechanical and cold allodynia) effect No increased pERK in the spinal cord	Son and Kwon 2010
Neuropathic pain-streptozotocin-induced diabetes		Antinociceptive and antiallodynic (mechanical allodynia) effects	Ohsawa *et al*. 2011
Neuropathic pain-partial sciatic nerve ligation	No development of mechanical and cold allodynia Development of thermal hyperalgesia No increased pERK in the spinal cord	Antiallodynic (mechanical and cold allodynia) effect Antihyperalgesic effect (thermal hyperalgesia)	Romero *et al*. 2012; Bura *et al*. 2013; de la Puente *et al*. 2009
Neuropathic pain-paclitaxel treatment	No development of mechanical and cold allodynia	Antiallodynic (mechanical and cold allodynia) effect	Nieto *et al*. 2012
Wind-up response	Reduced wind-up responses in the spinal cords sensitized by repetitive nociceptive stimulation	Reduced wind-up responses in the spinal cords sensitized by repetitive nociceptive stimulation	Romero *et al*. 2012; de la Puente *et al*. 2009

1.7 S1RA

Eva Drews and Andreas Zimmer (Drews and Zimmer 2009) published a commentary in Pain journal entitled "Central sensitization needs sigma receptors", and concluded with the following sentence: "The demonstration that σ_1R antagonists have any efficacy in reverting central sensitization and thereby in reducing the increased pain sensitivity is still missing". Therefore, although the σ_1R could be considered a good molecular target for pain treatment, no conclusive data using a selective σ_1R antagonist was available.

Data on selectivity and pharmacology of σ_1R ligands used as pharmacological tools are scarce, and most antagonists used in published researches (i.e., NE-100, haloperidol, BMY-14802, BD-1047, BD-1063, (+)-MR200, (-)-(1S,2R)-methyl 2-(bromomethyl)-1-phenylcyclopropanecarboxylate) have been shown to bind also σ_2R at nanomolar range, among other receptors (Matsumoto *et al.* 1995; Matos *et al.* 1996; Ronsisvalle *et al.* 2000; Marrazzo *et al.* 2006, 2011). Consequently, the selectivity (especially σ_1R over σ_2R) of σ_1R ligands used as pharmacological tools was not properly achieved.

ESTEVE has developed a new chemical entity exerting selective σ_1R antagonism: the S1RA (Fig. 12). Our recent publications (Romero *et al.* 2012; Díaz *et al.* 2012) describe the synthesis and pharmacological profile of S1RA.

The chemical design was performed taking into account the known pharmacophoric features for the σ_1R. A basic amine was shown to be necessary in accordance with known receptor pharmacophores. Although the S1RA molecular structure does not fulfil all well-established pharmacophoric requirements of some σ_1R ligands (missing fundamental hydrophobic requirement), it presents a high affinity for the σ_1R because it is able to compensate it by fulfilling all other pharmacophoric requirements and by gaining in solvation energy (Laurini *et al.* 2013).

S1RA showed high affinity for human (K_i = 17 nM) and guinea pig σ_1R (K_i = 23.5 nM). S1RA behaved as a σ_1R antagonist based on the shift to lower-affinity values when incubated in the presence of phenytoin (Cobos *et al.* 2005, 2006; Nahas *et al.* 2008). The compound had a σ_1/σ_2 selectivity ratio >550 and failed to show significant affinity (inhibition % at 1 μM of <50%) for the 170 molecular targets tested (receptors, enzymes, transporters and ion channels). The only exception to this cut-off was the binding affinity for the human serotonin 5-HT_{2B} receptor (K_i = 328 nM) but it acted as a very low potency antagonist (IC_{50} value = 4700 nM).

S1RA displayed analgesic activity after systemic administration in different animal models of pain and enhanced opioid antinociception without increasing opioid-side effects (some of the results obtained from this thesis) at doses devoid of side effects and penetrated into the CNS and occupied CNS σ_1Rs. Interestingly, a close correspondence (significant correlation) has been found between the extent of CNS receptor occupancy and the antinociceptive efficacy of S1RA on different pain models (Romero *et al.* 2012).

S1RA is metabolised *in vitro* by multiple human CYP's (CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2D6, CYP2E1 and CYP3A4) and FMO's (FMO1 but also FMO3). It is thus unlikely that S1RA 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.

Due to the overall pharmacological, physicochemical, and ADME profile, S1RA was selected as a preclinical candidate. Upon successful completion of the preclinical regulatory toxicological and pharmacodynamic package, it was selected as a clinical candidate. S1RA has recently completed single and multiple dose phase I clinical studies and it showed a good safety, tolerability and pharmacokinetic profile in single- (EudraCT 2008-000751-94) and multiple-dose (EudraCT 2009-009424-37) studies compatible with a good absorption and once-daily oral administration (Abadias *et al.* 2013). Also, a drug interaction and safety study with morphine (EudraCT 2010-023993-38) has recently been completed in volunteers. These clinical results supported proceeding to proof of concept phase II studies for the treatment of neuropathic pain of different aetiologies and potentiation of opioid analgesia. Interestingly, it is the first-in-class compound to be developed for the indication of pain.

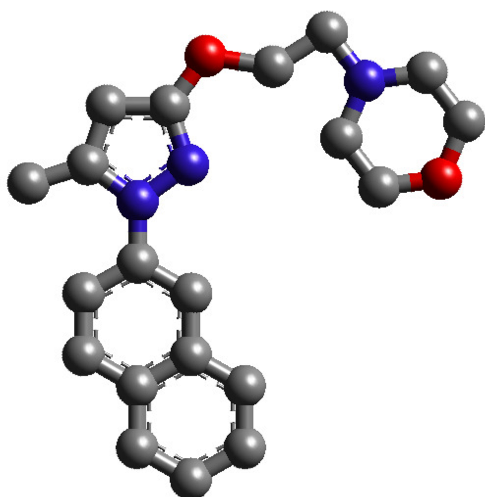


Fig. 12. Chemical structure in 3D layout of the selective σ_1R antagonist S1RA. Nitrogen atoms are represented in blue and oxygen atoms are represented in red.

2. HYPOTHESIS

The involvement of the σ_1R in the modulation of opioid analgesia in classical models of thermal acute antinociception is known from long time ago (Chien and Pasternak 1993, 1994; Marrazzo *et al.* 2006; Prezzavento *et al.* 2008). Unlike opioids, σ_1R ligands did not affect acute pain perception but σ_1R antagonists enhanced morphine antinociception in these models. However, there are still gaps of information that preclude the generalization of these findings. Thus, i) most ligands used in these studies had compromised selectivity as they also bind to σ_2R and/or other receptors at the nanomolar range; ii) only morphine was used as a μ-opioid ligand; iii) the site and mechanism of action were poorly explored; and iv) other opioid-induced phenomena, including opioid-related adverse events were not extensively investigated. Thus, further studies are needed in order to validate previous observations as well as to explore the use of σ_1R antagonists as an opioid adjuvant strategy.

On the other hand, the knowledge about the role of σ_1R in modulating pain behaviour in the absence of opioids is still incomplete. There are some reports pointing to the use of σ_1R antagonists as analgesics, mainly in models involving central sensitization (Cendán *et al.* 2005a; Cobos *et al.* 2008; Entrena *et al.* 2009a). However, very few information is available regarding the site and the mechanism of action of σ_1R antagonism on pain modulation.

Studies focused on clarifying these knowledge gaps will be important to validate σ_1R antagonism as a new strategy for pain treatment. These studies will take advantage of using S1RA, a potent and selective σ_1R antagonist developed by ESTEVE. Thus, based on previous findings reported in the literature and in the context of the Sigma-1 receptor project at ESTEVE, which is looking for new approaches for pain treatment, **we hypothesise that the use of selective σ_1R antagonists could result in two therapeutic uses for pain treatment, probably involving different sites and mechanisms of action, i.e. the enhancement of analgesia when combined with opioids and their use as analgesics *per se* in certain pain conditions.**

3. OBJECTIVES

The global objective of this thesis was to explore the therapeutic interest of selective blockade of σ_1Rs both as monotherapy and opioid adjuvant strategy, comparing possible different mechanisms and sites of action.
In order to accomplish it we addressed the following specific objectives:

1. To study the efficacy of σ_1R antagonism on opioid-induced thermal antinociception in the tail-flick test in mice and rats.

2. To explore the advantage of using σ_1R antagonists as opioid adjuvant strategy comparing opioid-induced antinociception *versus* adverse effects in mice.

3. To set up the *in vivo* concentric microdialysis technique in rats in order to assess the release of neurotransmitters in the intra-dorsal horn of the spinal cord in awake, freely-moving rats that can be used to evaluate the effect of σ_1R inhibition and explain the effect of σ_1R antagonism in modulating opioid analgesia or eliciting analgesia *per se.*

4. To evaluate the modulation of spinal release of pain-related neurotransmitters and the sites of action of selective σ_1R antagonism on opioid-induced antinociception in the tail-flick test in rats.

5. To study the analgesic efficacy of σ_1R antagonism on the formalin-evoked pain in rats.

6. To evaluate the modulation of spinal release of pain-related neurotransmitters and the sites of action of selective σ_1R antagonism on formalin-evoked pain in rats.

4. RESULTS

4.1 Set-up of the concentric microdialysis technique in awake-freely moving rats in the formalin model.

- **4.1.1 Article 1.** Evaluation of formalin-induced pain behaviour and glutamate release in the spinal dorsal horn using in vivo microdialysis in conscious rats. *Journal of Pharmacological Sciences* 2012;120(2):129-32.

4.2 Exploring the advantage of using sigma-1 receptor antagonists as opioid adjuvant strategy comparing opioid-induced antinociception versus adverse effects in mice.

- **4.2.1 Article 2.** Sigma-1 receptor antagonism as opioid adjuvant strategy: Enhancement of opioid antinociception without increasing adverse effects. *European Journal of Pharmacology* 2013;711(1-3):63-72.
- **4.2.2 Annex 1.** Supplementary results.

4.3 Exploring the analgesic efficacy and the spinal modulation of pain-related neurotransmitters by σ_1R antagonism on the formalin-evoked pain in rats.

- **4.3.1 Article 3.** Effects of the selective sigma-1 receptor antagonist S1RA on formalin-induced pain behavior and neurotransmitter release in the spinal cord in rats. *Journal of Neurochemistry.* (Submitted).
- **4.3.2 Annex 2.** Supplementary results.

4.4 Exploring the site and mechanism of action of sigma-1 receptor modulation of opioid antinociception in rats.

- **4.4.1 Article 4.** Supraspinal and peripheral but not intrathecal σ_1R blockade by S1RA enhances morphine antinociception. (Manuscript in preparation).

4.1 Set-up of the concentric microdialysis technique in awake-freely moving rats in the formalin model

4.1.1 Article 1

Vidal-Torres A, Carceller A, Zamanillo D, Merlos M, Vela JM, Fernández-Pastor B. Evaluation of formalin-induced pain behavior and glutamate release in the spinal dorsal horn using in vivo microdialysis in conscious rats. *J Pharmacol Sci.* 2012;120(2):129-32. Epub 2012 Sep 15. PubMed PMID: 22986364. DOI: 10.1254/jphs.12105SC

4.2 Exploring the advantage of using sigma-1 receptor antagonists as opioid adjuvant strategy comparing opioid-induced antinociception versus adverse effects in mice

4.2.1 Article 2

Vidal-Torres A, de la Puente B, Rocasalbas M, Touriño C, Andreea Bura S, Fernández-Pastor B, Romero L, Codony X, Zamanillo D, Buschmann H, Merlos M, Baeyens JM, Maldonado R, Vela JM. Sigma-1 receptor antagonism as opioid adjuvant strategy: enhancement of opioid antinociception without increasing adverse effects. *Eur J Pharmacol.* 2013 Jul 5;711(1-3):63-72. doi:10.1016/j.ejphar.2013.04.018. Epub 2013 Apr 28. PubMed PMID: 23632394.

4.2.2 Annex 1

The Annex 1 describes some additional studies which support and extend some of the conclusions obtained from *Article 2*. In *Article 2*, we used the new selective σ_1R antagonist S1RA (E-52862) to characterize the effect of selective σ_1R blockade on opioid-induced efficacy using the tail-flick test. S1RA (40 mg/kg) had no effect in the tail-flick test but it did enhance the antinociceptive potency of several opioids by a factor between 2 and 3.3. In addition, as compared to WT mice, σ_1R-KO mice were equally sensitive to morphine antinociception.

In the present annex we completed the studies on the modulation that σ_1R antagonism exerts on opioid-mediated antinociception in the acute pain model of tail-flick by using two approaches:

1. Characterizing the effect of selective σ_1R blockade on opioid-induced antinociception using a fixed dose of morphine in the tail-flick test and S1RA and BD-1047 as σ_1R antagonists.
2. Characterizing the opioid antinociception of sufentanyl, fentanyl, buprenorphine, and oxycodone using σ_1R-KO mice.

1. Characterization of the effect of selective σ_1R blockade on opioid-induced antinociception

We previously studied the effect of the combination of a fixed S1RA dose (40 mg/kg) with different doses of µ-opioids used in clinics, including tramadol, morphine, buprenorphine, codeine, oxycodone, and fentanyl. The effect of the combination always resulted in an increase of the opioid antinociceptive potency (ED_{50}). In the present annex we studied a different approach: we combined a fixed dose of the opioid (morphine) with increasing doses of the σ_1R antagonist S1RA both in mice and rats, to explore whether this other complementary approach also results in a potentiation of the opioid antinociception. In addition, morphine was also combined with BD-1047 to further confirm the σ_1R involvement on opioid antinociception modulation by using another σ_1R antagonist.

The material and methods (*animals*, *drug administration*, *antinociceptive test*) are the same as in *Article 2* for mouse studies and as in *Article 4* for rat studies.

The results showed that S1RA and BD-1047 were inactive when administered alone in the tail-flick test at doses that do not exert any significant effect on motor coordination after i.p. administration in mice and rats (Fig. 1). These results are in agreement with previous reports (Chien and Pasternak 1995a,b; Cendán *et al.* 2005a; Kim *et al.* 2008; Díaz *et al.* 2009; Entrena *et al.* 2009a; Romero *et al.* 2012; Vidal-Torres *et al.* 2013a). The highest tested dose of BD-1047 (80 mg/kg) resulted in a slight significant effect that may be consequence of non-selective σ_1R effects.

In mice, the subcutaneous injection of 2 mg/kg of morphine alone produced a slight significant effect (25% in Fig. 1A, and 20% in Fig. 1B), very similar to that obtained in our previous studies (24% in Fig. 1B, *Article 2).* The combination of the fixed morphine dose with increasing doses of the selective S1RA resulted in a marked enhancement of morphine antinociception in a dose-dependent manner: morphine 2 mg/kg, s.c. + S1RA 10, 20, or 40 mg/kg, i.p. produced 26, 32, and 68% of antinociception, respectively (Fig. 1A). The combination of morphine with increasing doses of another σ_1R antagonist, BD-1047, also resulted in a marked enhancement of morphine antinociception: morphine 2 mg/kg, s.c. + BD-1047 20, 40 and 80 mg/kg, i.p. produced 50, 70, and 91% of antinociception, respectively (Fig. 1B).

In rats, the subcutaneous injection of 2.5 mg/kg of morphine alone produced non-significant effect (10%). The combination of the fixed dose of morphine with increasing doses of the selective S1RA resulted in a marked enhancement of morphine antinociception in a dose-dependent manner: morphine 2.5 mg/kg, s.c. + S1RA 10, 20, 40, and 80 mg/kg, i.p. produced 24, 33, 45, and 51% of antinociception, respectively (Fig. 1C).

These studies extend previous work (*Article 2*) and show that combining a fixed subactive dose of opioid with increasing σ_1R antagonist doses, an enhancement of the opioid antinociception is also produced. These results support the use of σ_1R antagonists as an adjuvant therapy to opioid analgesics.

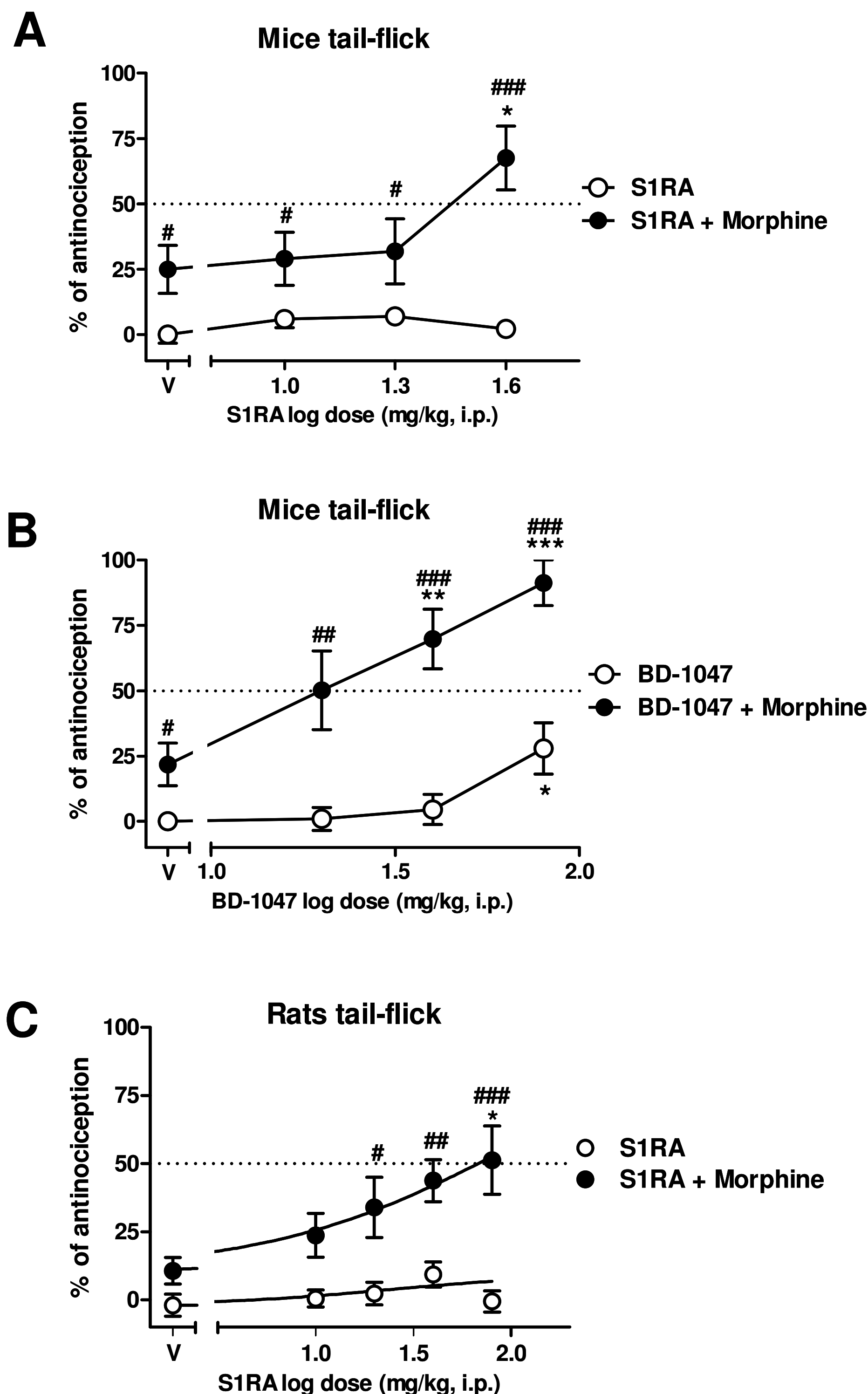


Fig. 1. Dose-response antinociception curves of S1RA (A, C) and BD-1047 (B) in presence or absence of morphine in the tail-flick test. Mice received i.p. S1RA (A) or BD-1047 (B) with (●) or without (○) 2 mg/kg of morphine administered s.c. and the tail-flick latency was evaluated 30 min later. Rats received i.p. S1RA (C) with (●) or without (○) 2.5 mg/kg of morphine administered i.p. and the tail-flick latency was evaluated 30 min later. Note that σ_1R antagonists increased the antinociceptive effect of morphine both in mice and rats. Each point and vertical line represents the mean ± SEM percentage of antinociception (n=7-13 per group). *$p<0.05$, **$p<0.01$, ***$p<0.001$ *vs.* respective vehicle group (Newman-Keuls multiple comparison test post one-way ANOVA). $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ *vs.* corresponding morphine dose (unpaired t-test).

2. Characterization of the potentiating effect of S1RA on opioid antinociception using σ_1R-KO mice

In order to unambiguously attribute the potentiating effect of S1RA to σ_1R, we previously investigated the effect of S1RA on morphine antinociception in mice lacking σ_1Rs and the results showed that S1RA did not increase the potency of morphine in σ_1R-KO mice, indicating that the σ_1R mediates the enhancing effects of S1RA. The same study evidenced that σ_1R-KO mice perceived normally acute thermal nociceptive stimuli and were equally sensitive to morphine as compared to WT mice. Herein, we examined the effect elicited by other opioids used in clinics in order to better characterize the opioid response in σ_1R-KO mice. Thus, the potency (ED_{50}) of sufentanyl, fentanyl, buprenorphine, and oxycodone were evaluated in σ_1R-KO and compared to WT mice.

The material and methods (*animals*, *drug administration*, *antinociceptive test*) are the same as in *Article 2*.

As in the case of morphine (*Article 2*) the ED_{50} for sufentanyl, fentanyl, and buprenorphine in σ_1R-KO mice was not statistically different respect to WT mice (Fig. 2, Fig. 3 and Table 1). These results confirm that the absence of the regulatory mechanism in KO mice is not equivalent to the decrease or gain of function promoted by σ_1R ligands through conformational changes that are relayed to and affect the activity of the target protein (opioid receptor) the σ_1R is interacting with. However, in the case of oxycodone, a significant difference was obtained. Oxycodone resulted in a significant higher potency in WT respect to σ_1R-KO mice (ED_{50} = 0.65 ± 0.06 and 0.96 ± 0.06 mg/kg, respectively). Some speculations can be performed to explain the different sensitivity between WT and σ_1R-KO mice to oxycodone:

1. The analgesic response of oxycodone may be interfered by non-selective activities of either the parent compound or oxycodone metabolites (noroxycodone, oxymorphone and 6α/β-oxycodol). However, the principal metabolites of oxycodone have shown restricted brain barrier penetration (Lalovic *et al.* 2006). Consequently, the contribution of oxycodone metabolites to central analgesic effect may not be important. However a potentiation effect at the peripheral level should not be discarded as we have demonstrated that σ_1R can potentiate the peripheral opioid loperamide in the tail-flick assay (*Article 4*).

2. Oxycodone or its metabolites could have σ_1R antagonism properties that enhance the antinociceptive effect of the parent opioid compound. *In vitro* binding assays (data not shown) revealed that oxycodone does not bind σ_1R, but the possibility that its metabolites potentiate opioid activity through σ_1R antagonism in the WT mice should not be underestimated.
3. σ_1R-KO mice may metabolize faster the parent compound (oxycodone).
4. σ_1R-KO mice may differently express (up- or down-regulation) proteins involved in the antinociceptive effect of oxycodone.

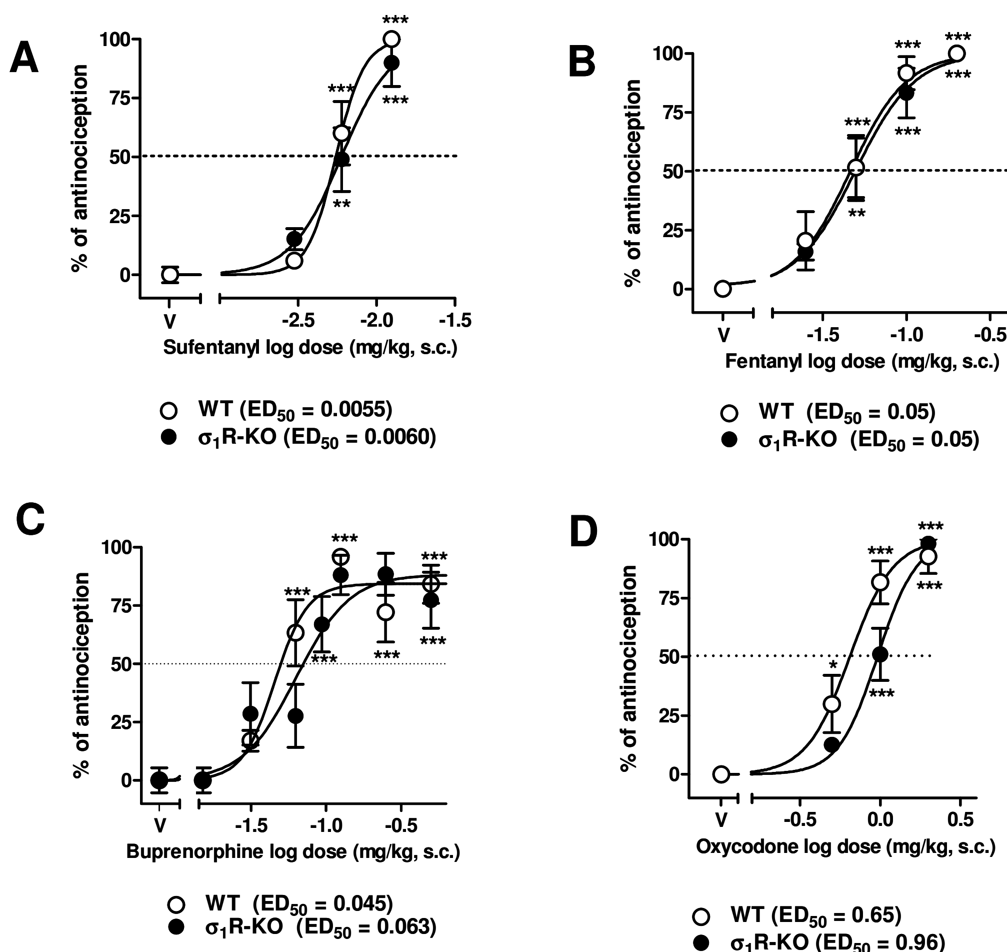


Fig. 2. Comparison of the effect of sufentanyl, fentanyl, buprenorphine and oxycodone in WT and σ_1R-KO mice in the tail-flick test. WT (○) and σ_1R-KO (●) mice received a s.c. opioid administration and the tail-flick latency was evaluated 30 min after treatments. Sufentanyl (A), fentanyl (B), and buprenorphine (C) produced a similar dose-dependent antinociceptive effect in WT and σ_1R-KO mice. However, oxycodone dose-response curve revealed a right shift in σ_1R-KO mice (D). Each point and vertical line represents the mean ± SEM percentage of antinociception (n=8-12 per group). *$p<0.05$, **$p<0.01$, ***$p<0.001$ *vs*. corresponding vehicle (v) group (Newman-Keuls multiple comparison test post one-way ANOVA).

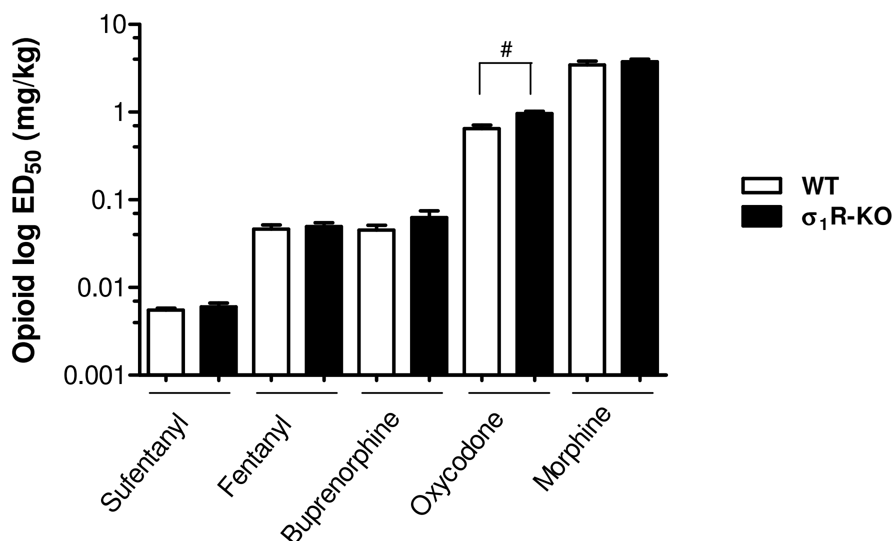


Fig. 3. Comparison of the opioid ED_{50} of sufentanyl, fentanyl, buprenorphine, oxycodone, and morphine in WT and σ_1R-KO mice in the tail-flick test. WT (○) and σ_1R-KO (●) mice received a s.c. opioid administration and the tail-flick latency was evaluated 30 min after treatments. ED_{50} values were calculated from the dose-response curves. Sufentanyl, fentanyl, buprenorphine, and morphine produced similar ED_{50} in WT and σ_1R-KO mice. However, oxycodone had an ED_{50} significantly different in σ_1R-KO mice. Each bar and vertical line represents the mean ± S.E.M. of ED_{50} (n=8-12 per group). $^{\#}p<0.05$ *vs*. corresponding WT opioid ED_{50} (unpaired t-test).

Table 1. Summary of the antinociceptive potency of different opioids in WT and σ_1R-KO mice in the tail-flick test. WT and σ_1R-KO mice received a s.c. opioid (sufentanyl, fentanyl, buprenorphine, oxycodone, and morphine) administration and the tail-flick latency was evaluated 30 min after treatments. ED_{50} values were calculated from the dose-response curves. $^{*}p<0.05$ *vs*. corresponding WT opioid ED_{50} (unpaired t-test). CI confidence intervals.

ED_{50} (95% CI)	WT	σ_1R-KO
Sufentanyl	0.0055 (0.0048-0.0062)	0.0060 (0.0048-0.0074)
Fentanyl	0.05 (0.04-0.06)	0.05 (0.04-0.06)
Buprenorphine	0.045 (0.036-0.057)	0.063 (0.044-0.090)
Oxycodone	0.65 (0.53-0.79)	0.96 (0.85-1.09) *
Morphine	3.5 (2.8-4.3)	3.7 (3.3-4.3)

4.3 Exploring the analgesic efficacy and the spinal modulation of pain-related neurotransmitters by σ1R antagonism on the formalin-evoked pain in rats

4.3.1 Article 3

Vidal-Torres A, Fernández-Pastor B, Carceller A, Vela JM, Merlos M, Zamanillo D. Effects of the selective sigma-1 receptor antagonist S1RA on formalin-induced pain behavior and neurotransmitter release in the spinal cord in rats. *Journal of Neurochemistry.* (Submitted).

Vidal-Torres A, Fernández-Pastor B, Carceller A, Vela JM, Merlos M, Zamanillo D. Effects of the selective sigma-1 receptor antagonist S1RA on formalin-induced pain behavior and neurotransmitter release in the spinal cord in rats. J Neurochem. 2014 May;129(3):484-94. doi: 10.1111/jnc.12648

4.3.2 Annex 2

The Annex 2 details complementary experiments that support and extend some of the conclusions obtained from *Article 3.* In *Article 3*, we studied the systemic effect of S1RA administration in the behavioural formalin model in rats. The results revealed that systemic S1RA-induced antinociceptive effect was concomitant with an enhancement on NA spinal levels and an attenuation of formalin-evoked Glu release.

In the present annex we show:

1. Supplementary graphs of the behavioural effect of systemic S1RA administration in the formalin model performed in *Article 3.*
2. The behavioural effect of systemic BD-1063 and morphine administration in the formalin test. We used BD-1063 as a σ_1R antagonist reference compound and morphine as a positive control of an analgesic drug in the formalin model.
3. The spinal neurochemical effect of systemic BD-1063 and morphine administration in the formalin model.

1. Supplementary graphs of behavioural studies of S1RA in the formalin model.

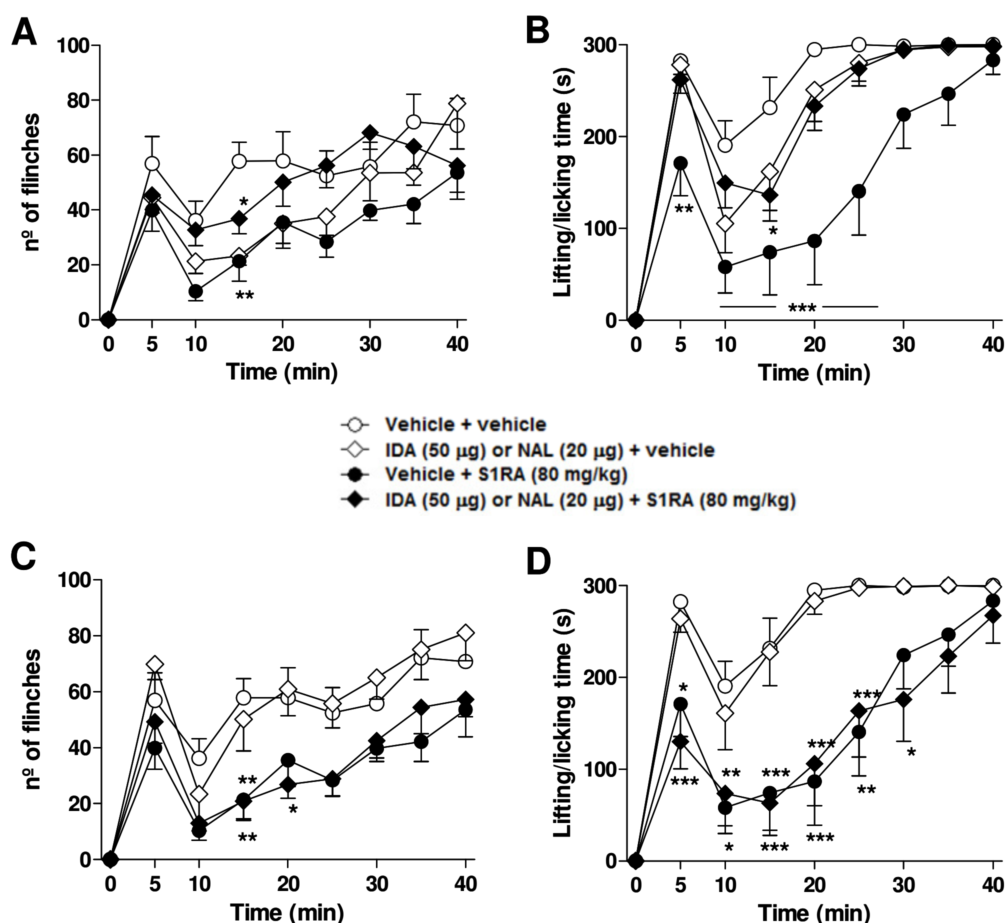


Fig. 1. Time-course effects of systemic S1RA in rats pre-treated with intrathecal idazoxan or naloxone in the formalin test. Rats received an i.t. administration of idazoxan (IDA) (50 µg) or naloxone (NAL) (20 µg) 10 min before i.p. administration of S1RA (80 mg/kg). 15 min after i.p. administration, 5% formalin was injected and behavioural time-course effects were evaluated during 40 min. Data are presented as the mean ± SEM of the number of flinches (A and C) and as the mean ± SEM of the lifting/licking time (B and D) of the injected paw (n=8-9 per group). Note that both idazoxan and naloxone *per se* did not affect behavioural responses. The antinociceptive effect of S1RA was blocked by i.t. pre-treatment with idazoxan but not i.t. naloxone. *p<0.05, **p<0.01, ***p<0.001 *vs*. vehicle group (HPMC + CSF) (Bonferroni test post two-way ANOVA).

In *Article 3* some of the behavioural results are shown as time-course graphs and some others are summarized in AUC graphs. In this annex we complement the graphs of *Article 3*. Thus, Fig. 1 shows the whole time course effect of i.t. idazoxan and naloxone pre-treatment on S1RA systemic antinociceptive effect presented in *Article 3 Figure 3* as AUC graphs. The next figures of this annex show the time course effect of i.t. (Fig. 2), i.c.v. (Fig. 3) and i.pl. (Fig. 4) S1RA administration in the formalin test which was presented by using AUC graphs in *Article 3 Figure 4.*

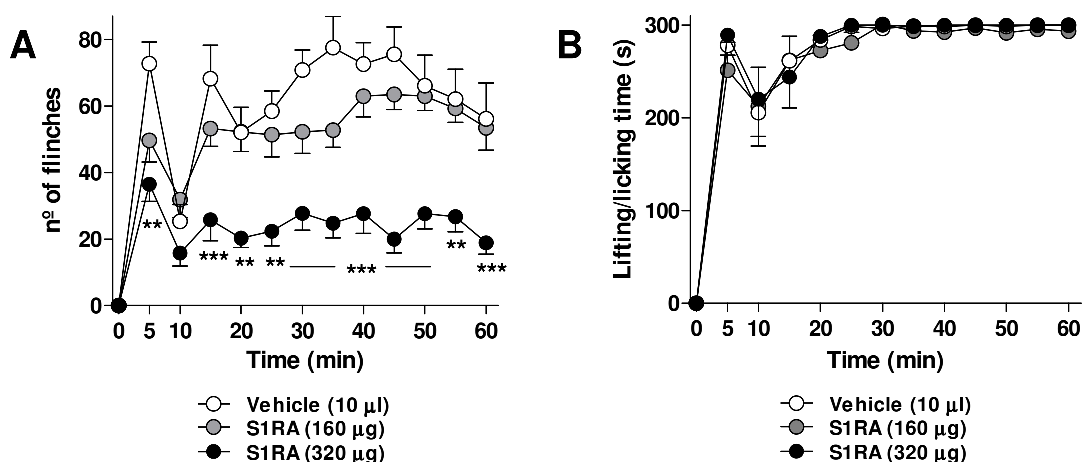


Fig. 2. Time-course effects of intrathecal S1RA administration in the formalin test in rats. Rats received an i.t. administration of S1RA (160 and 320 µg) 15 minutes before 5% formalin injection and time-course effects were evaluated. Data are presented as the mean ± SEM of the number of flinches (A) and as the mean ± SEM of the lifting/licking time (B) of the injected paw (n=8-11 per group). Note that S1RA attenuated both the number of flinches and the lifting/licking time. **p<0.01, ***p<0.001 *vs.* vehicle group (HPMC + CSF) (Bonferroni test post two-way ANOVA).

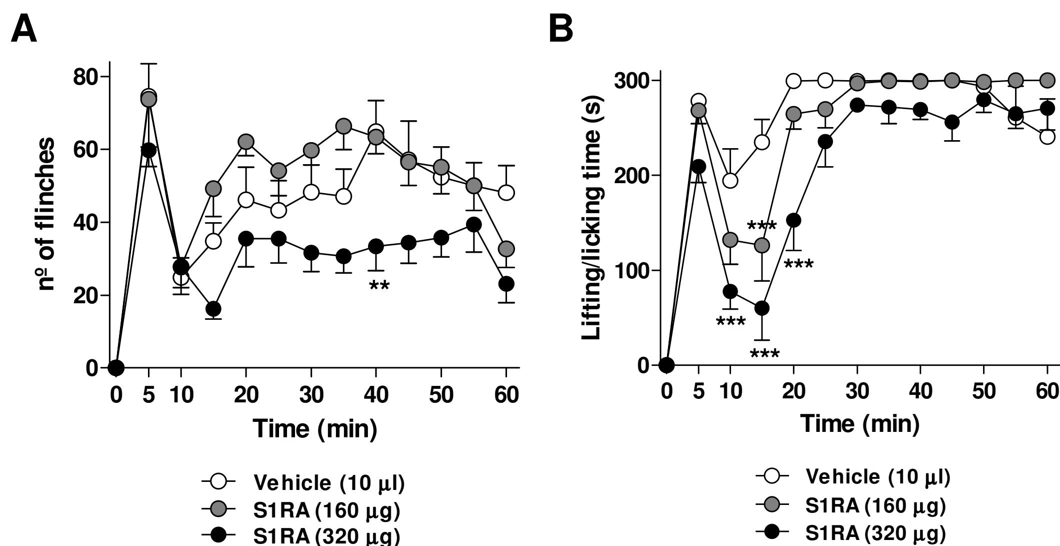


Fig. 3. Time-course effects of intracerebroventricular S1RA administration in the formalin test in rats. Rats received an i.c.v. administration of S1RA (160 and 320 μg) 10 minutes before 5% formalin injection and time-course effects were evaluated. Data are presented as the mean ± SEM of the number of flinches (A) and as the mean ± SEM of the lifting/licking time (B) of the injected paw (n=7-10 per group). Note that S1RA attenuated both the number of flinches and the lifting/licking time. **$p<0.01$, ***$p<0.001$ *vs*. vehicle group (HPMC + CSF) (Bonferroni test post two-way ANOVA).

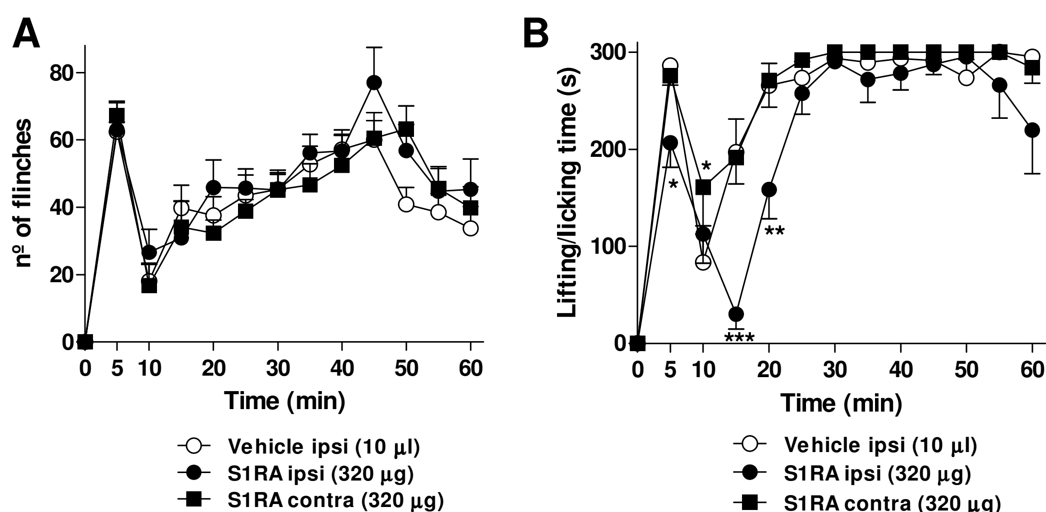


Fig. 4. Time-course effects of intraplantar S1RA administration in the formalin test in rats. Rats received an i.pl. administration of S1RA (320 μg) 10 minutes before 5% formalin injection and time-course effects were evaluated. Data are presented as the mean ± SEM of the number of flinches (A) and as the mean ± SEM of the lifting/licking time (B) of the injected paw (n=8-11 per group). Note that S1RA attenuated both the number of flinches and the lifting/licking time. *$p<0.05$, **$p<0.01$, ***$p<0.001$ *vs*. vehicle group (HPMC + Saline) (Bonferroni test post two-way ANOVA).

2. Behavioural effect of systemic BD-1063 and morphine administration in the formalin test.

We previously reported that systemic S1RA reduced the number of flinches and the lifting/licking time behaviours of both phase I and II of the formalin test in rats. In this annex we show the behavioural effect of BD-1063 and morphine in the formalin test in rats.

The material and methods used in these experiments were the same as in *Article 3*.

Our results indicate that, as observed with the selective S1RA, i.p. administration of BD-1063 (40 mg/kg) and morphine (10 mg/kg) 15 min before intraplantar formalin injection also reduced the number of flinches (Fig. 5A) and the lifting/licking time (Fig. 5B) when compared to vehicle-treated rats. AUC analysis revealed a significant reduction in the number of flinches for BD-1063 (ns min 0-5 and $p<0.01$ min 5-40) and for morphine ($p<0.01$ min 0-5 and $p<0.001$ min 5-40) (Fig. 6 A and C). AUC values for lifting+licking time also revealed an attenuation for BD-1063 (ns min 0-5 and $p<0.05$ min 5-40) and for morphine ($p<0.001$ min 0-5 and $p<0.001$ min 5-40) (Fig. 6 B and D). The results with BD-1063 are in accordance to those previously reported in the formalin test by using other σ_1R antagonists (Cendán *et al.* 2005a; Kim *et al.* 2006; Romero *et al.* 2012) or obtained in σ_1R-KO mice (Cendán *et al.* 2005b). Similarly, morphine (i.p.) significantly reduced formalin-induced pain behaviour as expected (Wheeler-Aceto and Cowan 1993).

Administration of BD-1063 (40 mg/kg, i.p.) and morphine (10 mg/kg, i.p.) 15 min before intraplantar formalin injection in implanted animals also attenuated the formalin-induced pain behaviours (Fig. 7 and 8) as observed in non-implanted animals (Fig. 5).

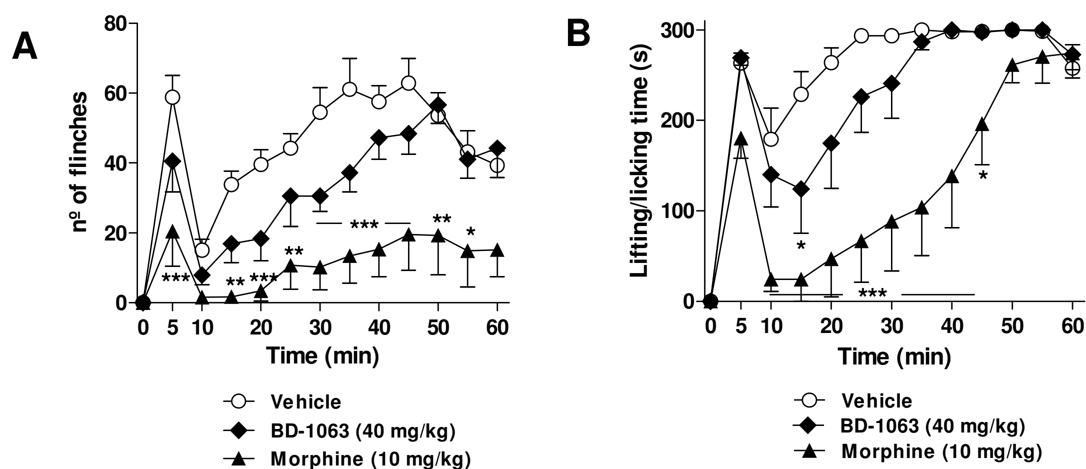


Fig. 5. Time-course effects of systemic BD-1063 and morphine administration in the formalin test in naïve rats. Rats received an i.p. administration of BD-1063 (40 mg/kg) or morphine (10 mg/kg) 15 min before 5% formalin intraplantar injection and time-course antinociception was evaluated. Data are presented as the mean ± SEM of the number of flinches (A) and as the mean ± SEM of the lifting/licking time (B) of the injected paw (n=7-13 per group). Note that BD-1063 and morphine attenuated both the number of flinches and the lifting/licking time. *p<0.05, **p<0.01, ***p<0.001 *vs*. vehicle group (HPMC) (Bonferroni test post two-way ANOVA).

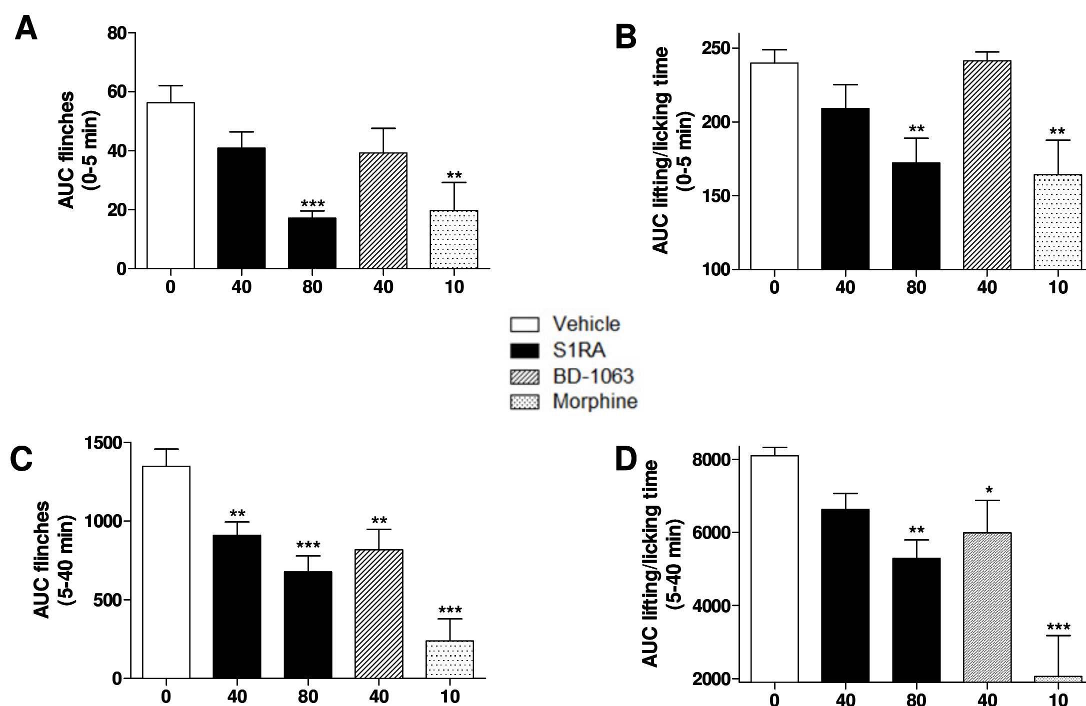


Fig. 6. Behavioural effects of systemic S1RA, BD-1063 and morphine administration in the formalin test in naïve rats. Rats received an i.p. administration of S1RA (40 and 80 mg/kg), BD-1063 (40 mg/kg) or morphine (10 mg/kg) 15 min before 5% formalin intraplantar injection and time-course antinociception was evaluated. Data are presented as the AUC values for flinches min 0-5 (A), min 5-40 (C); and for lifting/licking time min 0-5 (B), min 5-40 (D) (n=7-13 per group). *p<0.05, **p<0.01, ***p<0.001 *vs*. vehicle group (HPMC) (Bonferroni test post one-way ANOVA).

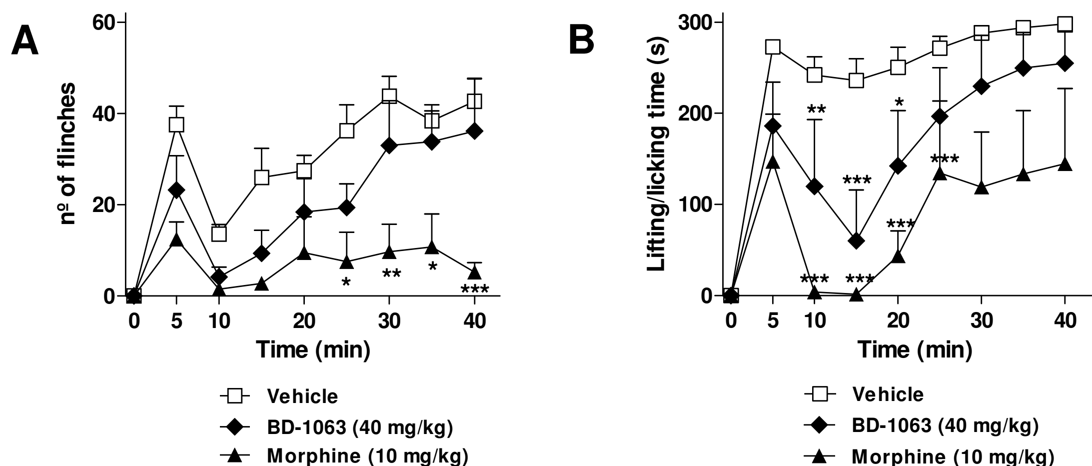


Fig. 7. Time-course effects of systemic BD-1063 and morphine administration in the formalin test in operated rats. DH concentric microdialysis implanted animals received an i.p. vehicle, BD-1063 (40 mg/kg) or morphine (10 mg/kg) administration 15 min before 5% formalin intraplantar injection and formalin–induced behaviour was evaluated. Behavioural data are presented as the mean ± SEM of the number of flinches (A) and of the lifting/licking time (B) of the injected paw (n=3-7 per group). $*p<0.05$, $**p<0.01$, $***p<0.001$ *vs*. vehicle group (Bonferroni test post two-way ANOVA).

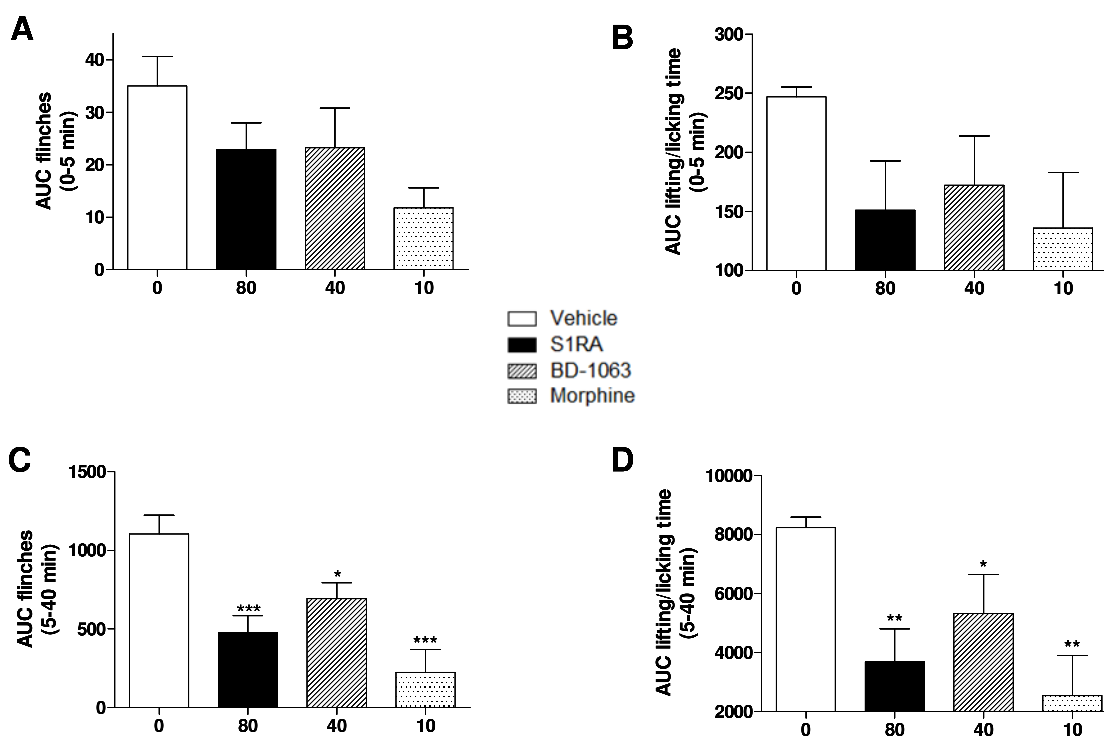


Fig. 8. Behavioural effects of systemic S1RA, BD-1063 and morphine administration in the formalin test in operated rats. DH concentric microdialysis implanted animals received an i.p. vehicle, S1RA (80 mg/kg), BD-1063 (40 mg/kg) or morphine (10 mg/kg) administration 15 min before 5% formalin intraplantar injection and formalin–induced behaviour was evaluated. Data are presented as the AUC values for flinches min 0-5 (A), min 5-40 (C); and for lifting/licking time min 0-5 (B), min 5-40 (D) (n=5-7 per group). $*p<0.05$, $**p<0.01$, $***p<0.001$ *vs*. vehicle group (HPMC) (Bonferroni test post one-way ANOVA).

3. Spinal neurochemical studies using BD-1063 and morphine in the formalin model.

In *Article 3* we reported that systemic S1RA-induced antinociceptive effect is concomitant with an enhancement on NA spinal levels and an attenuation of formalin-evoked Glu release. In this annex we studied the modulation of Glu levels after systemic BD-1063 and morphine administration in the formalin test. The material and methods used in these experiments were the same as in *Article 3.*

As an example, we also show two representative chromatograms of those obtained in our neurochemical studies for Glu, GABA and NA quantification. In our chromatographic conditions optimized for analysing Glu levels, detection was performed by fluorescence system. As observed in Fig. 9, Glu and GABA eluted at 15.8 and 24.9 minutes, respectively. In our chromatographic conditions optimized for analysing NA levels, detection was performed by electrochemical detection. NA and 5-HT eluted at 2.2 and 4.9 minutes, respectively (Fig. 10).

Our results indicate that systemic BD-1063 (40 mg/kg) inhibited the formalin-evoked Glu release (Fig. 11). These results support that it is a σ_1R-mediated effect as it is observed with both S1RA and BD-1063. As discussed in *Article 3*, the NA enhancement observed with S1RA could induce the inhibition of formalin-evoked Glu release. However, we did not evaluate NA levels after BD-1063 administration. On the other hand, systemic morphine (10 mg/kg, i.p.) also inhibited formalin-evoked Glu release (Fig. 11) as we previously described with lower morphine doses (3 mg/kg, s.c.) (Vidal-Torres *et al.* 2012). These results are similar to that reported in literature (Malmberg and Yaksh 1995b) by using other microdialysis approaches (intrathecal microdialysis).

When performing the neurochemical studies for spinal Glu detection, in the chromatograms obtained, we were also able to quantify GABA levels. As stated in *Results* section of *Article 3,* systemic administration of S1RA failed to modify the extracellular concentration GABA. However, the graph was not showed. In this annex we show the GABA levels after treatment not only with S1RA but also with BD-1063 and morphine (Fig. 12). In vehicle-treated animals and in S1RA-, BD-1063-, and morphine-treated groups, no modification of GABA levels in the DH of the spinal cord was observed.

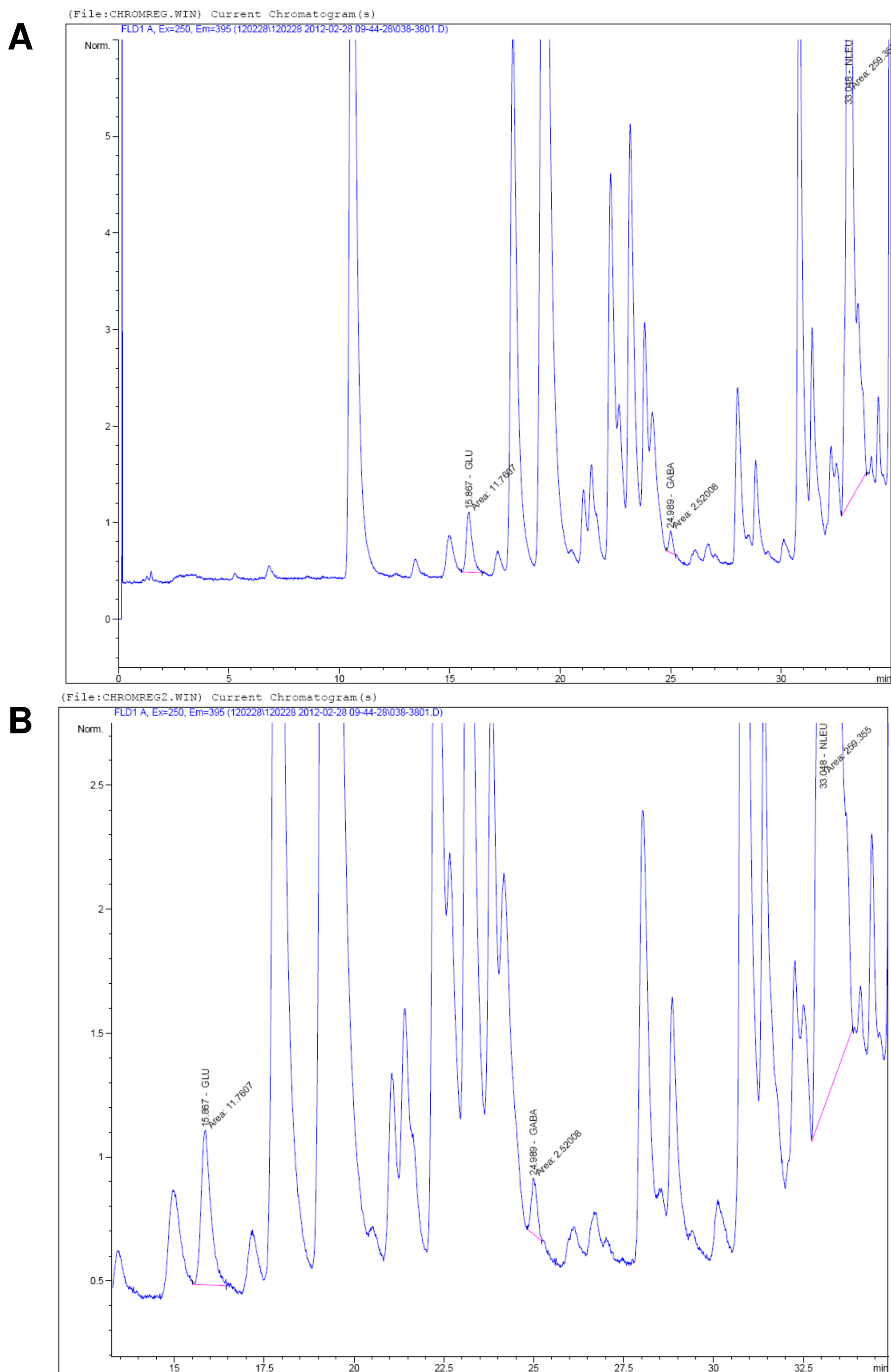


Fig. 9. Retention time of Glu and GABA in fluorescence HPLC detection. DH concentric microdialysis implanted animals received an i.p. vehicle administration 15 min before 5% formalin intraplantar injection and extracellular concentration of Glu and GABA levels was determined. Note that formalin in this sample (vehicle-treated group) induced a significant increase of Glu levels but GABA concentration was not modified *vs* baseline levels. (A) General overview of the whole running with the elution time for each amino acid. (B) Detail of the Glu and GABA peaks.

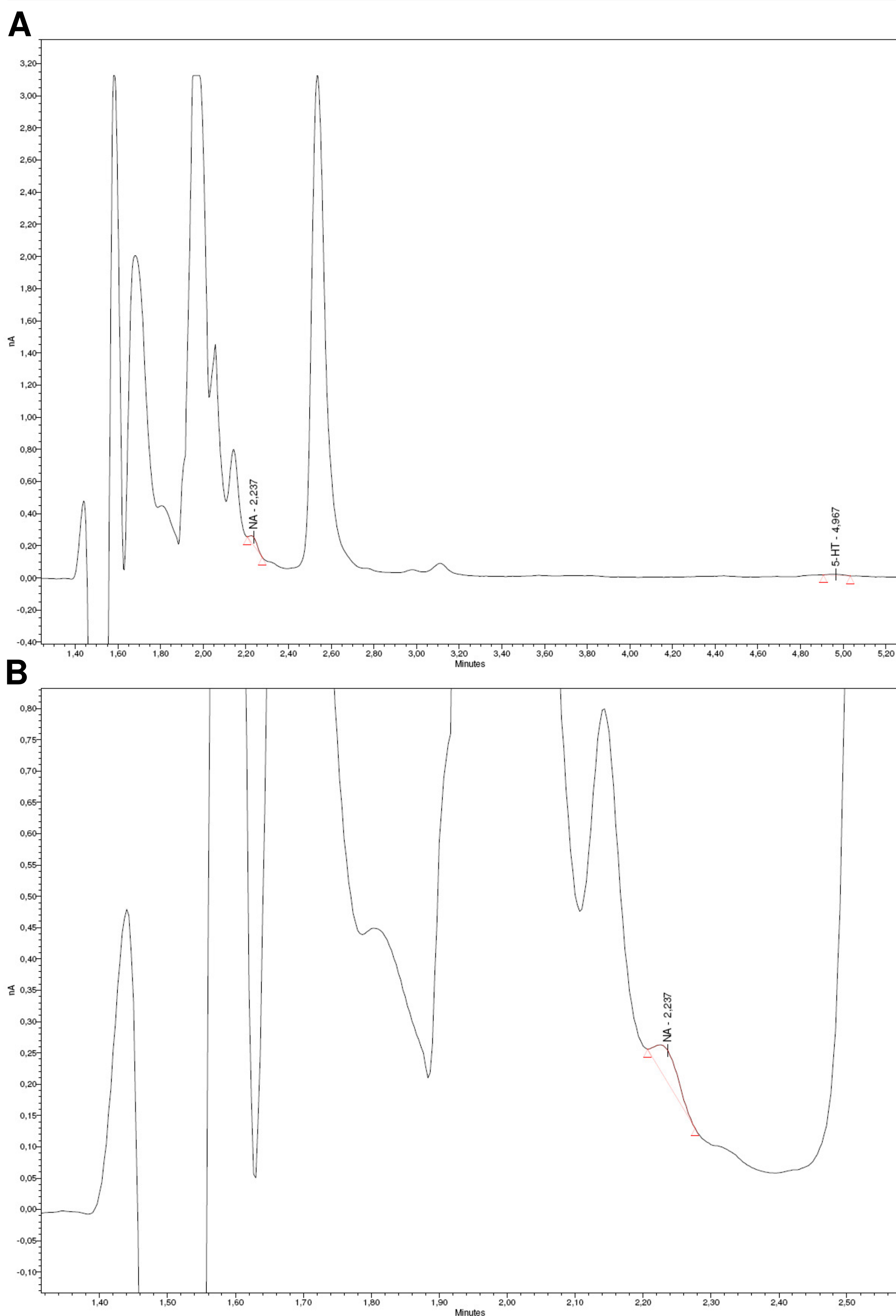


Fig. 10. Retention time of NA and 5-HT in electrochemical HPLC detection. DH concentric microdialysis implanted animals received an i.p. S1RA (80 mg/kg) administration 15 min before 5% formalin intraplantar injection and extracellular concentration of NA was determined. Note that S1RA enhanced NA levels *vs* baseline levels. (A) General overview of the whole running with the elution time for each amine. (B) Detail of the NA peak.

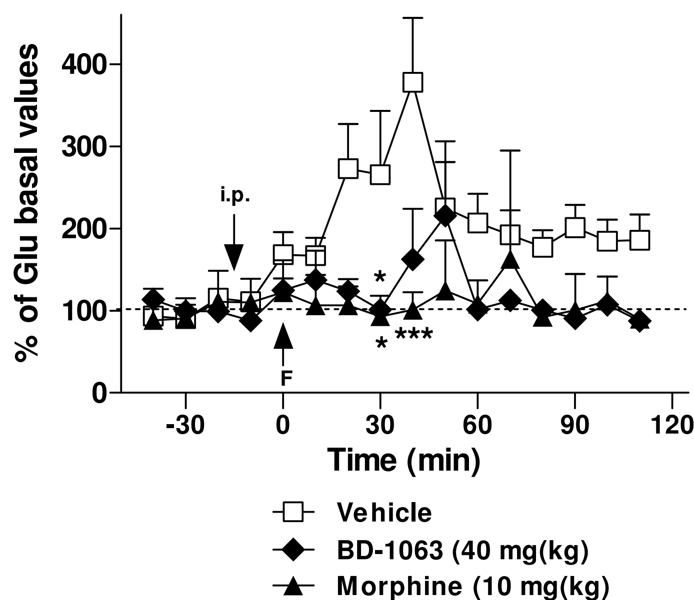


Fig. 11. Neurochemical effect of systemic BD-1063 and morphine administration in presence of formalin (F). Extracellular concentration of Glu in the DH-implanted animals from Fig. 3 was determined (n=3-7 per group). Points are means ± SEM values and are expressed as percentages of the respective baseline values. Note that BD-1063 (40 mg/kg) and morphine (10 mg/kg) attenuated formalin-induced Glu release when compared to vehicle-treated animals. These effects where concomitant to the behavioural antinociceptive effects. **p*<0.05, ****p*<0.001 *vs.* vehicle group (Bonferroni test post two-way ANOVA).

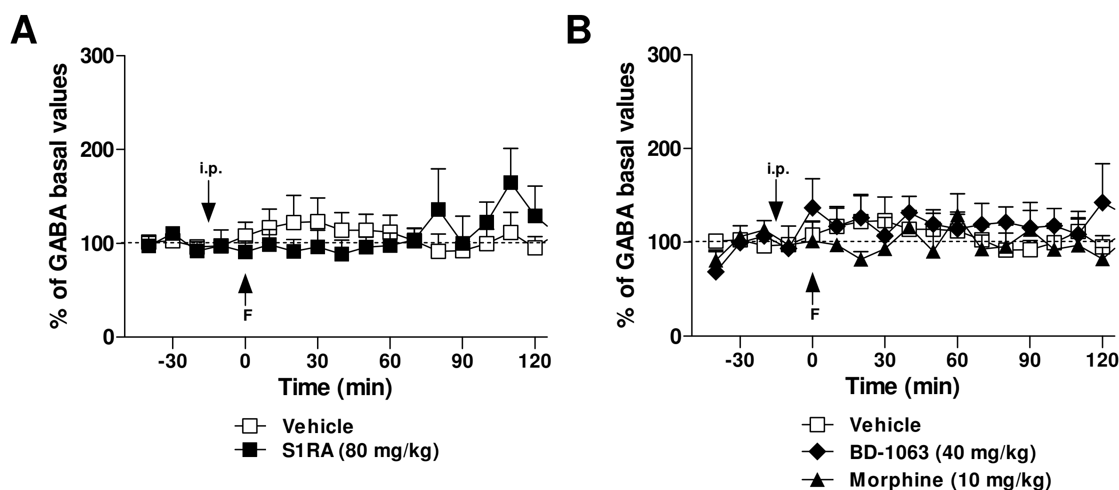


Fig. 12. Effect of systemic S1RA, BD-1063 and morphine administration on spinal GABA levels in presence of formalin (F). DH concentric microdialysis implanted animals received an i.p. vehicle, S1RA (80 mg/kg), BD-1063 (40 mg/kg) or morphine (10 mg/kg) administration 15 min before 5% formalin intraplantar injection and extracellular concentration of GABA in the DH of the spinal cord was determined (n=4-7 per group). Points are means ± SEM values and are expressed as percentages of the respective baseline values. Note that no modification of GABA levels was obtained neither in vehicle nor in treated groups.

4.4 Exploring the site and mechanism of action of sigma-1 receptor modulation of opioid antinociception in rats

4.4.1 Article 4

Vidal-Torres A, Fernández-Pastor B, Carceller A, Vela JM, Merlos M, Zamanillo D. Supraspinal and peripheral but not intrathecal σ_1R blockade by S1RA enhances morphine antinociception. (Manuscript in preparation).

Supraspinal and peripheral but not intrathecal σ_1R blockade by S1RA enhances morphine antinociception

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Keywords:
Sigma-1 receptor
S1RA
Concentric microdialysis
Antinociception
Morphine

Abstract

S1RA (E-52862) is an analgesic compound with a new mechanism of action: the selective blockade of sigma-1 receptors. We previously reported that systemic, peripheral, intrathecal or supraspinal administration of S1RA alone exerts an antinociceptive effect in the formalin test in rats, and that increased noradrenaline levels in the spinal cord account for its antinociceptive effect. Conversely, systemic S1RA failed to show an antinociceptive effect by itself in the tail-flick test in mice, but it potentiated the antinociceptive effect of opioids in this acute pain model. The present study aimed to investigate the site of action and the involvement of spinal noradrenaline on the potentiation of opioid antinociception by S1RA in the tail-flick test in rats. Systemic S1RA alone had no effect in the tail-flick test but enhanced the antinociceptive effect of morphine in rats, as observed in mice. Similarly, spinal and supraspinal administration of S1RA alone did not produce an antinociceptive effect in the tail-flick test. However, supraspinal, but not spinal, S1RA administration potentiated the antinociceptive effect of morphine. Neurochemical studies revealed that noradrenaline levels in the dorsal horn of the spinal cord were not increased at doses exerting a clear potentiation of the antinociceptive effect of the opioid. Additionally, the potentiation by S1RA of the opioid-induced antinociceptive effect at the periphery was studied by combining systemic S1RA with the peripherally restricted opioid agonist loperamide. Loperamide was devoid of antinociceptive effect but produced antinociception when combined with S1RA. We conclude that the site of action of S1RA on opioid antinociception modulation is located at the peripheral and supraspinal levels, and that its opioid-potentiating effect is independent on the spinal noradrenaline increase produced by S1RA.

1. Introduction

The sigma-1 receptor (σ_1R) has been described as the first ligand-regulated molecular chaperone whose activity is regulated in an agonist-antagonist manner. The σ_1R gene encodes a protein of 223 amino acids anchored to the endoplasmic reticulum and plasma membranes (Su and Hayashi 2003; Cobos *et al.* 2008; Maurice and Su 2009; Tsai *et al.* 2009). The σ_1R is expressed in key areas for pain control such as the superficial layers of the dorsal horn (DH), the dorsal root ganglia (DRG), the periaqueductal gray (PAG) matter, the locus coeruleus (LC) and rostroventral medulla (RVM) (Walker *et al.* 1992; Alonso *et al.* 2000; Kitaichi *et al.* 2000; Palacios *et al.* 2003; Bangaru *et al*. 2013). There is cumulative evidence supporting an implication of the σ_1R mainly in two kinds of pain conditions: (1) those involving sensitisation, e.g. after sensitisation with capsaicin or formalin or following nerve injury (Cendán *et al.* 2005; Cobos *et al.* 2007; Entrena *et al.* 2009; de la Puente *et al.* 2009; Romero *et al.* 2012; Vidal-Torres *et al.* 2013b) where σ_1R antagonists by themselves inhibit pain behaviours in the absence of opioids; and (2) in acute pain after mechanical (paw pressure test) or thermal (tail-flick and hot plate tests) stimuli, where σ_1R ligands by themselves fail to modify the nociceptive pain but treatment with σ_1R antagonists enhances the antinociception induced by opioids (Chien and Pasternak 1993; Chien and Pasternak 1994; Tseng *et al.* 2011; Vidal-Torres *et al.* 2013a; Sánchez-Fernández *et al.* 2013).

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S1RA is a σ_1R antagonist with high affinity for the σ_1R, excellent σ_1/σ_2 selectivity ratio (>550) and selective against a panel of 170 receptors, enzymes, transporters and ion channels (Romero *et al*. 2012). We previously reported that S1RA co-administration with several opioids used in clinics results in an enhancement of the antinociception but not of undesired opioid-induced phenomena such as the development of morphine analgesic tolerance, physical dependence, or inhibition of gastrointestinal transit. Moreover, S1RA restored morphine antinociception in tolerant mice and reversed the reward effects of morphine (Vidal-Torres *et al.* 2013a). These results indicate that a dissociation exists between the modulatory effect mediated by σ_1R on opioid-induced antinociception and the rest of opioid-induced effects. S1RA has successfully completed phase I clinical studies (Abadias *et al.* 2012) and a drug interaction and safety study with morphine (EudraCT 2010-023993-38), and it is now in phase II clinical trials in order to explore its potential as an opioid adjuvant analgesic.

Regarding the site of action, recent studies from our group demonstrated that S1RA exerts by itself an antinociceptive effect after spinal, supraspinal and peripheral administration in the formalin-induced pain model in rats (Vidal-Torres *et al.* 2013b). In contrast, available information points to a supraspinal modulation of σ_1R ligands on opioid antinociception (Mei and Pasternak 2002; Mei and Pasternak 2007). It was found that the antinociceptive action of morphine decreased when it was co-administrated with (+)-pentazocine in RVM, LC or PAG, and enhanced when was co-administrated with haloperidol or σ_1R antisense oligodeoxynucleotide only in the RVM, pointing out that the RVM was the only region with evidence for tonic σ_1 activity (Mei and Pasternak 2007). At the neurochemical level, S1RA increased noradrenaline (NA) levels in the dorsal horn of the spinal cord after formalin intraplantar injection, and intrathecal pre-treatment with the selective α_2-adrenoceptor (α_2-AR) antagonist idazoxan blocked the antinociceptive effect of S1RA. No studies addressing this issue are available in relation to the opioid potentiating effect.

To gain further insight into the mechanisms underlying the modulatory effect of S1RA on opioid antinociception we evaluated the S1RA effect by using different routes of administration in the tail-flick acute thermal nociceptive pain model in rats. The possible involvement of spinal NA in the potentiating effect was also investigated by using the concentric microdialysis technique in awake, freely-moving rats.

2. Methods

2.1. Animals

All animal husbandry and experimental procedures complied with the European guidelines for the protection of animals used for experimental and other scientific purposes (Council Directive of 24 November 1986, 86/609/ECC), and were approved by the local Ethics Committee. The results are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath *et al.* 2010). Male Wistar rats weighing 230-330 g (Charles River, France) were used. Naïve animals were housed in groups of four and housed individually after surgery. They had free access to food and water and were kept in controlled laboratory conditions with the temperature at 21 ± 1 ºC and a light-dark cycle of 12 h (lights on at 7:00 a.m.). Experiments were carried out in a soundproof and air-regulated experimental room during the light phase. Each animal was only used in a single experiment.

2.2. Drugs and drug administration

Morphine hydrochloride was obtained from the Spanish Drug Agency (Agencia Española de Medicamentos y Productos Sanitarios, Area Estupefacientes (Madrid, Spain)). Loperamide hydrochloride and naloxone-methiodide were obtained from Sigma-Aldrich. 4- (2- (5- methyl-1- (naphthalen-2- yl)- 1H-pyrazol-3- yloxy) ethyl) morpholine hydrochloride (S1RA; E-52862) (Díaz *et al.* 2012) was synthesized at Laboratories Esteve (Barcelona, Spain). Morphine (2.5, 5, and 10 mg/kg), naloxone-methiodide (4 mg/kg) and S1RA (10, 20, 40, and 80 mg/kg) were dissolved in 0.5% hydroxypropyl-methylcellulose (HPMC) (Sigma-Aldrich), and loperamide (1, 2, and 4 mg/kg) in HPMC containing 0.5% Tween 80; and administered intraperitoneally (i.p.) at 2 ml/kg. Naloxone-methiodide was administered 5 minutes prior to loperamide and S1RA. Baseline responses were always obtained prior to treatment administration. For intrathecal (i.t., volume: 10 µL), intracerebroventricular (i.c.v., volume: 10 µL bilaterally) and rostroventral medulla (RVM, volume: 1 µL) administrations, S1RA (80, 160 or 320 µg) was dissolved in cerebrospinal fluid (CSF, Perfusion Fluid CNS, CMA) and co-administered with systemic morphine (i.p., 2.5 or 5 mg/kg). I.t. and i.c.v. S1RA doses where selected based on a previous

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study where S1RA showed antinociceptive effects in the formalin-induced pain model (Vidal-Torres *et al.* 2013b). The doses of drugs refer to their salt forms.

2.3. *Antinociceptive assay (tail-flick test)*

To evaluate the acute antinociceptive effects of the drugs and their combination, the nociceptive responses to acute thermal (heat) stimulation were assessed by the tail-flick test as previously described (d'Amour and Smith 1941). Briefly, animals were gently restrained with a cloth to orient their tails toward the source of heat of the tail-flick apparatus (Panlab, Barcelona, Spain). A noxious beam of light was focussed on the tail about 5 cm from the tip, and the tail-flick latency (TFL, latency to remove the tail as of the onset of the radiant heat stimulus) was recorded automatically to the nearest 0.1 s. The intensity of the radiant heat source was adjusted to yield baseline latencies between 2 and 4 s and a cut-off time was set at 10 s to avoid heat-related tail damage.

The effect of treatments on TFL was calculated by the formula %Antinociception=((Individual test latency – Individual baseline latency)/(Cut-off latency – Individual baseline latency))x100.

When corresponded, the ED_{50} value was estimated from the dose-response curve.

2.4. Intrathecal catheterization and administration

Catheterization of the spinal subarachnoid space was conducted as previously described (Storkson *et al.* 1996; Pogatzki *et al.* 2000) with i.t. catheters (No. 0007740, Alzet) under anaesthesia with pentobarbital (i.p., 60 mg/kg, 2 ml/kg). The lower dorsal pelvic area corresponding to vertebral L3-S3 was shaved and prepared with povidone-iodine. A midline longitudinally skin incision was made (2-3 cm) and the space between the lumbar vertebrae L5 and L6 was punctured with a 22G hypodermic needle. Tail-flick or hind paw retraction indicated an i.t. position. A 28G PU catheter (10 cm length, 0.36 mm OD; 0.18 mm ID, Alzet), reinforced with a teflon-coated stainless steel stylet, was advanced cranially 4 cm through the needle to reach the L4-L5 medullar area. The needle and the stylet were removed and the catheter withdrawn so that 5 cm extended outside of the lumbar muscles. Superglue-3 gel (Loctite®) was used to fix the catheter to the fascia. The distal end of the 28G PU catheter was connected with super glue to an 8-cm length tube (0.84 mm OD; 0.36 mm ID) ended with an ALZET connection (1.02 mm OD; 0.61 mm ID). The catheter was tunnelled under the skin to the cervical region, flushed with CSF and sealed with a cautery pen. The skin was then closed and animals were allowed to recover in individual cages 7 days. Catheterized rats had no detectable motor deficit. S1RA or CSF were injected i.t. with a 50 µL Hamilton syringe at a volume of 10 µL over a period of 20 s, followed by 20 µL of CSF to flush the catheter. The animals were killed by CO_2 inhalation, and 10 µL of fast green was i.t. injected, and the level and side position of the catheter tip were determined. Epidural catheterizations were discarded and only i.t. catheters were considered.

2.5. Intracerebroventricular and RVM cannulae implantation and administration

Bilateral i.c.v. administration guide cannulae (26 GA, 0.46 mm OD, 0.24 mm ID, 5 mm up long, Plastics One) or a RVM administration guide cannulae (26 GA 20 mm, C315G/SPC, Plastics One) were stereotaxically implanted in rats anaesthetized with pentobarbital (i.p., 60 mg/kg, 2 ml/kg). With the incisor bar set at 0 or -5 mm, coordinates from bregma were -0.8 AP, +-1.6 L, and -3.5 DV; or -10.8 AP, 0.0 L, and -4.3 DV (from the dura matter) for i.c.v. and RVM, respectively. Stainless steel guide cannulae were secured to the skull with two anchor screws and dental acrylic. Animals were housed in individual cages, disinfected daily with povidone-iodine and allowed 6-7 days to recover from surgery. In RVM-implanted rats, 18 hours prior to the test, after removing the dummy cannulae (Plastics One), an internal cannula (33 GA, C315IA/SP, Plastics One) which extended 6 mm past the guide cannula was introduced under isofluorane anaesthesia. S1RA or CSF were injected i.c.v. with a 10 µL Hamilton syringe at a volume of 5 µL (per cannula) over a period of 20 s, followed by 1.8 µL of CSF to flush the cannula. S1RA or CSF were injected RVM with a 5 µL Hamilton syringe in a volume of 1 µL over a period of 20 s, followed by 1.8 µL of CSF to flush the cannula. After experimental testing, the animals were killed by CO_2 inhalation and fast green was injected for cannula placement examination. Only animals with correct cannula placements were included in data analyses.

2.6. Microdialysis surgical procedures/Microdialysis probe implantation in spinal cord

Rats were anaesthetised with chloral hydrate (i.p., 440 mg/kg) and placed in a David Kopf stereotaxic frame. The dorsal zone

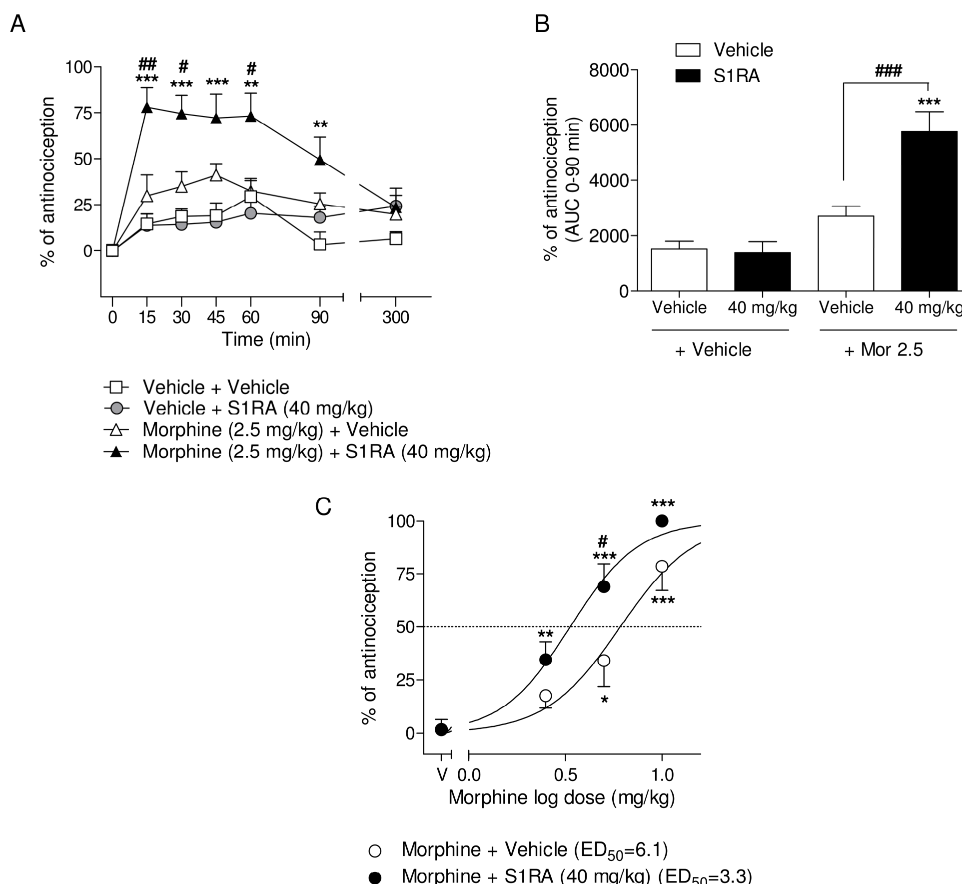

Fig. 1. Effects of systemic co-administration of S1RA with morphine in the tail-flick test in rats. (A) Rats received morphine (2.5 mg/kg, i.p.), S1RA (40 mg/kg, i.p.), their combination or respective vehicles and the tail-flick latency was evaluated along the time. Note that the enhancement of the antinociceptive effect was clear 15 min post-treatment and lasted till 90 min post-treatment. Each point and vertical line represents the mean ± S.E.M. percentage of antinociception (n=9-10 per group). $^{**}P<0.01$, $^{***}P<0.001$ *vs*. respective basal value; $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs*. morphine group (Bonferroni post-hoc test post two-way ANOVA). (B) AUC of 0 to 90 min interval evaluation. $^{***}P<0.001$ *vs*. vehicle group; $^{\#\#\#}P<0.001$ *vs*. morphine group (Newman-Keuls multiple comparison test post one-way ANOVA). (C). Rats received increasing doses of morphine (i.p.) or vehicle + a fixed dose of S1RA (40 mg/kg, i.p.) or vehicle and the tail-flick latency was evaluated 30 min later. Note that S1RA increased the morphine antinociceptive effect. Each point and vertical line represents the mean ± S.E.M. percentage of antinociception (n=8-10 per group). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs*. respective vehicle group (Newman-Keuls multiple comparison test post one-way ANOVA); $^{\#}P<0.05$ *vs*. corresponding morphine dose (unpaired t-test).

corresponding to the thoracic vertebra (Th13) was shaved and prepared with povidone-iodine. An incision was made along the dorsal midline such that the muscle overlaying the Th13 and the first lumbar vertebra (L1) could be removed. Th13 was then immobilised on the horizontal plane by using a transverse process clamp and a burr hole created in the dorsal surface. The exposed dura matter was then carefully opened and a microdialysis probe of concentric design (CMA/11) was inserted into the spinal cord at an angle of 45º from the vertical plane. The microdialysis probes (exposed tip 2.0 mm x 0.24 mm) were implanted into the medial DH of the L4 lumbar region of the spinal cord. The probe was fixed by application of superglue-3 gel (Loctite®) and dental cement around the probe and by a stainless steel anchorage screw located in the Th13 vertebra. The skin was then closed and rats were allowed to recover overnight, one per cage, with free access to food and water. Only implanted rats showing normal behaviour after the recovery period (no walking dysfunction, normal weakened extension withdrawal reflex of the hindlimb, no reduced toe spread, normal food and water intake, no piloerection or apparent stress signals) were considered in the study and the rats were used only once. At the end of the experiment, animals were killed by CO_2 inhalation and spinal cords were dissected out for histological

examination to verify that microdialysis probes were correctly implanted.

2.7. Sample collection in awake rats

Around 20 h after probe implantation, rats were placed individually in a system for freely moving animals. The dialysis probes were connected to a CMA microdialysis system and then perfused with CSF perfusion fluid at 1.5 µl min/1 flow rate and consecutive samples were collected into vials every 15 min. The probe was perfused for 1 h for stabilization of baseline NA release. This was followed by a 90-min period to collect baseline samples. Animals received systemic (i.p.) morphine (5 and 10 mg/kg) or vehicle + systemic (i.p.) S1RA (40 and 80 mg/kg) or vehicle and were perfused during a period of 180 min.

2.8. Analytical procedures

Dialysate samples were assayed for NA content by reversed-phase high-performance liquid chromatography (HPLC) coupled with electrochemical detection. The mobile phase was 75 mM phosphate buffer (pH 6) containing 0.35 mM octanesulfonic acid and 0.2 mM ethylendiamine tetra acetic acid (EDTA) with 25% of methanol. Separation was carried out with a Gemini C18 110A (3 µm) column, connected to a Waters 2465 electrochemical detector at 35ºC and operated at a flow rate of 0.2 ml/min. Detection was performed by oxidation at 0.45 V. Values were not corrected for *in vitro* recovery through the dialysis probe.

2.9. Statistical analysis

Data were expressed as means ± S.E.M. The mean values of four dialysate samples obtained before treatment administration were considered as the 100% baseline values. The extracellular NA concentration of dialysate samples collected during an experiment were normalized as percentage of the baseline values. Treatment groups were compared with appropriate control groups using one-way or two-way ANOVA analysis of variance followed by the Newman-Keuls multiple comparison test or followed by the Bonferroni post-hoc test, respectively, and as appropriate. ED_{50} values were determined using a four-parameter logistic equation (sigmoidal dose-response curve, variable slope) with the top or bottom fixed (DeLean *et al.* 1978). The ED_{50} was defined as the dose that produced 50% of the maximum possible effect. ED_{50} values with 95% confidence intervals (CI), calculation of Area Under the Curve (AUC) values and statistical analyses were computed using GraphPad Prism version 5 software (San Diego, CA, USA).

3. Results

3.1. Systemic S1RA enhanced the antinociceptive effect of systemic morphine in the tail-flick test in rats

We first investigated the antinociceptive effects elicited by the systemic co-administration of an opioid with a σ_1R antagonist in the tail-flick test in rats. For this purpose, we evaluated the tail-flick latencies obtained from the co-administration of morphine (2.5 mg/kg, i.p.) + S1RA (40 mg/kg, i.p.) over a time course (Fig. 1A). In vehicle + vehicle treated rats, the tail-flick latencies did not change significantly from the baseline values over the entire time course (300 min). Morphine (2.5 mg/kg, i.p.) exerted a discrete, non-significant antinociceptive effect during the first 60 min post-treatment whereas S1RA (40 mg/kg, i.p.) was devoid of antinociceptive effect at any evaluated time. Co-administration of S1RA with morphine produced a significant increase in the tail-flick latency over time, with maximum effect at 15-60 min post-treatment and return to baseline values 300 min post-treatment (Fig. 1A). AUC analysis revealed a significant antinociception enhancement ($P<0.001$) in the co-treated group respect to the morphine-treated group (Fig. 1B).

To further assess the potentiation of the antinociceptive effect, we next combined different doses of morphine (2.5, 5, and 10 mg/kg, i.p.) with a fixed dose of S1RA (40 mg/kg, i.p.) and tail-flick latencies were evaluated 30 min after the co-administration. The combination induced a displacement to the left of the dose-response curve of morphine, resulting in an enhancement of the antinociceptive potency of the opioid by a factor of 1.8. The ED_{50} were 6.1 (95% CI, 4.8-7.8) and 3.3 (95% CI, 2.7-4.1) mg/kg for morphine alone and morphine plus 40 mg/kg of S1RA, respectively (Fig. 1C). The morphine dose that resulted in a higher enhancement by S1RA was 5 mg/kg and it was, therefore, the first selected dose for the next set of experiments.

3.2. S1RA and morphine systemically co-administered failed to modify spinal noradrenaline (NA) levels

As we previously reported that S1RA (80 mg/kg) increased NA levels in the dorsal horn of the spinal cord and this effect correlated to the antinociceptive effect of S1RA in the formalin-induced pain model (Vidal-Torres *et al.* 2013b), we addressed herein whether S1RA

A. Vidal-Torres et al, 2013

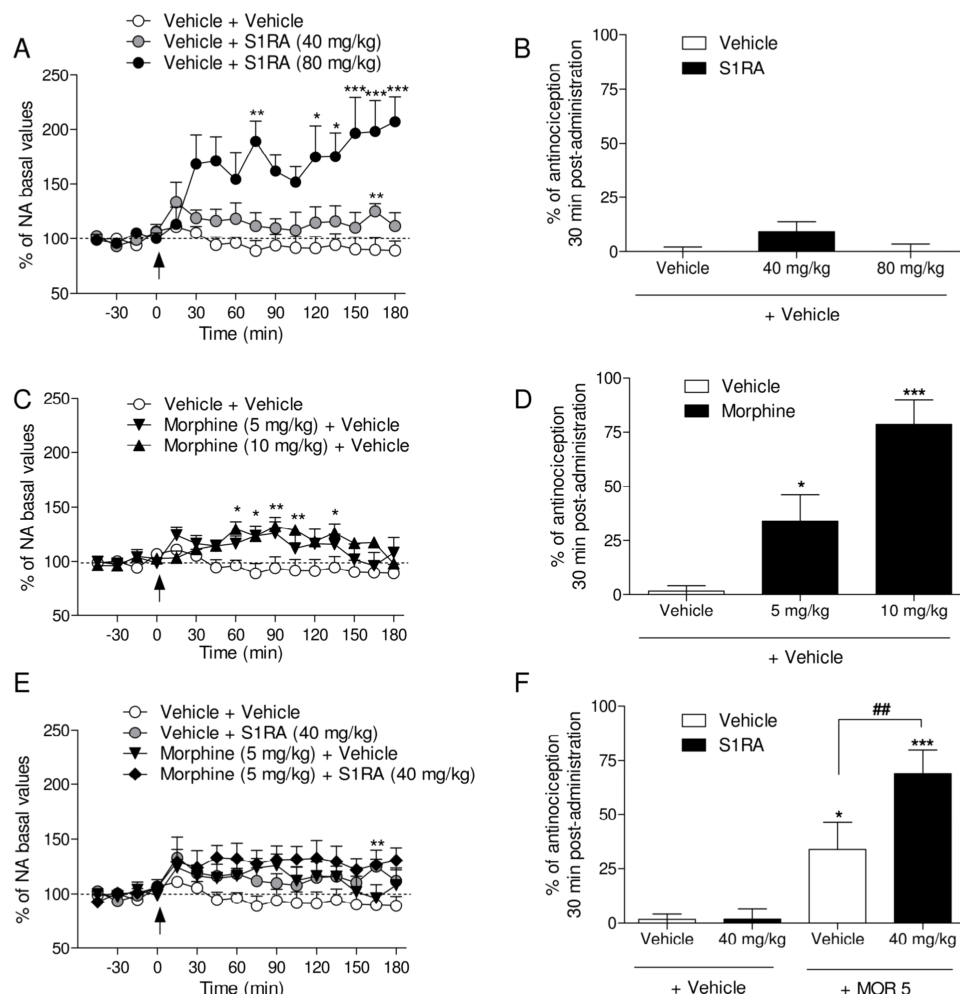

Fig. 2. Behavioural antinociceptive effects and noradrenaline (NA) levels in the dorsal horn of the spinal cord following systemic S1RA, morphine and their combination in rats. Implanted rats received i.p. S1RA (40 and 80 mg/kg) or vehicle (A), i.p. morphine (5 and 10 mg/kg) or vehicle (C), or the combination of morphine (5 mg/kg) and S1RA (40 mg/kg) (E), and were perfused during a period of 180 min to evaluate the effect on extracellular concentration of NA in the dorsal horn of the spinal cord. Dots are means ± S.E.M. values and are expressed as percentages of the respective baseline values (n=4-8 per group). **P*<0.05, ***P*<0.01, ****P*<0.001 *vs*. respective baseline value (Bonferroni post-hoc test post two-way ANOVA). Naïve rats received the same treatments and 30 min later tail-flick latencies were evaluated and the percentage of antinociception elicited by treatments was calculated (B, D, and F). Note that S1RA at 80 mg/kg increased NA levels (A) but failed to produce an antinociceptive effect (B). In contrast, 5 and 10 mg/kg of morphine, although did not change NA levels 30 min post-administration (C), resulted in antinociception (D). The combination of S1RA (40 mg/kg) and morphine (5 mg/kg) failed to significantly modify NA values (E) but produced an enhancement of antinociception (F). Each point and vertical line represents the mean ± S.E.M. percentage of antinociception (n=8-10 per group). **P*<0.05, ****P*<0.001 *vs*. respective vehicle group; ##*P*<0.01 *vs*. morphine 5 mg/kg group (Newman-Keuls multiple comparison test post one-way ANOVA).

enhancement of opioid antinociception is associated with a potentiation of the increase in NA spinal levels.

Vehicle-treated animals showed stable NA spinal levels (Fig. 2). NA spinal levels increased following i.p. administration of S1RA at 80 mg/kg (171% increase respect to baseline values was found 30 minutes post-administration), but not at 40 mg/kg (Fig. 2A). However, both doses of S1RA, when administered alone, were devoid of antinociceptive effects at 30 minutes post-administration (Fig. 2B). Thirty minutes post i.p. administration of 5 and 10 mg/kg of morphine, NA spinal levels did not significantly differ from baseline values (114 and 115%, respectively), although significantly increased levels were attained 60 minutes post-administration (Fig. 2C). Interestingly, both morphine doses resulted in an antinociceptive

A. Vidal-Torres et al, 2013

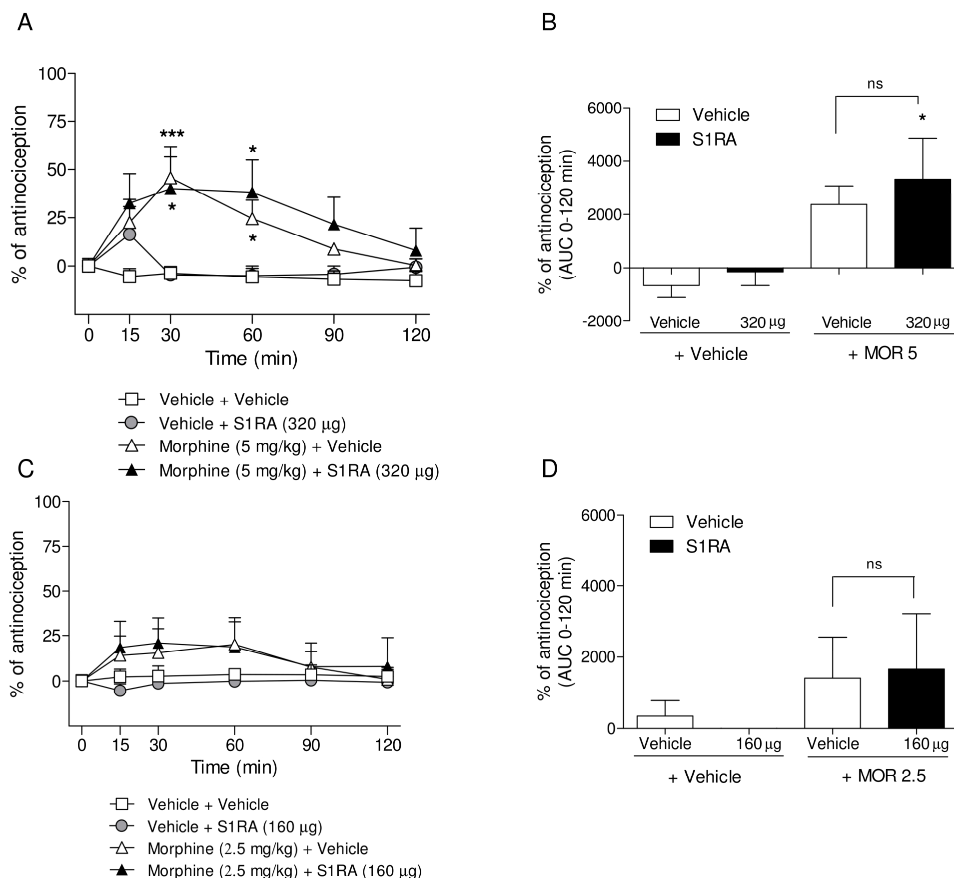

Fig. 3. Time course effects of intrathecal S1RA administration with systemic morphine in the tail-flick test in rats. Rats received i.p. morphine (2.5 or 5 mg/kg) or vehicle + i.t. S1RA (160 or 320 µg) or vehicle and the tail-flick latencies were assessed along the time. Note that morphine elicited significant antinociceptive effect (30 and 60 min post-administration) that this effect was not increased by i.t. S1RA. (A, C) Each point and vertical line represents the mean ± S.E.M. percentage of antinociception (n=5-10 per group). **P*<0.05, ****P*<0.001 *vs*. respective basal value (Bonferroni post-hoc test post two-way ANOVA). (B, D) AUC of 0 to 120 min interval evaluation. **P*<0.05 *vs*. vehicle group; ns *vs*. morphine group (Newman-Keuls multiple comparison test post one-way ANOVA).

effect (*P*<0.05 and *P*<0.001, respectively) 30 minutes post-administration (Fig. 2D). The combination of S1RA (40 mg/kg) and morphine (5 mg/kg), doses that resulted in an enhancement of the opioid antinociception (Fig. 2F), did not modify NA values significantly with respect to baseline levels (133%) (Fig. 2E). The tail-flick assessment of operated animals and dialysis sampling were performed separately in different groups of rats (spinal operated and naïve animals) in order to avoid excessive handling of the rats that could interfere the NA level determination.

3.3. Intrathecal S1RA failed to enhance the antinociceptive effect of systemic morphine in the tail-flick test in rats

We have previously shown that i.t. administration of 160 and 320 µg of S1RA dose-dependently reduced formalin-induced flinching but not licking/lifting behaviours. In order to investigate whether spinal σ_1R antagonism is involved in the modulation of opioid antinociception, rats were i.t. administered with S1RA in combination with systemic morphine. S1RA administered alone through i.t. route at 160 and 320 µg was inactive in the tail-flick test. Morphine (5 mg/kg, i.p.) exerted significant antinociceptive effects (*P*<0.001) 30 min post-administration, but S1RA (320 µg) co-administered i.t. was unable to increase its analgesic effect (Fig. 3A). A lower morphine dose (2.5 mg/kg) was also not enhanced by S1RA (160 µg) (Fig. 3C). AUC analysis confirmed no enhancement of morphine antinociception in the co-treated groups respect to that in morphine-treated groups (Fig. 3B and 3D).

A. Vidal-Torres et al, 2013

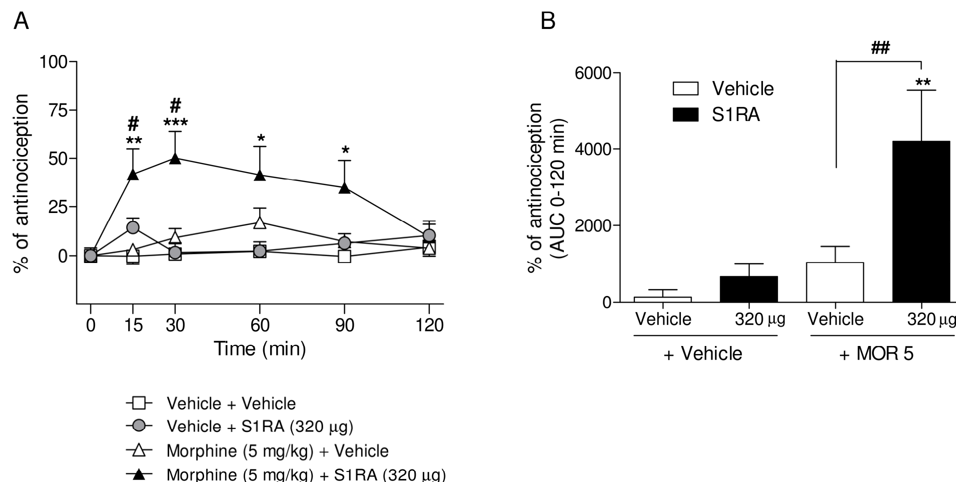

Fig. 4. Time course effect of intracerebroventricular S1RA administration with systemic morphine in the tail-flick test in rats. Rats received i.p. morphine (5 mg/kg) or vehicle + i.c.v. S1RA (320 µg) or vehicle and the tail-flick latencies were evaluated along the time. Note that i.c.v. S1RA increased the antinociceptive effect of systemic morphine. (A) Each point and vertical line represents the mean ± S.E.M. percentage of antinociception (n=7-9 per group). **P*<0.05, ***P*<0.01, ****P*<0.001 *vs*. respective basal values; #*P*<0.05 *vs*. morphine group (Bonferroni post-hoc test post two-way ANOVA). (B) AUC of 0 to 120 min interval evaluation. ***P*<0.01 *vs*. vehicle group; ##*P*<0.01 *vs*. morphine group (Newman-Keuls multiple comparison test post one-way ANOVA).

3.4. Intracerebroventricular but not rostroventral medullar S1RA enhanced the antinociceptive effect of systemic morphine in the tail-flick test in rats

We had previously shown that 320 µg of i.c.v. S1RA significantly reduced formalin-induced pain behaviours. Herein, we also assessed whether supraspinal σ_1R antagonism may mediate potentiation of morphine's antinociception. For this purpose, S1RA (320 µg) was combined with systemic morphine (5 mg/kg, i.p.). S1RA administered i.c.v. and morphine systemically administered did not significantly modify tail-flick latencies in i.c.v. implanted rats when both compounds were administered alone. However, their combination resulted in a significant enhancement (*P*<0.05) of the antinociception at 15 and 30 minutes post-administration (Fig. 4A). AUC analysis revealed a significant enhancement of antinociception (*P*<0.01) in the co-treated group respect to morphine-treated group (Fig. 4B).

RVM was reported to be a key area in the opioid modulation by some σ_1R ligands (Mei and Pasternak, 2007). To further explore the supraspinal site for σ_1R-mediated potentiation of opioid antinociception, we investigated the involvement of the RVM in the potentiation seen following i.c.v. S1RA administration. Thus, intra-RVM administration of S1RA (80 µg) was combined with systemic morphine (2.5 and 5 mg/kg, i.p.). RVM microinjection of S1RA (80 µg) alone exerted a significant pronociceptive effect in the tail-flick test when evaluated 15 and 30 minutes post-administration. Morphine at 2.5 mg/kg i.p. was devoid of effect and exhibited a significant antinociceptive effect when administered at 5 mg/kg i.p. only at 30 min post-treatment (*P*<0.05) (Fig. 5A). When S1RA (80 µg, intra-RVM) and morphine (2.5 and 5 mg/kp, i.p.) were combined, no significant modification of the effect was observed. AUC analysis revealed no significant enhancement of antinociception in the co-treated groups respect to that in the morphine-treated groups (Fig. 5B and D).

3.5. Systemic S1RA enhanced the antinociceptive effect of systemic loperamide in the tail-flick test in rats

In order to address the involvement of the σ_1R on opioid antinociception at the periphery we co-administered different doses of the peripheral µ-opioid agonist loperamide (1, 2, and 4 mg/kg, i.p.) with a fixed dose of S1RA (40 mg/kg, i.p.) in the tail-flick test in rats. Loperamide did not produce significant antinociceptive responses alone but dose-dependently produced antinociception when combined with S1RA over the time with the maximum effect being observed at 30 min post-treatment (Fig. 6A and 6B). In another set of experiments, animals where only measured for the baseline and 30 minutes after the administration, and the potentiating effect was blocked by i.p. pre-treatment with the peripherally acting µ-opioid receptor antagonist naloxone-methiodide (4 mg/kg) (Fig. 6C).

A. Vidal-Torres et al, 2013

4. Discussion

We have previously reported that i) acute systemic, spinal, supraspinal or peripheral S1RA administration results in an antinociceptive effect in the formalin-induced pain model (Vidal-Torres *et al.* 2013b); ii) systemic S1RA-induced antinociceptive effect is concomitant with an enhancement in intra-dorsal horn spinal NA levels (Vidal-Torres *et al.* 2013b); and iii) co-administration of systemic S1RA with several opioids used in clinics results in an enhancement of the analgesia in the acute thermal nociceptive model of tail-flick in mice, where S1RA *per se* is devoid of activity (Vidal-Torres *et al.* 2013a). The present study extends previous work demonstrating that supraspinal and peripheral, but not spinal, S1RA administration enhances opioid antinociception and that such a potentiating effect occurs without a concomitant increase of spinal NA release.

The acute tail-flick response to nociceptive thermal (heat) stimulation was used to assess the potentiating effect of S1RA on opioid antinociception in rats, as it was previously described in mice (Vidal-Torres *et al.* 2013a). In agreement with these previous results, 40 mg/kg of systemic S1RA had no antinociceptive effect when given alone but significantly increased the antinociceptive effect induced by morphine (2.5 mg/kg) till 90 min post-administration. The maximum antinociceptive effect of the combination was observed between 15 and 60 min, which is consistent to the previously reported time course for the non-selective σ_1R antagonist haloperidol in rats (Chien and Pasternak 1995). The enhancement of the morphine potency was also determined by the ED_{50} ratio value obtained for morphine alone and in combination with S1RA (40 mg/kg, i.p.) 30 minutes after administration. This value was similar to that obtained previously for S1RA in mice (1.8 and 2.4 in rats and mice, respectively)

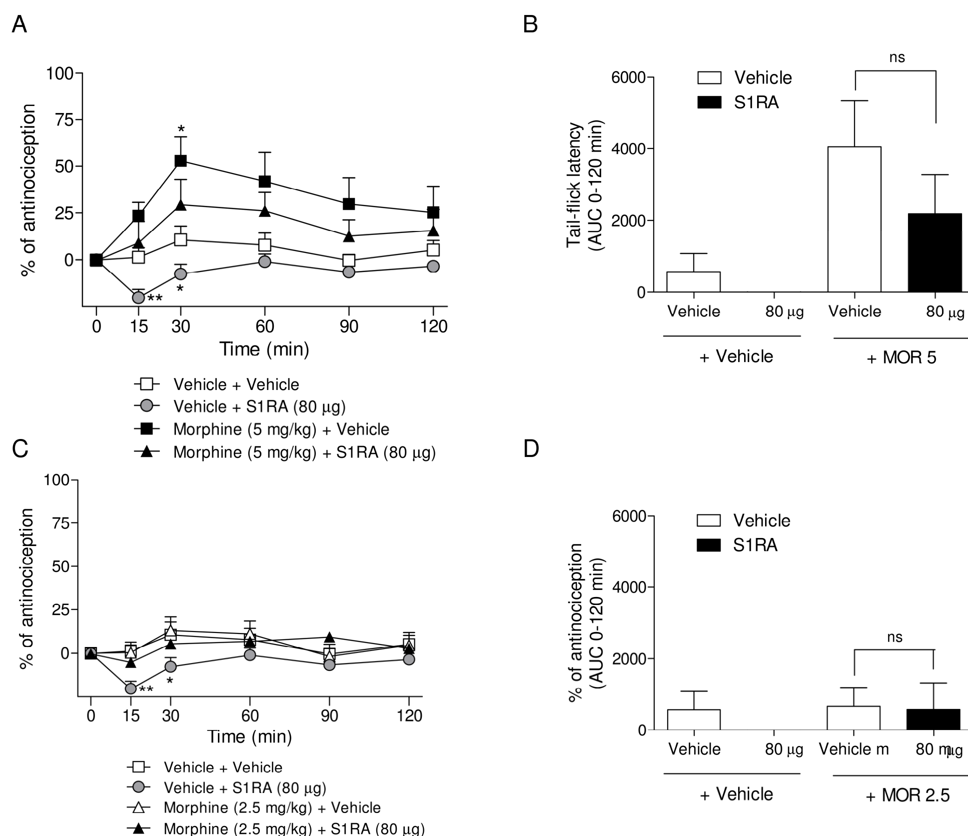

Fig. 5. Time course effects of rostroventral medulla S1RA administration with systemic morphine in the tail-flick test in rats. Rats received i.p. morphine (2.5 or 5 mg/kg) or vehicle + RVM S1RA (80 µg) or vehicle and the TFL was evaluated along the time. Note that morphine exhibit significant antinociceptive effects (30 min post-administration) that were not increased by RVM S1RA. (A, C) Each point and vertical line represents the mean ± S.E.M. percentage of antinociception (n=6-8 per group). *P<0.05, **P<0.01 *vs*. respective basal values (Bonferroni post-hoc test post two-way ANOVA). (B, D) AUC of 0 to 120 min interval evaluation. ns *vs*. morphine group (Newman-Keuls multiple comparison test post one-way ANOVA).

A. Vidal-Torres et al, 2013

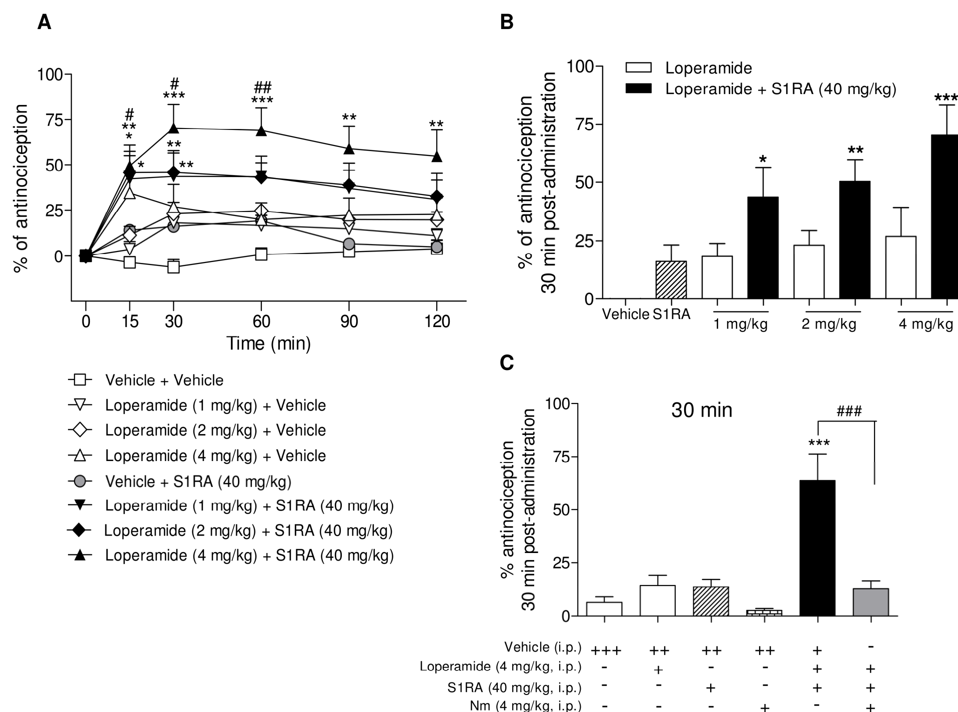

Fig. 6. Time course effects of systemic S1RA administration with systemic loperamide in the tail-flick test in rats. (A) Rats received i.p. loperamide (1, 2, and 4 mg/kg) or vehicle + i.p. S1RA (40 mg/kg) or vehicle and the tail-flick latencies were evaluated along the time. Note that loperamide effects were enhanced by systemic S1RA. Each point and vertical line represents the mean ± S.E.M. percentage of antinociception (n=6-10 per group). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs*. vehicle-treated group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs*. corresponding loperamide dose (Bonferroni post-hoc test post two-way ANOVA). (B) Effects at 30 minutes post-administration. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs*. vehicle-treated group (Newman-Keuls multiple comparison test post one-way ANOVA). (C) Animals were pre-treated with i.p. naloxone-methiodide (4 mg/kg) 5 minutes prior to i.p. loperamide (4 mg/kg) and i.p. S1RA (40 mg/kg), and evaluated at 30 min post-administration. Note that enhancement of the loperamide effect by S1RA was blocked by naloxone-methiodide. Each point and vertical line represent the mean ± S.E.M. percentage of antinociception (n=8-12 per group). $^{***}P<0.001$ *vs*. respective basal value; $^{\#\#\#}P<0.001$ *vs*. loperamide + S1RA group (Newman-Keuls multiple comparison test post one-way ANOVA).

and in rats for haloperidol (2 ratio value) (Chien and Pasternak 1994).

As we previously argued that S1RA modulates the analgesic effect in the formalin test by increasing spinal NA levels (Vidal-Torres *et al.* 2013b), we dialysed the dorsal horn of the spinal cord after co-administration of morphine and S1RA at doses exerting an antinociceptive effect. We described this technique as very sensitive for the study of spinal neurochemical modulation in awake, freely-moving rats (Vidal-Torres *et al.* 2012). Subactive doses of S1RA and morphine, when combined, produced an enhancement of opioid antinociception in the tail-flick test, but failed to modify NA concentration respect to baseline levels. In fact, morphine produced a dose-dependent antinociceptive effect without concomitant increase of NA spinal levels, and S1RA (80 mg/kg) *per se* increased NA levels but failed to evoke antinociceptive effects in the tail-flick test. Therefore, opioid antinociception and potentiation of opioid antinociception did not correlate with an enhancement of NA levels in the dorsal horn of the spinal cord, which discards the change in spinal NA levels as a key mechanism underlying opioid antinociception and σ_1R antagonism-mediated potentiation of opioid antinociception in the reflex tail-flick response to an acute thermal stimulation. This contrasts with previous findings suggesting that increased NA levels lie behind the antinociceptive effect of S1RA in the formalin test (Vidal-Torres *et al.* 2013b).

It is well documented that morphine exerts its antinociceptive effects in part by an hyperpolarisation of both the presynaptic primary afferent C-fibre terminals and the postsynaptic ascending projection neurons (North and Yoshimura 1984; Fleetwood-Walker

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et al. 1985), and by the activation of the descending NA inhibitory pathway (Wigdor and Wilcox 1987; Ossipov *et al.* 1989; Tseng and Tang 1990; Ohsawa *et al.* 2000). α_2-ARs are present at high density in the superficial laminae, including the substantia gelatinosa, of the dorsal horn (Nicholas *et al.* 1993) and many studies involve spinal NA and selective α_2-AR agonists as mediators of antinociceptive effects in the acute thermal tail-flick test (Howe and Yaksh 1982; Milne *et al.* 1985; Pedersen *et al.* 2005). Our results do not substantiate a major role for increased NA levels in the dorsal horn in morphine antinociception in the tail-flick test. In addition, although S1RA treatment results in an enhancement on spinal NA, it seems insufficient to produce antinociception in the tail-flick test.

The site of action of σ_1R modulation of opioid analgesia were addressed in a few studies and using non-selective sigma compounds (Mei and Pasternak 2002; Marrazzo *et al.* 2006; Mei and Pasternak 2007; Tseng *et al.* 2011). In the present study we took advantage of using the selective σ_1R antagonist S1RA to investigate the contribution of peripheral, spinal, and supraspinal σ_1R blockade on morphine antinociception enhancement in the acute thermal nociceptive pain model of tail-flick in rats.

Firstly, we found that i.t. and i.c.v. S1RA treatment alone failed to produce antinociception in the tail-flick test. These results are not surprising given that systemic S1RA did not produce antinociceptive effects by itself in the tail-flick test and agrees with previous studies reporting that σ_1R antagonism elicits antinociception in sensitizing conditions (formalin, capsaicin, partial sciatic nerve ligation, paclitaxel-induced neuropathy) but it does not affect perception of normal nociceptive stimuli (e.g., perception of thermal stimulation in the tail-flick test) (de la Puente *et al.* 2009; Nieto *et al.* 2012; Romero *et al.* 2012). In fact, S1RA failed to produce antinociception in the tail-flick test at the same doses that induced clear cut antinociceptive effects in the formalin-induced pain model (Vidal-Torres *et al.* 2013b). In particular, i.t. S1RA attenuated the flinching behaviour (phase I and II) but not the lifting/licking response in the formalin test. These results in the formalin test can be reconciled if we consider that the lifting/licking response requires supraspinal integration, whereas the flinching behaviour is essentially a spinal response that does not require the integrative action of higher brain centres. Accordingly, σ_1R antagonists acting locally at the spinal cord level seem to modulate the spinal reflex output but not the motorneuron responses evoked by descending, supraspinally processed outputs. This fits well with data in the formalin test, but i.t. S1RA did not inhibit the tail withdrawal response in the tail-flick test, which is also considered to be a spinal response (Irwin 1962). Differences in the nociceptive stimuli (thermal *vs.* chemical), which recruit different spinal pathways/mechanisms being differentially regulated (or not regulated at all) by σ_1R, could provide an explanation. In this way, i.t. administration of the σ_1R antagonist BD-1047 is known to attenuate mechanical allodynia but not thermal hyperalgesia in a neuropathic pain model (Roh *et al.* 2008). Alternatively, the difference could be related to the duration of the stimulus as thermal stimulation in the tail-flick test evokes immediate withdrawal/guarding responses whereas formalin-induced pain, even phase I, lasts for some minutes, and thus some degree of sensitization may occur, this giving to σ_1R antagonists the opportunity to exert an effect.

Secondly, our results revealed that i.c.v. but not i.t. administration of S1RA in combination with systemic morphine produced an enhancement of morphine antinociception in the co-treated group respect to that in the morphine-treated group. These results are consistent with those previously described by Mei and Pasternak in mice (Mei and Pasternak 2002). They found diminished systemic morphine antinociception when the σ_1R agonist (+)pentazocine was given i.c.v., but no effect of (+)pentazocine against morphine when both were given spinally. In the same way, down-regulation of supraspinal σ_1R using an antisense approach potentiated systemic and i.c.v. morphine effects (Mei and Pasternak 2002). The supraspinal regional localization relevant for σ_1R-mediated modulation of opioid antinociception just starts to be clarified. PAG, LC and RVM, areas with σ_1R expression (Walker *et al.* 1992), showed to be potent morphine-sensitive sites (Rossi *et al.* 1993; Rossi *et al.* 1994). Morphine antinociception was found when microinjected in all three regions and it was lowered by co-administration of low doses of (+)-pentazocine in the three regions (although the PAG was far less sensitive than the others), implying a highly sensitive σ_1 system. Only the RVM seems to have a tonic σ_1 activity based upon the ability of the σ_1R antagonist haloperidol and the antisense treatment to enhance morphine actions (Mei and Pasternak 2007). Furthermore, it is known that the blockade of σ_1R in the RVM enhances the antinociception from morphine microinjected into the PAG (Mei and Pasternak 2007). On this basis, we examined the possibility that S1RA was able to enhance the antinociceptive effect of

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systemic morphine when given locally in the RVM. Microinjection studies revealed that S1RA (80 µg) when administered into the RVM failed to modify the tail-flick latency when given alone and also failed to enhance the effects of morphine. Thus, our results (absence of effects of S1RA when given into the RVM) strongly suggest that the σ_1R system in this brainstem region (RVM) does not modulate systemic morphine antinociception. In contrast to the study of Mei and Pasternak (2007), in which morphine was microinjected together with the σ_1R ligand, in our experiment morphine was systemically administered. This and other methodological differences (e.g., use of different σ_1R ligands) could explain the apparently discrepant results, that otherwise seem difficult to reconcile.

We found that morphine's activity varied among the different experiments, possibly as a consequence of altered sensitivity resulting from the distinct surgeries. In naïve rats as well as in i.t.- and RVM-cannulated animals, morphine 5 mg/kg induced a significant antinociceptive effect 30 min post-administration (34, 45 and 52%, respectively). However, the same morphine dose was devoid of significant effect (9%) in i.c.v.-implanted rats. To guarantee that the lack of potentiation effect by S1RA in the i.t.- and RVM-cannulated animals was not consequence of different sensitivity to morphine, we also assayed the combination of S1RA with an inactive dose of morphine (2.5 mg/kg) in the i.t. and RVM local administration experiments.

Finally, we showed that σ_1R plays an important role on peripheral opioid-mediated thermal antinociception. In addition to centrally acting morphine, we also tested the effects of S1RA on the modulation of analgesia by the peripherally acting µ-opioid agonist loperamide (Heykants *et al.* 1974; Schinkel *et al.* 1996), which is routinely prescribed as an antidiarrheal drug as its effects are restricted to the periphery (i.e., inhibition of gastrointestinal transit but no central effects) (Layer and Andresen 2010; Gallelli *et al.* 2010). Loperamide (1, 2, and 4 mg/kg) was devoid of antinociceptive effects in the tail-flick in mice, in agreement with previous reports (Menéndez *et al.* 2005; Sevostianova *et al.* 2005) and consistent with the view that analgesic effects of opiates on acute pain are primarily mediated through receptors located in the central nervous system (Yaksh and Rudy 1978; McNally 1999).

Interestingly, systemic loperamide produced a marked antinociceptive effect when combined with S1RA (40 mg/kg). The recruitment of peripheral opioid receptors in the antinociception produced by loperamide in presence of S1RA was confirmed by its sensitivity to the reversion by the peripherally restricted opioid antagonist naloxone methiodide (Russell *et al.* 1982). To our knowledge, this is the first study supporting the involvement of σ_1R blockade on peripheral opioid thermal antinociception. In the same way, it has recently been reported an important role of peripheral σ_1R antagonism on morphine-induced mechanical antinociception (Sánchez-Fernández *et al.* 2013). It seems thus clear that the σ_1R exerts an inhibitory role precluding opioid-induced peripheral antinociception and that σ_1R antagonism "releases the brake" enabling opioids to exert clear cut antinociceptive effects by acting at the periphery.

Opioids exert peripheral antinociceptive effects that are more pronounced under tissue injury such as inflammation, neuropathy or bone damage (Stein *et al.* 2001; Sehgal *et al.* 2011). Inflammation triggers migration of opioid containing immunocytes to the inflamed tissue and the release of endogenous opioid peptides (Mousa 2003). Inflammatory mediators (e.g. bradykinin) stimulate the expression of peripheral opioid receptors, and the specific milieu (low pH, prostanoid release) of inflamed tissue may increase opioid agonist efficacy. Altogether, these mechanisms contribute to increase opioid receptor function with subsequent inhibition of Ca^{2+} ion channels and thus attenuation of substance P and calcitonin gene related peptide release (Stein *et al.* 2003). Local opioid application is used for the treatment of inflammatory pain in arthritis, burns, skin grafts, and chronic wounds (Kalso *et al.* 2002; Stein and Kuchler, S. 2012). The σ_1R-mediated peripheral opioid potentiation points to topical application as a promising therapeutic potential that would enhance opioid efficacy and suppress undesirable central (respiratory depression, dysphoria, sedation, nausea or addiction) and also peripheral (constipation) opioid-mediated side effects, thus increasing the opioid benefit-to-risk window. Our results were obtained under non tissue injury conditions, but we hypothesize that potentiation of peripheral opioid analgesia may also be expected under these conditions. Further studies are needed at this regard.

In conclusion, studies herein suggest that S1RA produces enhancement of opioid antinociception in acute thermal pain conditions by the sum/integration of the supraspinal and peripheral effect by a mechanism independent on spinal NA levels.

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5. GENERAL DISCUSSION

NSAIDs, opioids, triptans, anticonvulsants, and antidepressants are the most common analgesic drugs. However, they have a limited effectiveness in certain pain conditions (e.g., neuropathic pain) and important adverse effects. Because there is an unmet need of new drug treatments with new mechanisms of action or new drug combination approaches, in the present thesis we explored the therapeuthic interest, the neurochemical changes at the spinal cord, and the site of action of selective σ_1R antagonism in the formalin-induced pain model and in the modulation of opioid-induced behavioural responses. These results, presented in four manuscripts and two annexes, are grouped for discussion as follows:

- 5.1 Sigma-1 modulation of opioid-induced behavioural responses
- 5.2 Sigma-1 modulation of formalin-induced behavioural responses
- 5.3 Neurochemical studies
- 5.4 Site of action of σ_1R blockade

5.1 Sigma-1 modulation of opioid-induced behavioural responses

As commented in section *1.6.9.1 (σ_1R modulation of opioid-induced antinociception)* the evidence involving the ability of σ_1R to modulate opioid antinociceptive effects is strong, but based exclusively in studies using morphine as a μ-opioid ligand and non-selective σ_1R ligands. In addition, it is still unclear if potentiation of opioid analgesia occurs in parallel with a potentiation of undesirable opioid-induced effects. Thus, part of the experiments of the present thesis aimed to study the effect of selective blockade of σ_1R on opioid antinociception and on some undesirable opioid-induced adverse effects.

5.1.1 Sigma-1 modulation of opioid-induced antinociception

The acute thermal nociceptive test of tail-flick was used to explore the modulatory effect of S1RA on opioid antinociception. In this nociceptive model the analgesics used for treating moderate to severe pain in the clinical situation work quite well (Le Bars *et*

al. 2001). The selective σ_1R antagonist S1RA failed to produce any effect when it was administered alone but it was able to potentiate the analgesic effect of systemic morphine in the radiant tail-flick test in mice and rats (*Article 2, Annex 1, Article 4*). These results are consistent with previous reports (Chien and Pasternak 1995a,b; Kim *et al.* 2008; Díaz *et al.* 2009; Romero *et al.* 2012). In rats, we also studied the duration of this enhancement, and the results showed that the potentiation was obtained till 90 minutes post co-administration (*Article 4),* suggesting both an increase in morphine antinociceptive intensity and duration, findings also in agreement with previous works (Chien and Pasternak 1995b). Regarding the improvement of the morphine potency, the potentiating factor achieved in rats was similar to that reached in mice (factor of 1.8 and 2.4, respectively) which is also consistent with the results obtained using other non-selective σ_1R antagonists such as haloperidol in mice (factor 2) (Chien and Pasternak 1994). Apart from S1RA, we also investigated the effect of BD-1047, another σ_1R antagonist employed in many studies, but to our knowledge, not investigated by systemic administration in thermal opioid antinociception modulation studies. BD-1047 was also devoid of effect when administered alone, but greatly potentiated the effects of systemic morphine (*Annex 1*). All these results are consistent with the research recently published reporting that systemic treatment with σ_1R antagonists enhanced morphine-induced mechanical antinociception as measured following punctuate mechanical stimulation (Sánchez-Fernández *et al.* 2013a).

Given the potential widespread application in clinics of σ_1R antagonists as adjuvants for opioid treatment, we tested whether the S1RA capability to potentiate morphine analgesia is extended to other μ-opioid receptor agonists. Our studies revealed that S1RA enhanced the analgesic potency of tramadol, buprenorphine, codeine, oxycodone, and fentanyl by a factor between 2 and 3.3 (*Article 2*). The highest enhancement (x3.3) was found for tramadol. Tramadol is a μ-opioid agonist that also inhibits monoamine reuptake (Grond and Sablotzki 2004). This additional mechanism could be involved in the superior analgesia factor observed when combined with S1RA.

The potentiating effect of opioid antinociception cannot be explained by direct interaction of S1RA and BD-1047 with μ-opioid receptors since both compounds fail to bind to μ-opioid receptors. Alternatively, a possible explanation could be that a pharmacokinetic interaction may enhance the opioid bioavailability. We discarded this possibility based on pharmacokinetic studies and because intravenous administration of SR1A with systemic morphine also revealed a significant enhancement of morphine's

effects in the tail-flick test (data not shown), thus suggesting a pharmacodynamic interaction. Likewise, σ_1R antisense oligodeoxynucleotides microinjection studies also enhanced opioid antinociception (King *et al.* 1997; Mei and Pasternak 2007). Altogether, these results point to a functional interaction between σ_1 and opioid receptors. In this direction, based on an immunoprecipitation study, it was described a physical interaction between the μ-opioid and the σ_1R, whereby the σ_1R antagonists (but not agonists) potentiated opioid-induced G-protein-coupled signal transduction without influencing opioid receptor binding. In addition, when administered alone, neither σ_1R agonists nor antagonists stimulated GTPγS binding (Kim *et al.* 2010).

We took advantage of a genetic approach using σ_1R-KO mice in order to unambiguously prove that the potentiating effect of S1RA is due specifically to σ_1R (*Article 2*). It was previously reported that deletion of this gene results in viable and fertile mice with no detectable abnormalities (Langa *et al.* 2003). As compared to WT mice, our results showed that σ_1R-KO perceived normally acute thermal nociceptive stimuli, as has also recently been described for mechanical stimuli (Sánchez-Fernández *et al.* 2013a), suggesting that basic mechanisms for transduction, transmission and perception of nociceptive and sensory inputs are intact in mice lacking σ_1R. In addition, the analgesic effect produced by opioids (morphine, sufentanyl, fentanyl, and buprenorphine), was similar in deficient σ_1R and WT mice in terms of efficacy and potency (*Article 2 and Annex 1*). However, S1RA failed to increase the analgesic potency of morphine in σ_1R-KO mice discarding off-target effects and consequently attributing to σ_1R the modulatory effects of S1RA. The observation that the potency of the opioids studied (with the exception of oxycodone) was similar in WT and σ_1R-KO mice suggests that the absence of a modulatory system (as is the case with KO mice) precludes ligand-mediated regulation but does not mimic the modulatory (enhancing) effect elicited by an antagonist acting on the σ_1R. The σ_1R is an intracellular chaperone protein associated with ER and mitochondrial membranes (Hayashi and Su 2007). Thus, this finding can be explained by the chaperone nature of the σ_1R, which exerts its action by physical protein-protein interactions. Accordingly, the absence of regulatory mechanisms in KO mice is not equivalent to the decrease or gain of function promoted by σ_1R ligands through conformational changes that affect the activity of the target protein (opioid receptor) with which the σ_1R is interacting (Hayashi *et al.* 2011). Neutral antagonism-like (KO situation) *versus* inverse agonism-like (ligand effect)

activities or plastic adaptive changes of gene expression in the KO mice could also explain the lack of enhancement of opioid effect in σ_1R-KO mice.

Conversely, recent studies (Sánchez-Fernández *et al.* 2013a) reported that the mechanical antinociceptive effect of morphine was markedly enhanced in σ_1R genetically inactivated mice, indicating that morphine effects in KO mice differ depending on the stimulus modality. These differences could be explained by the distinct subsets of primary sensory fibres that mediate behavioural responses to noxious thermal and mechanical stimuli (Cavanaugh *et al.* 2009). In addition, different mechanisms have been reported for opioid-induced thermal and mechanical antinociception (Kuraishi *et al.* 1985a; Wegert *et al.* 1997). However, independently on the measured parameter, the potentiation that σ_1R exerts on opioid antinociception cannot be interfered by adaptative changes of μ-opioid receptors in σ_1R-KO mice since [^{3}H]DAMGO binding in forebrain, spinal cord and hind-paw skin membranes was unaltered in σ_1R-KO mice (Sánchez-Fernández *et al.* 2013a).

Summarizing, we confirmed and extended prior observations of the functional interaction between σ_1R and opioid antinociception. All these data point, as already suggested, to the presence of an antiopioid σ_1 regulatory system where σ_1R can exert a tonic inhibitory control on the opioid receptor-mediated antinociception, which can be pharmacologically counteracted by using σ_1R antagonists to increase the response to opioids.

5.1.2 Sigma-1 modulation of opioid-induced adverse effects

Another main purpose of the present thesis was the assessment of not only analgesic potency but also safety endpoints to explore the advantage of using a selective σ_1R antagonist as an opioid adjuvant therapy. That is, is the σ_1R able to modulate opioid receptor just only in pain pathways? Does the σ_1R modulate also other opioid-induced effects? The effect of S1RA was evaluated on morphine-induced antinociceptive tolerance, physical dependence, reward, constipation, and mydriasis, using the tail-withdrawal test, the naloxone-precipitated withdrawal test, the conditioned place preference (CPP) paradigm, the charcoal meal test, and the pupillary diameter determination, respectively (*Article 2*).

We first addressed the possibility that S1RA could modulate morphine **tolerance.** Morphine tolerance occurs on continued use of the drug such that the amount of drug

must be increased to compensate for diminished responsiveness, and consequently it may limit the effective long term analgesia (Adriaensen *et al.* 2003; Jage 2005; Morgan and Christie 2011). Tolerance to morphine analgesia is manifested as a shift to the right of the dose-response curve or as a decrease in the intensity of the response when a constant dose is repetitively administered. Our results showed that acute S1RA is able to restore the analgesic effect of morphine in morphine-tolerant mice, which stands along with the enhancement of the analgesic effect of morphine, suggesting that σ_1R-mediated mechanisms, recruited by S1RA to enhance morphine antinociception, remain active in tolerant mice. We also investigated the effect of S1RA on the development of morphine tolerance but the results showed that the co-administration of the σ_1R antagonist S1RA with morphine neither potentiated nor significantly reduced the development of tolerance. The restoration of the analgesic effect of morphine could be explained by phosphorylation mechanisms. Phosphorylation of the μ-opioid receptor by calcium dependent PKC, calcium/calmodulin-dependent protein kinase II (CaMKII), and cAMP dependent PKA is known to play a critical role in receptor desensitisation and opioid tolerance (Liu and Anand 2001; Wang and Wang 2006; Gabra *et al.* 2008; Garzón *et al.* 2008; Bailey *et al.* 2009). Interestingly, these kinases are recruited by downstream activation not only of opioid but also of other neurotransmitter receptors including NMDARs (Garzón *et al.* 2008; Rodríguez-Muñoz *et al.* 2012). It is known that the μ-opioid and the NR1 subunit of the NMDAR associate postsynaptically in neurons involved in pain control (Rodríguez-Muñoz *et al.* 2011), and that μ-opioid activation increases NMDAR function (Martin *et al.* 1997; Przewlocki *et al.* 1999) while NMDAR activation inhibits the μ-opioid receptor function (Rodríguez-Muñoz *et al.* 2011). Indeed, morphine disrupts this complex by PKC-mediated phosphorylation of the NR1 C1 segment, which potentiates the NMDAR–CaMKII pathway involved in morphine tolerance. In turn, activation of the NMDAR also separates the μ-opioid-NR1 complex and induces PKA-mediated phosphorylation of the μ-opioid receptor, which reduces its association with G-proteins and diminishes the antinociceptive effect of morphine (Rodríguez-Muñoz *et al.* 2011). Although the role played by σ_1R in this cross-talk involving kinases activation is presently not defined, it is well-known that:

- σ_1R physically associate with μ-opioid receptors (Kim *et al.* 2010).
- Antagonists acting at σ_1R reduce NMDAR function (Monnet *et al.* 1990; Hayashi *et al.* 1995; Martina *et al.* 2007) and inhibit both PKC- and PKA-

dependent phosphorylation of the NMDAR NR1 subunit (Kim *et al.* 2006, 2008; Roh *et al.* 2008, 2010).

Both mechanisms would result in the restoration of morphine antinociception in tolerant animals.

On the other hand, it is well known that the tail-flick reflex although being a spinal phenomenon, it is under a descending modulatory influence from supraspinal sites (Zemlan *et al.* 1980). Morphine inhibits this spinal reflex both supraspinally, by activating descending modulatory influences, and spinally, by directly inhibiting nociceptive input. It is well established that morphine antinociception is increased by the administration of i.t. α_2-AR agonists such as clonidine. In fact, clonidine retains full antinociceptive efficacy in morphine-tolerant mice (Ossipov *et al.* 1989) and full α_{2C}-AR activation appears advantageous in reducing morphine tolerance expression but not morphine tolerance acquisition (del Bello *et al.* 2010). In this way, it has been reported that the activation of spinal α_2-AR reduces the i.t. NMDA-induced increase on spinal NR1 subunit phosphorylation and nociceptive behaviours in the rat (Roh *et al.* 2010). Results from *Article 4* by using *in vivo* microdialysis in the spinal cord of non-tolerant rats showed that systemic acute S1RA administration (80 mg/kg) results in an enhancement on NA levels, although antinociception in the tail-flick test was not observed (*Article 2, Article 4*). On this basis, if a NA enhancement also occurs in tolerant animals, this could explain the rescue of morphine antinociception seen once tolerance has developed, but not in tolerance development, as we observed (*Article 2*).

We also investigated the effect of S1RA on morphine-induced physical dependence and reward effects. **Physical dependence**, induced by repeated morphine administration and precipitated by naloxone was not modified by co-administration of S1RA. Thus, S1RA co-administration modified neither the severity of somatic manifestations of naloxone-precipitated morphine withdrawal nor the development of morphine tolerance. In line with this, many studies show that these two phenomena (development of tolerance and dependence) are highly correlated. The development of morphine tolerance and dependence has been linked to the cAMP pathway. μ-opioid receptor activation leads to inhibition of adenylate cyclase (Sharma *et al.* 1975; Wang and Gintzler 1994). However, the repeated administration of morphine may lead to up-regulation of adenylate cyclase, increased cAMP levels and PKA activation, which contribute to the development of tolerance via cAMP response element-binding protein, a transcription

factor that regulates genes responsible for the development of physical dependence (Lilius *et al.* 2009).

The **reward** effect of morphine evaluated by the CPP paradigm was antagonized. When administered alone, S1RA produced neither reward nor aversive effects and it blocked the reward effects of morphine. Our studies are the first to report that a σ_1R antagonist blocks the CPP produced by an opioid. The σ_1R has been pointed up as a molecular target for the treatment of drug addiction (Matsumoto 2009; Robson *et al.* 2012). In particular, σ_1R agonists are reported to augment and antagonists block the reward effects of drugs of abuse, including cocaine and ethanol.

- The involvement of σ_1R on cocaine abuse has been extensively studied (Maurice *et al.* 2002; Maurice and Romieu 2004). The σ_1R antagonists NE-100 and BD-1047 had no effects when administered alone but decreased both acquisition and expression of cocaine-induced reward (Romieu *et al.* 2000, 2002). Similarly, the reduction of σ_1R expression following antisense treatment prevents the acquisition of cocaine CPP (Romieu *et al.* 2000). In turn, the σ_1R agonists igmesine or PRE-084 failed to induce CPP when injected alone, suggesting that the σ_1R receptor modulates but it is not sufficient to induce CPP.
- Similarly, in the case of ethanol, the σ_1R antagonist BD-1047 dose-dependently blocked the development, expression and reinstatement of ethanol-induced CPP in mice, whereas the σ_1R agonist PRE-084 dose-dependently enhanced the CPP and reinstated the extinguished response (Maurice *et al.* 2003; Bhutada *et al.* 2012).
- Data are much less clear regarding opioid reward as σ_1R activation (instead of σ_1R antagonism) has been suggested to attenuate morphine-induced CPP based on studies using low doses (microgram/kg range) of the non-selective σ_1R agonist dextromorphine, which does not have μ-opioid receptor activity, or dextromethorphan, a low affinity NMDAR antagonist which is also a non-selective σR agonist (Wu *et al.* 2007; Chen *et al.* 2011).

Our results showing blockade of opioid-induced CPP by a σ_1R antagonist are thus in line with studies describing the ability of σ_1R antagonists to inhibit the reward produced by other drugs of abuse. Methodological differences and most probably the use of compounds with non-delineated selectivity and functionality in previous studies may explain the apparent discrepancy with previous studies.

A key mechanism that contributes to addiction is the increase of the extracellular levels of DA in the nucleus accumbens, where activation of dopamine D_1 receptor (D_1R) is a requirement for cellular and behavioural responses to drugs of abuse (Xu *et al.* 1994a,b; Anderson and Pierce 2005). In this way it is known that D_1 and σ_1 receptors heteromerize and that D_1R-mediated signalling is inhibited by σ_1R antagonist administration, and strongly diminished in cells transfected with σ_1R small interfering RNA and in brain slices of mice lacking the σ_1R (Navarro *et al.* 2010). Modulation of the DA neurotransmission by σ_1R could provide a common mechanism allowing the inhibition of the reward effects of different drugs of abuse by σ_1R antagonists.

Because **constipation** is the most frequently-reported adverse event in patients receiving opioid treatment (Coluzzi and Pappagallo 2005) and can lead patients to reduce or even discontinue their opioid therapy (Kurz and Sessler 2003), in the present thesis we explored the possible modulation of opioid-induced constipation by σ_1R. Our results showed that S1RA, when administered alone, produced no significant reduction of the distance travelled by a charcoal meal through the gastrointestinal tract. As expected (Paul and Pasternak 1988; Tavani *et al.* 1990), morphine administration produced significant reduction of the intestinal transit. When given in conjunction with S1RA, neither potentiation nor attenuation of the morphine-induced inhibition of intestinal transit was found with respect to morphine alone, although a chronic study should be performed in order to also assess it under repeated administrations. The same results but with a σ_1R agonist were obtained when (+)pentazocine was co-administered with morphine (Chien and Pasternak 1994). Morphine reduction of gastrointestinal transit was insensitive to (+)pentazocine at a dose that reduced systemic morphine antinociception by approximately 50%. These results further support that, although widely distributed in the digestive tract (Samovilova and Vinogradov 1992), the σ_1R does not modulate the opioid-induced inhibition of gastrointestinal transit. In line with this, a very recent study has reported that morphine-induced inhibition of gastrointestinal transit is not modified in σ_1R-KO respect to WT mice (Sánchez-Fernández *et al.* 2013a).

It has been reported that opioids affect **pupillary** responses by changing the centrally mediated parasympathethic outflow to the iris. However, these effects on the pupil differ markedly between species:

- In some animals, including the dog, rabbit and human, morphine and other opioids produce miosis.
- In most other animals, including cats, mice and rats, mydriasis occurs.

The pupillary changes to morphine are dose related and are antagonized by naloxone, which suggests that they are actually mediated by opioid receptors (Robin *et al.* 1985). We assessed if σ_1R modulates the opioid-induced pupillary changes. When administered alone morphine produced a dose-response mydriatic effect in mice, as previously reported (Robin *et al.* 1985). However, no modification of pupillary diameter was found when S1RA was administered alone, and the morphine-induced mydriasis was not modified by S1RA co-administration.

5.1.3 σ_1R antagonism as opioid adjuvant therapy

The reduction of adverse effects of opioid analgesics represents an important clinical target. Attempts to exploit different opioid receptor types have had low success. Opioids that are more selective agonists for μ-opioid receptors have had limited success because both the most potent desired actions (analgesia) and adverse effects, including dependence and addiction, are mediated by μ-opioid receptors (Kieffer and Gaveriaux-Ruff 2002). Recent promising preclinical approaches to limit tolerance, dependence and addiction include simultaneous activation of more than one opioid receptor type (e.g. μ and δ receptors), selective targeting of heteromultimers, or μ-opioids that differentially activate distinct intracellular signalling cascades, particularly G-protein activation versus endocytosis (Berger and Whistler 2010).

In this thesis we addressed another therapeutic approach: the potential of σ_1R antagonism as an opioid adjuvant therapy. An opioid adjuvant (or co-analgesic) is a drug that increases the therapeutic index of opioids by a dose-sparing effect, reduces opioid adverse effects, or adds a unique analgesic action in opioid-resistant pain (Khan *et al.* 2011). Our results indicate that a dissociation exits between the modulatory effect mediated by σ_1R on opioid-induced antinociception and on the rest of opioid-induced effects (Table 6). There could be multiple causes for such dissociation. Firstly, although no comparative or double labelling studies on the distribution of σ_1 and μ-opioid receptors are presently available, the regional distribution of both receptors differs clearly (Moriwaki *et al.* 1996; Alonso *et al.* 2000) and, even if they co-localize, coupling of σ_1 and μ-opioid receptors would not necessarily -and thoroughly- occur,

which could explain why certain but not all the effects are modulated. As an example of a similar dissociation, it has been recently described that inhibition of Gβγ-subunit signalling to PLC β3 by gallein potentiates morphine-mediated acute antinociception while attenuating the development of tolerance and not enhancing respiratory depression, constipation, locomotion and reward (Hoot *et al.* 2013). This study suggests that different and specific μ-opioid receptor-mediated signalling pathways mediate morphine-induced antinociception and side effects. A possible hypothesis is thus that σ_1R antagonism could inhibit opioid signalling through some pathways (e.g., the above mentioned Gβγ-subunit one or other yet unknown) without interfering others involved in mediating side effects. A second cause for this dissociation could be that the σ_1R modulates the function of proteins others than μ-opioid receptors (i.e., D_1 and NMDA receptors), and this could account for complementary, counteracting activities.

As a summary, S1RA co-administration restored morphine antinociception in tolerant mice and reversed the reward effects of morphine in the CPP paradigm. In addition, enhancement of antinociception was not accompanied by potentiation of other opioid-induced effects, such as the development of morphine analgesic tolerance, physical dependence, inhibition of gastrointestinal transit, or mydriasis. Based on this preclinical data obtained with S1RA, showing that opioid antinociception but not adverse effects are potentiated, it seems clear that lower and safer doses of opioids could potentially provide satisfactory pain relief if combined with a σ_1R antagonist. The use of σ_1R antagonists as opioid adjuvants could thus represent a promising pharmacological strategy to enhance opioid potency and, most importantly, to increase the safety margin of opioids.

Table 6. Summary of interactions between morphine and S1RA in different behavioural tests. S1RA potentiates morphine antinociception by a factor of 2.4 (tail-flick). In addition, S1RA was able to restore morphine antinociception in morphine-tolerant mice. The antinociceptive potentiation was not accompanied by potentiation of some other morphine-induced effects, like tolerance development, physical dependence, inhibition of gastrointestinal transit and mydriasis. Furthermore, S1RA reversed the reward properties of morphine. N.D. not determined; CI confidence intervals.

Opioid Induced Phenomena	Assay	Endpoint	ED_{50} (Mean, 95% CI)		ED_{50} Ratio	Interaction
			Morphine +Vehicle	Morphine +S1RA		
Analgesia	Tail-flick test	Latency withdrawal in naïve mice	3.2 (2.6-3.9)	1.3 (1.0-1.7)	2.4	Potentiation
		Latency withdrawal in morphine tolerant mice (protocol I)	N.D.	N.D.	N.D.	Potentiation
Tolerance Development	Tail-immersion test	Latency withdrawal after daily administration (day 1-10, protocol II)	N.D.	N.D.	N.D.	No Interaction
		Reduction of morphine effect in tolerant mice (day 11, protocol II)	90 (66-126)	59 (44-79)	1.5	No Interaction
Physical Dependence	Naloxone-precipitated morphine withdrawal test	Wet dog shakes, jumping, body tremor, forepaw tremor, ptosis, piloerection and mastication	N.D.	N.D.	N.D.	No Interaction
Reward	CPP	Time spent in the drug-paired compartment	N.D.	N.D.	N.D.	Reversion
Gastrointestinal Transit	Charcoal meal test	Intestinal transit length	3.3 (2.4-4.6)	2.5 (1.7-3.7)	1.3	No Interaction
Mydriasis	Pupillary size determination	Pupil diameter	1.9 (1.5-2.3)	2.0 (1.6-2.6)	1.0	No Interaction

5.2 Sigma-1 modulation of formalin-induced behavioural responses

As commented in section *1.6.9.2 ($σ_1$R modulation of pain behaviour in the absence of opioids),* $σ_1$R antagonists by themselves attenuate pain hypersensitivity in different pain models, particularly in sensitizing and chronic pain conditions (Zamanillo *et al.* 2013). In the present thesis we further addressed the effects and explored some potential mechanisms involved in $σ_1$R blockade in the formalin-induced pain model in rats (*Article 3 and Annex 2*). The formalin model is characterised by an initial acute pain response (phase I) followed by a relatively short quiescent period and then by a prolonged tonic phase (phase II) (Wheeler-Aceto and Cowan 1991; Abbott *et al.* 1995). The acute phase is believed to correlate with peripheral pain pathways, whereas the second phase is indicative for a centralization and sensitization of pain at higher pain conducting pathways comparable to pain pathways activated in neuropathic pain models (Vissers *et al.* 2003).

Phase I starts immediately after formalin chemical injection and it is characterized by paw flinching, lifting, and licking and it is caused by activation of peripheral nociceptors. After a short period of quiescence this is followed by a second phase (**phase II**) of paw flinching, lifting and licking which is attributed to ongoing activity in primary afferents and increased sensitivity of DH neurons. The initial barrage of C-fibre input in phase I may produce an NMDA- and substance P-mediated sensitisation of DH neurones that leads to phase II. Consistent with this hypothesis, administration of local anaesthetics, NMDA antagonists or SP antagonists prior to phase I significantly reduces phase II behavioural responses and/or DH neuronal activity (Coderre *et al.* 1990, Murray *et al.* 1991). Phase II presumably involves local release of neuroactive substances, including 5-HT, histamine, bradykinin, and PGs that are responsible for the sensitisation of primary afferent fibres and subsequent activation and sensitization of the DH neurons in the spinal cord (Dickenson and Sullivan 1987).

Licking behaviour is the regular endpoint evaluated for the formalin test in mice. In our experiments performed in rats we evaluated the lifting/licking time after formalin injection but also, independently, the number of flinches. Some studies suggest that the flinching behaviour is organized at the spinal level while the lifting/licking behaviour is mainly organized supraspinally (Coderre *et al.* 1994). As these behaviours can be

clearly differentiated and quantified in rats, both measurements allowed us to obtain further mechanistic information in our experiments.

Systemic administration of 10 mg/kg of morphine, evaluated as a positive control, reduced the formalin-induced pain behaviour in both phases (*Article 3*), as expected (Wheeler-Aceto and Cowan 1993).

We evaluated the effects of S1RA and BD-1063 in the formalin-induced pain model in rats at doses devoid of significant effects on locomotion (measured by locomotor activity and rotarod tests) and other apparent behavioural side effects. Systemic administration of S1RA and BD-1063 reduced the nociceptive effects (flinching and lifting/licking) induced by intraplantar injection of formalin in rats in both phase I and II (*Article 3 and Annex 2*):

- Regarding **phase I**, previous data indicated that systemic σ_1R antagonists did not show antinociceptive effects on acute thermal and mechanical pain models (*Article 2; Annex 1; Article 4;* Sánchez-Fernández et al. 2013a). On this basis, should we expect an antinociceptive effect of σ_1R antagonists in the phase I of the formalin test? Our results are in line with the literature reporting that systemically administered haloperidol (non-selective σ_1R antagonist) reduced the licking behaviour in phase I in mice (Cendán *et al.* 2005a), and an attenuation of the licking behaviour was also observed in σ_1R-KO mice (Cendán *et al.* 2005b). It can be speculated that differences depend on the nature of the nociceptive stimulus (thermal or mechanical *vs.* chemical), which recruit different pathways/mechanisms that are differentially regulated (or not regulated at all) by σ_1R in acute conditions. In addition, thermal and mechanical stimulation tests usually evoke immediate withdrawal/guarding responses whereas phase I pain following intraplantar injection of formalin lasts for some minutes, and thus some degree of sensitization (probably peripheral but perhaps also central) may occur, this giving to σ_1R antagonists the opportunity to exert a modulatory activity.
- Regarding **phase II**, our results are coherent with previous ones using haloperidol and its metabolites in mice (Cendán *et al.* 2005a). A prolonged tonic period and neuronal sensitization are known to occur at the spinal cord in phase II of formalin-evoked pain. Therefore, inhibition of formalin induced pain in phase II by σ_1R antagonists is consistent with the well-described role played by σ_1R in sensitization states (Drews and Zimmer 2009; Zamanillo *et al.* 2013). In

fact, S1RA has shown efficacy in several neuropathic pain models, including partial sciatic nerve ligation (Romero *et al.* 2012) and paclitaxel-induced neuropathy (Nieto *et al.* 2012).

5.3 Neurochemical studies

The analgesic effect of σ_1R antagonists has been studied mainly on the spinal cord as the primary site of action in central sensitization (Kim *et al.* 2006, 2008; de la Puente *et al.* 2009; Romero *et al.* 2012). Electrophysiological data point to a modulatory role for σ_1R on spinal excitability, whereby blocking of the receptor inhibits the amplified spinal response that would normally arise from repetitive nociceptor stimulation (de la Puente *et al.* 2009; Romero *et al.* 2012; Zamanillo *et al.* 2013).

At the moment, there is no available literature regarding the spinal modulation of neurotransmitters induced by a systemic administration with σ_1R antagonists that could explain their mechanism of action. Thus, in the present thesis we first aimed to set-up the *in vivo* concentric microdialysis technique in conscious rats to investigate later on the possible mechanisms that may underlie both the systemic σ_1R antagonism antinociceptive effect *per se* in the formalin model and in the modulation of opioid analgesia.

5.3.1 Set-up of the dorsal horn *in vivo* concentric microdialysis technique in the formalin-induced pain model in conscious rats

In an effort to identify neurochemical markers of nociceptive processing that could be correlated to behavioural antinociceptive efficacy of σ_1R antagonists, we developed an *in vivo* microdialysis method in conscious rats in order to study neurochemical events in the DH of the spinal cord in parallel with pain behaviours.

As explained in section *1.4 (Spinal cord microdialysis approaches),* three spinal microdialysis methods have been described based on the location of the fibre (transversal, intrathecal, and concentric). Only the concentric approach allows ipsilateral intra-DH microdialysis, which is relevant in most animal models of pain to focus on ipsilateral pain-related changes. Although some pain studies are available in anaesthetized animals (Tzschentke *et al.* 2012), we assayed the concentric method in

absence of the suppressant action of the anaesthesia allowing simultaneously microdialysis sampling and behavioural nociception assessment.

We selected the formalin-induced pain model to set-up the concentric microdialysis technique in our laboratory. It is known that following chemical subcutaneous administration of formalin, Glu is released in the DH leading to increased neuron sensitivity (Marsala *et al.* 1995; Malmberg and Yaksh 1995a,b) mainly in the DH of the spinal cord ipsilateral to the site of the injury, and thus the concentric microdialysis approach seems more appropriate to detect changes in neurotransmitter release in this region.

First of all, in order to validate the methodology we evaluated the nociceptive response and changes in Glu release in the ipsilateral spinal DH and their modulation by systemic morphine administration. Injection of formalin into the hind paw evoked the characteristic biphasic behavioural response in intra-DH implanted animals, although the flinching behaviour was reduced compared to the naïve group (*Article 1*). In this way, it is important to note that the concentric microdialysis approach implies a slight more invasive surgery than the intrathecal one:

- The Th13 is immobilized by using a transverse process clamp and a burr hole is done in the dorsal surface of the vertebrae for screw placement.
- The fibre is directly introduced in the spinal cord tissue.

However, the implanted animals kept the typical biphasic response on flinching behaviour and systemic morphine treatment inhibited both formalin-induced phases, as previously reported (Wheeler-Aceto and Cowan 1993; Yamamoto and Yaksh 1992). Morphine activates opioid receptors in the brainstem and spinal cord suppressing spinal nociceptive processing. At the spinal level, morphine inhibits Glu and neuropeptides (e.g. SP, CGRP) release from primary afferent C-fibre terminals and hyperpolarize ascending projection neurons (Fleetwood-Walker *et al.* 1985; North *et al.* 1987).

Microdialysis samples were assayed by HPLC coupled to fluorescence detection and the neurochemical analysis revealed that paw saline injection failed to modify Glu levels (*Article 1*), as previously described (Malmberg and Yaksh 1995b). In contrast, formalin injection increased Glu levels (maximum = 294% over baseline values), which were prevented by morphine treatment, as previously reported using intrathecal microdialysis (Malmberg and Yaksh 1995b). Glu levels in our study were higher (294%) respect to previous studies using the intrathecal approach (92%) (Malmberg and Yaksh 1995b)

suggesting a higher sensitivity of this technique (samples come from the primarily affected spinal DH laminae).

A common strategy to induce neurotransmitter release involves the inclusion of high concentrations of K^+ in the microdialysis perfusion fluid. Passive diffusion of these ions across the dialysis membrane and then into the tissues generates depolarization, leading to neurotransmitter release. At the end of the experiment a high K^+ infusion (100 nM) significantly increased Glu release suggesting that neuronal release appears to be the source for Glu increases detected in the DH.

One point to take into consideration is that during *in vivo* microdialysis neurotransmitters diffuse across the dialysis membrane from the extracellular space to the CSF within the concentric microdialysis probe. The results of our experiments are, therefore, presented as the content of neurotransmitter for each 10 μL dialysate sample and are interpreted as being representative of the extracellular space in the DH. However, this interpretation must be done with caution as the microdialysis technique may only collect a small fraction of the extracellular space, and differences in the internal and external milieu of the probe may lead to different perfusion profiles for each measured substance. The recovery rate of the microdialysis probes for Glu was 10% in our experimental conditions.

5.3.2 σ_1R antagonism modulation of DH Glu and GABA levels

Glu and GABA, involved in the modulation of pain transmission, are both amino acids and can be simultaneously quantified with the same HPLC sample running.

As commented, Glu is critical for spinal excitatory synaptic transmission and for the generation and maintenance of spinal states of pain hypersensitivity (Liu and Salter 2010). On the other hand, GABA is a key inhibitory neurotransmitter in the modulation of nociceptive processing. $GABA_A$ and $GABA_B$ sites are both enriched in superficial laminae of the DH, wherein they inhibit the terminals of small and large diameter primary afferent fibres and intrinsic DH neurons (Millan 2002). Thus, measurement of Glu and GABA levels in the DH could provide relevant information on pain signalling.

Remarkably, it is presently unknown if these aminoacidic neurotransmitters are modulated and if they play a role in pain relief elicited by σ_1R antagonists. In order to understand the neurochemical mechanism implicated in the inhibition that σ_1R blocking exerts on formalin-induced pain, we simultaneously evaluated the behavioural effect of

S1RA or BD-1063 and the release of Glu and GABA in the spinal DH of concentric microdialysis-implanted rats.

Our results confirmed that implanted animals exposed to formalin exhibited a biphasic behaviour similar to the non-implanted animals (*Article 3*), as previously observed (*Article 1*). The lifting/licking behaviour was not modified in implanted rats when compared to non-implanted, but they showed significantly less flinching behaviour, confirming our previous observation when setting up the technique (*Article 1*). Likewise, when Glu levels were analysed, an increase was observed (*Article 3*) similar to that in the setting-up experiments (*Article 1*). However the formalin-induced Glu increase was slightly different between studies in the vehicle group (*Article 1* vs *Article 3* and *Annex 2*). One possible explanation would be the different Glu baseline between experiments consequence of different level of injury during the surgery. In the first study (*Article 1*) the Glu baselines were higher and consequently the sample values obtained following formalin were lower (294%) when percentualizing. In the following study (*Article 3* and *Annex 2*), the Glu baseline levels were lower, allowing to detect a higher formalin-induced enhancement of Glu (368% over baseline values).

Systemic pre-treatment with S1RA (80 mg/kg) (*Article 3*) and BD-1063 (40 mg/kg) (*Annex 2*) attenuated formalin-evoked pain behaviours, as in non-implanted animals, indicating that implanted animals respond similarly to pharmacological treatment with σ_1R antagonists. Interestingly, systemic S1RA and BD-1063 prevented spinal formalin-induced Glu increase in contraposition to vehicle-treated animals (*Article 3* and *Annex 2*). No data are available in the literature regarding modulation of Glu release by σ_1R in the spinal cord, but an involvement of σ_1R on Glu release has been reported in other areas:

- PREG-S (endogenous σ_1R agonist) increased whereas haloperidol and BD-1063 blocked Glu release via presumable activation of a presynaptic $G_{i/o}$-coupled σR and elevation in intracellular Ca^{2+} levels in hippocampal neurons (Meyer *et al.* 2002).
- A DHEA-S (endogenous σ_1R agonist) presynaptic Glu-induced release was also reported in prelimbic cortex through presumable activation of D_1 and σ_1 receptors (Dong *et al.* 2007).
- On the opposite direction, SKF-10,047 (σ_1R agonist) has been described to inhibit Glu release evoked by a K^+ channel blocker from rat cortical nerve

terminals, an effect that was linked to a decrease in Ca^{2+} entry and the suppression of the PKC signalling cascade (Lu *et al.* 2012).

Altogether, although these studies do not go all in the same direction and were performed in cell cultures and in rat slices of supraspinal structures, they suggest that σ_1R could regulate in an inhibitory fashion the spinal Glu levels through a presynaptically mechanism. In our case, the inhibition of Glu release could be due to a presynaptic regulation exerted by σ_1R on the central nociceptor endings at the DH, but also at the peripheral nociceptor endings (inhibition of ascending pathway) or supraspinally (activation of the descending inhibitory pathway), which would equally result in an hyperpolarisation of the first order neuron (Fig. 12).

We also quantified GABA levels after S1RA and BD-1063 treatment. In contrast to Glu, GABA levels in the DH were not modified following intraplantar formalin injection and treatments did not exert any effect. There are data showing that the σ_1R agonist PREG-S produced an inhibitory effect on GABAergic synaptic transmission in cultured hippocampal pyramidal neurons (Mtchedlishvili and Kapur 2003), but our results, clearly showing that systemic administration of S1RA and BD-1063 did not modify the extracellular GABA concentration (*Annex 2*), indicate that GABA is not implicated in the analgesic effects of σ_1R antagonists, at least in the formalin model and at the spinal cord DH level. Thus, inhibition of the release of excitatory (i.e., Glu) but no control by inhibitory (i.e., GABA) amino acids at the DH of the spinal cord seems to be a mechanism by which σ_1R antagonists could exert their analgesic effect.

5.3.3 σ_1R antagonism involvement in DH NA levels

As stated in section (*1.3 Control of pain),* the descending pain modulatory system has a powerful influence on the modulation of nociceptive information transmitted from the periphery to the brain as the spinal cord DH is the site where the descending modulatory system impinges upon the ascending pain transmission pathway. This system uses NA and 5-HT as its main transmitters to exert its effects in the spinal cord. The role for NA appears to be predominantly inhibitory, while the role of 5-HT appears to be bidirectional, mediating inhibitory as well as excitatory effects (Suzuki *et al.* 2004). In the same sample running, both amines (NA and 5-HT) can be potentially detected. However, 5-HT could not be quantified in our experimental conditions (the technical

set-up should be optimised in order to simultaneously quantify this amine) and thus we focused on the involvement of σ_1R antagonism in DH NA levels.

Neurochemical results revealed that in formalin-treated rats systemic administration of the selective S1RA increased spinal NA levels, reaching a maximum effect of 181% over baseline values 30 minutes after the administration, while NA levels in formalin vehicle-treated animals receiving formalin were unchanged (*Article 3*). On this basis, we suggest that S1RA-mediated analgesic effect in the formalin-induced pain model involves activation of the spinal noradrenergic system. This is reinforced by two results:

- NA enhancement was concomitant with the behavioural antinociception.
- The systemic antinociceptive effect of S1RA was antagonized by the i.t. administration of the α_2-AR blocker idazoxan. In contrast, i.t. treatment with the non-selective opioid receptor antagonist naloxone failed to modify S1RA antinociception.

The mechanism by which the antagonism on σ_1R modulates NA levels is presently unknown. Only few reports are available exploring this issue. In rat hippocampal slices some studies have described that DHEA-S (endogenous σ_1R agonist) (Monnet *et al.* 1995) and (+)-pentazocine (Monnet *et al.* 1996) enhance the NMDA-stimulated NA release, which do not fit with our observations showing also an enhanced NA release but using a σ_1R antagonist. Multiple methodological (e.g., *in vitro vs. in vivo*) and regional differences (hippocampus *vs.* spinal cord) could account for the discrepancy. Possible potential explanations for this S1RA-mediated increase in NA levels can be proposed (Fig. 13):

- S1RA could directly enhance NA release acting at the DH level.
- S1RA could inhibit the enzymes responsible of the NA degradation such as monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT).
- Although S1RA does not show affinity for the NA reuptake transporter (NET), an indirect σ_1R inhibitory activity of the NET could not be discarded.
- S1RA could act supraspinally to promote NA release in the DH by activating descending inhibitory pathways.

In any case, σ_1R antagonism produces an effect that is comparable from the neurochemical point of view (increase of NA in the spinal cord) and in terms of behavioural outcome (analgesia) to that produced when the descending inhibitory pathway is activated. Therefore, we hypothesize that S1RA treatment may be a valuable

approach in restoring the disrupted balance produced under chronic pain conditions. A number of pain killers such as morphine, pregabalin and gabapentin have shown to enhance NA spinal levels, and this enhancement has been linked to the antinociceptive/antiallodynic/antihyperalgesic effect of these treatments in acute or chronic (i.e., neuropathic) pain conditions, usually via activation of α_2-AR (Takeuchi *et al.* 2007; Hayashida *et al.* 2007). Lot of studies show that spinal application of NA (Sullivan *et al.* 1992; Yaksh *et al.* 1995; Eisenach *et al.* 1996; Honoré *et al.* 1996; Millan *et al.* 1997; Supowit *et al.* 1998; Shinomura *et al.* 1999) and i.t. administration of selective α_2-AR agonists such as clonidine are antinociceptive while i.t. treatment with selective α_2-AR antagonists attenuate descending inhibition of pain (Green *et al.* 1998; Millan 2002). α_2-AR are present at high density in the superficial laminae of the DH (Nicholas *et al.* 1993). From a mechanistic point of view, α_2-AR agonists produce antinociception by acting on both presynaptic and postsynaptic receptors, basically by reducing the release of Glu and SP from central afferent terminals in the spinal cord (Kuraishi *et al.* 1985b; Ueda *et al.* 1995) and by hyperpolarizing DH neurons (North and Yoshimura 1984; Wolff *et al.* 2007). Our results (increased NA but reduced Glu and no changes in GABA levels, and reversion of the analgesic effect by i.t. administration of the α_2-AR blocker idazoxan) clearly point to a regulation by σ_1R antagonism whereby inhibitory NAergic, but not GABAergic, signalling is increased and excitatory Glu signalling is inhibited in the DH of the spinal cord (Fig. 13)

Moreover, clonidine enhances the neuropathic pain-relieving action of the NMDAR antagonist MK-801 (Jevtovic-Todorovic *et al.* 1998) suggesting that α_2-AR agonism modulates the abnormal excitability of spinal NMDAR in neuropathic rats. Further studies showed that activation of spinal α_2-AR reduces the i.t. NMDA-induced increase on spinal NR1 subunit phosphorylation and nociceptive behaviours in the rat (Roh *et al.* 2010) and that σ_1R antagonists reduce spinal NR1 subunit phosphorylation (Kim *et al.* 2006), which is known to play a major role in Glu-mediated sensitization phenomena.

All these data in combination with the current thesis studies would argue that the antinociceptive effect of σ_1R antagonism may also be linked to its ability to modulate the pain-induced enhancement of spinal NMDAR activity through a mechanism involving α_2-AR agonism. In this way, α_2-AR is a G_i-protein coupled receptor that, when activated, exerts its effects partially suppressing both the production of cAMP and the PKA activity (Karim and Roerig 2000; Smith and Elliott 2001), and it is known that NMDA current itself is dependent on PKA activity (Liu *et al.* 2007) (Fig. 14).

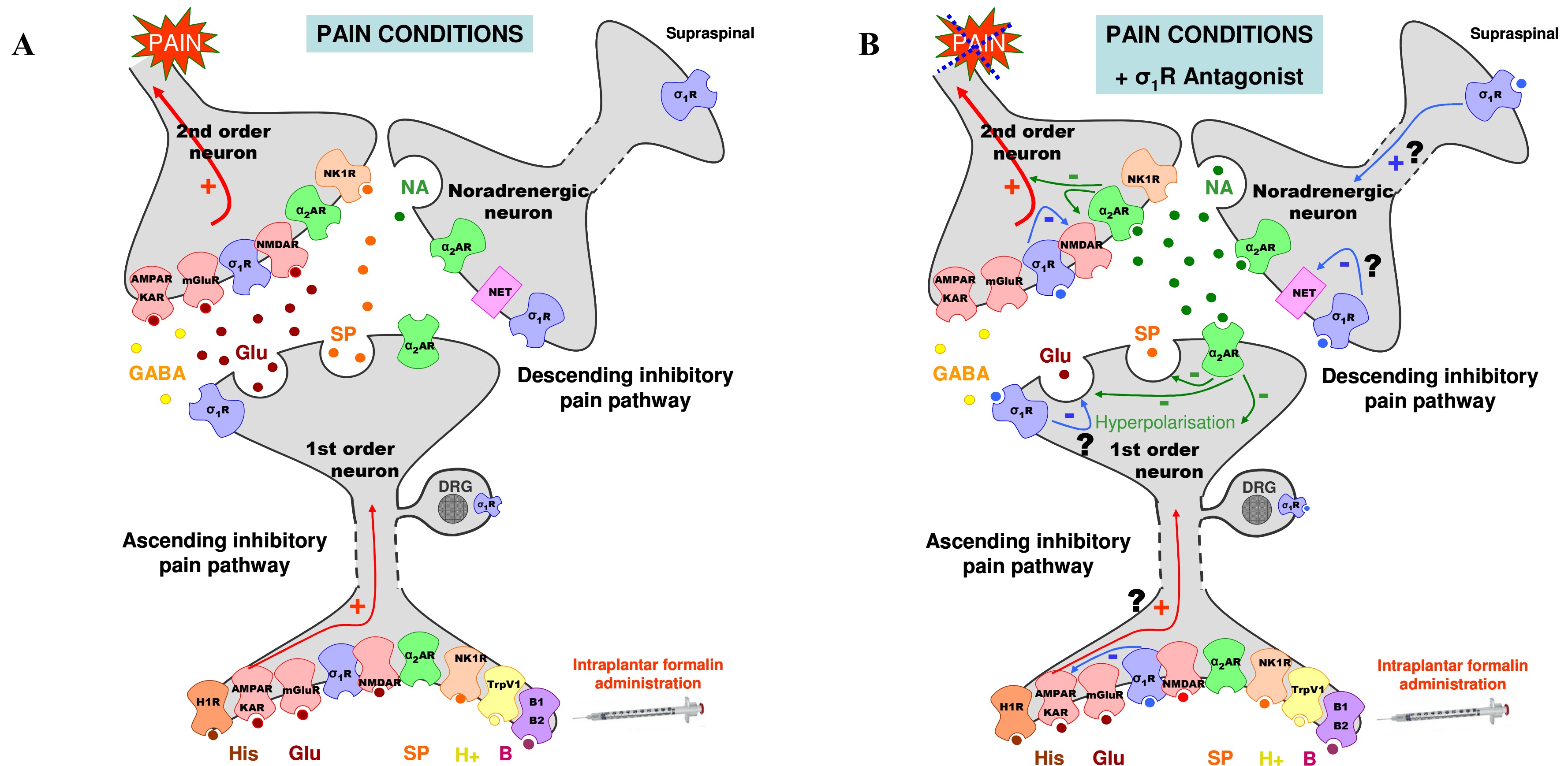
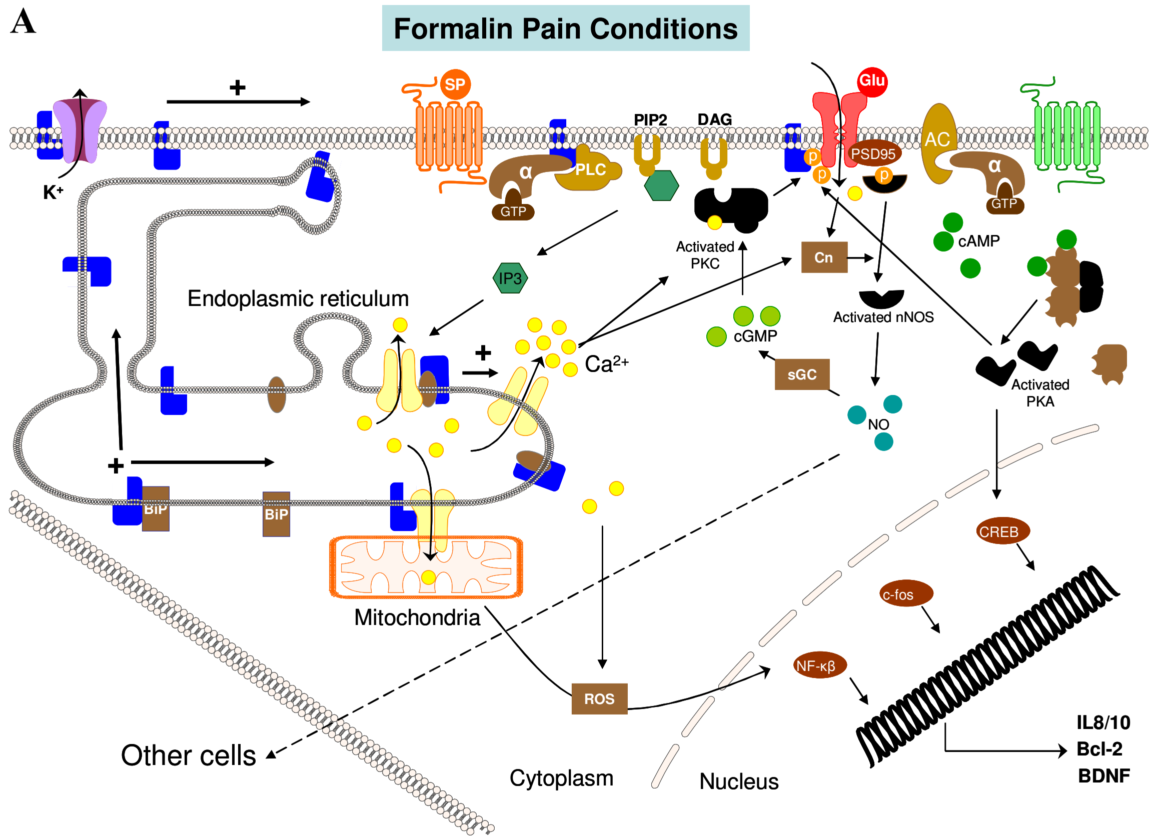
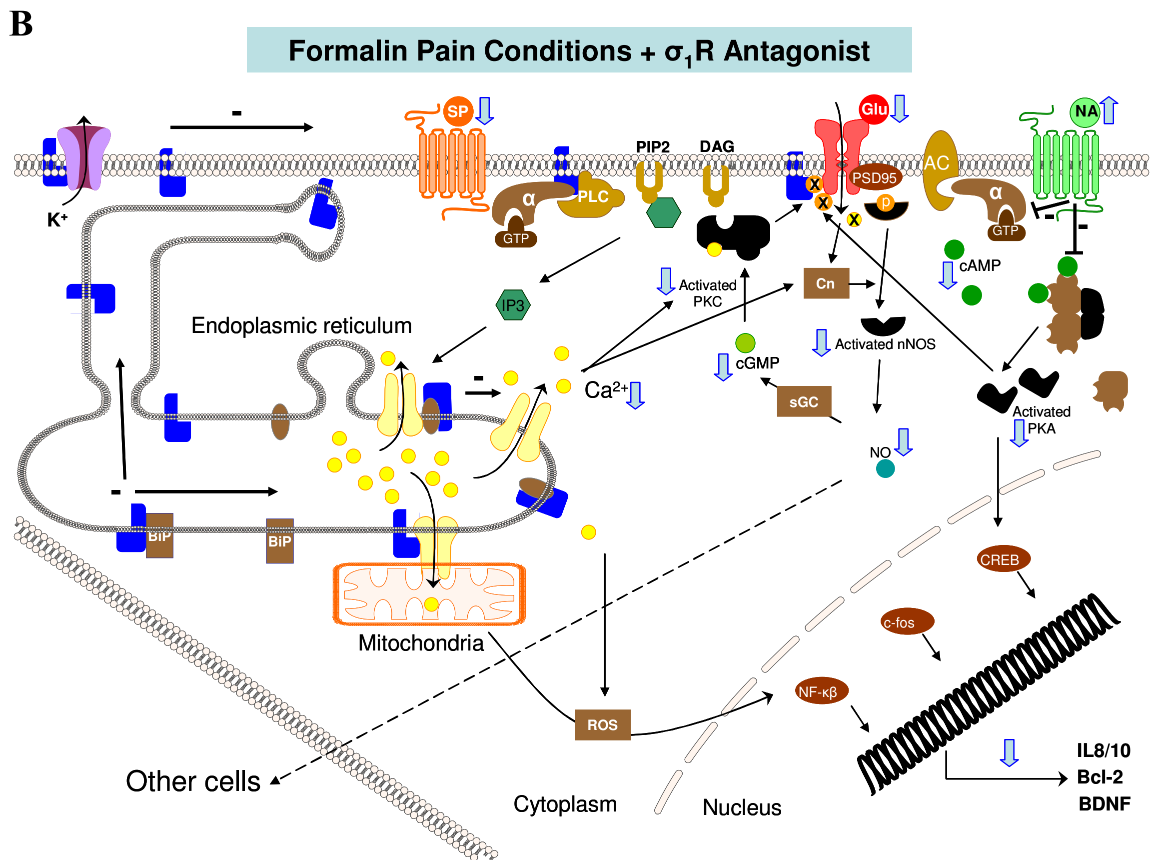


Fig. 13. We suggest that, at least, two major pathways are involved in the mechanism of action of σ_1R antagonism in the formalin-induced pain: the inhibition of the spinal excitatory synaptic transmission (Glu levels reduction) and the activation of descending inhibitory systems (NA levels enhancement). Regarding DH Glu levels reduction, we hypothesize that σ_1R antagonism reduces the formalin-induced increase on Glu levels by 1) a direct σ_1R-mediated inhibition of Glu release from the central DRG endings (modulated by σ_1R located presynaptically at the DH central endings or/and postsynaptically at the peripheral endings, which would equally involve hyperpolarisation of the first order neuron); or/and 2) an indirect presynaptic, NA-mediated inhibition of Glu release from central afferent endings through presynaptic α_2-AR. This inhibition on Glu release would result in lower activation of NMDAR in postsynaptic second order neurons transmitting pain to upper CNS areas. Regarding DH NA levels increase, we hypothesize that the σ_1R antagonism-induced enhancement of NA levels could be a consequence of direct σ_1R-mediated 1) increase of NA release, 2) NA degradation inhibition, 3) inhibition of NET or/and 4) activation of supraspinal NAergic neurons projecting to the DH. Accordingly, increased NA spinal levels are known to produce antinociception via activation of α_2-AR located presynaptically in primary central afferents (which ultimately results in a reduction of the release of Glu and SP from the central endings) and by postsynaptic activation of α_2-AR located on second order DH neurons (hyperpolarizing DH neurons and reducing the NMDAR-induced increase of NR1 subunit phosphorylation).

A



B



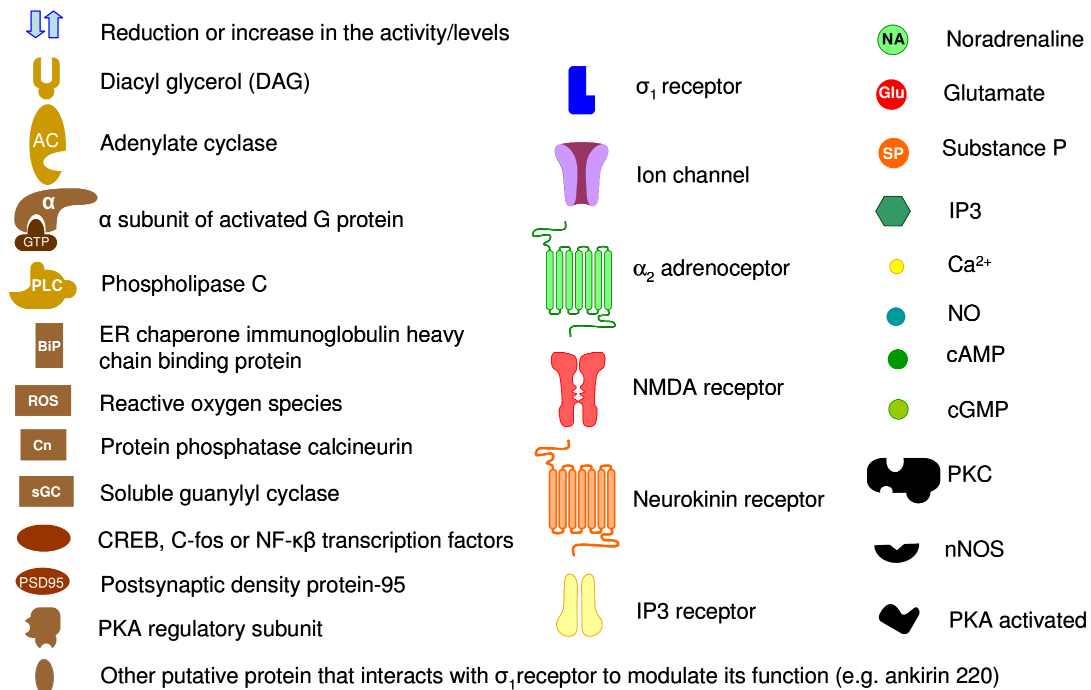


Fig. 14. **Under pain conditions,** as those after formalin administration, nociceptive mediators released from primary afferents in the DH (SP, bradykinin…) activate GPCRs and stimulate PLC enzymes to hydrolyze PIP_2 to produce DAG and IP_3. At the ER, activated σ_1R dissociates from BiP promoting its chaperone activity and redistribution to peripheral membranes. When activated, σ_1R in the ER binds to IP_3R to enhance Ca^{2+} influx into mitochondria and efflux into the cytosol. Raises of cytosolic Ca^{2+} are also produced by Ca^{2+} influx through NMDAR after activation by Glu. Cytosolic Ca^{2+} increase activates PKC (which phosphorylate NR1 subunit of NMDAR) and reduces nNOS phophorylation, resulting in increased NO, which additionally stimulates PKC activity. NO also diffuses to other cells facilitating pain. At the plasma membrane, σ_1R regulates the activity signal transduction components like PLC and PKC, K^+ and Ca^{2+} ion channels, NMDA, DA, GABA, and µ receptors. At the nucleus, σ_1R activation modulates transcriptional regulation of gene expression of the antiapoptotic protein Bcl-2, BDNF or IL 8-10 by NF-κβ, CREB, and c-fos, respectively. In summary, after its activation, σ_1R interacts with several molecular targets to ultimately facilitate pain signalling and sensitization at the DH neuronal level.
Treatment with σ_1R antagonist reduces spinal formalin-induced Glu release and increases NA levels at the DH. The Glu reduction would result in less activation of NMDAR and consequently lower cytosolic Ca^{2+} levels. σ_1R antagonism would also result in a reduced Ca^{2+} mobilization from ER stores (via PLC and IP_3R) after activation of some membrane GPCRs (e.g., neurokinin receptors activated by SP). The inhibition of cytosolic Ca^{2+} raises subsequent to reduced extracellular entry through NMDAR and ER mobilization through IP_3R ultimately results in inhibition of Ca^{2+}–dependent intracellular effectors such as PKC. Accordingly, σ_1R antagonists would reduce NMDAR sensitization by inhibition of PKC–dependent NR1 subunit phosphorylation. The increased NA levels would also contribute to reduce NMDAR sensitization by preventing PKA–dependent NR1 subunit phosphorylation via α_2-AR activation (α_2-AR is a G_i-protein whose activation reduces the activity of AC and thus reduces production of cAMP and PKA activity). Altogether, σ_1R antagonism would avoid upregulation of Ca^{2+}– and cAMP–dependent sensitizing intracellular cascades, which would reduce kinase-mediated receptor sensitization and transcriptional activation of key gene products involved in pain and hypersensitivity states. (Adapted from Zamanillo *et al.* 2013).

On the other hand, we next examined the possibility that spinal NA modulation could also be involved in the potentiation of opioid antinociception. Subactive doses of S1RA and morphine, when combined, produced an enhancement of opioid antinociception in the tail-flick test, but failed to modify NA concentration respect to baseline levels (*Article 4*). Therefore, potentiation of opioid antinociception did not correlate with an enhancement of NA levels in the DH of the spinal cord, which discards the change in spinal NA levels as a key mechanism underlying σ_1R antagonism-mediated potentiation of opioid antinociception in the reflex tail-flick response to acute thermal stimulation. This contrasts to previous findings suggesting that increased NA levels lie behind the antinociceptive effect of S1RA in the formalin test (*Article 3*). In fact, morphine produced a dose-dependent antinociceptive effect without concomitant increase of NA spinal levels, and S1RA (80 mg/kg) *per se* induced a NA enhancement but failed to evoke antinociceptive effects in the tail-flick test. It is well documented that morphine exerts its antinociceptive effects in part by a hyperpolarization of both the presynaptic primary afferent C-fibre terminals and the postsynaptic ascending projection neurons (Fleetwood-Walker *et al.* 1985; North *et al.* 1987), and by the activation of the descending NA inhibitory pathway (Wigdor and Wilcox 1987; Ossipov *et al.* 1989; Tseng and Tang 1990; Ohsawa *et al.* 2000). Altogether, our results do not substantiate a major role for increased NA levels in the DH in morphine antinociception in the tail-flick test. In addition, although S1RA treatment results in an enhancement on spinal NA, it seems insufficient to produce antinociception in the tail-flick test.

5.4 Site of action of σ_1R blockade

As we have already mentioned, the analgesic effect of σ_1R antagonists has been studied mainly on the spinal cord as the primary site of action in central sensitization (Kim *et al.* 2006, 2008; de la Puente *et al.* 2009; Romero *et al.* 2012). However, little is actually known about the site(s) of action of σ_1R antagonists. Therefore, in another set of experiments, we investigated the site of action of S1RA.

The σ_1R is expressed in key areas for pain control such as the two superficial layers of the DH (in dendritic processes and neuronal perikarya), DRG, PAG, LC and RVM (Walker *et al.* 1992; Alonso *et al.* 2000; Kitaichi *et al.* 2000; Palacios *et al.* 2003; Phan *et al.* 2005; Bangaru *et al*. 2013). In the present thesis, the effect of S1RA on the behavioural responses in the tail-flick and formalin tests was assessed in rats by using

different routes of administration, including intrathecal (i.t.), intracerebroventricular (i.c.v.), locally in the RVM and intraplantar (i.pl.) (Table 7).

5.4.1 Spinal involvement of σ_1R

There are two possible technical approaches for i.t. administration in rats: the direct lumbar puncture and the indwelling catheter methodology (Fairbanks 2003). Although the first one is rapid and avoids the impact of surgical procedures, its inability to conduct post-hoc verification of the injection site and the need for using anaesthesia, that could interfere the effect of the compounds under study, inclined us to choose the catheter approach.

S1RA administered i.t. failed to enhance opioid antinociception in the tail-flick test (*Article 4*) while attenuated the flinching behaviour (phase I and II) in the formalin model in rats (*Article 3*). Interestingly, in the formalin-induced pain model, i.t. S1RA dose-dependently reduced the number of flinches but it was not able to attenuate the lifting/licking time parameter. These results can be easily reconciled if we consider that lifting/licking behavioural response requires supraspinal integration, whereas flinching behaviour is essentially a spinal response that does not require the integrative action of higher brain centres. This is supported by a study using spinal transected rats (spinalized at the mid-thoracic level), where it was determined that the flinching behaviour is organized at the lumbar spinal level (Coderre *et al.* 1994). Accordingly, σ_1R antagonists acting locally at the spinal cord level seem to modulate the spinal reflex output but not the motorneuron responses evoked by descending, supraspinally processed outputs. This fits well with data in the formalin test, but i.t. S1RA did not inhibit the tail withdrawal response in the tail-flick test (*Article 4*), which is also considered to be a spinal response (Irwin 1962). Differences in the nociceptive stimuli (thermal *vs.* chemical), which recruit different spinal pathways/mechanisms being differentially regulated (or not regulated at all) by σ_1R, could provide an explanation. In this way, i.t. administration of the σ_1R antagonist BD-1047 is known to attenuate mechanical allodynia but not thermal hyperalgesia in a neuropathic pain model (Roh *et al.* 2008). Alternatively, the difference could be related to the duration of the stimulus as thermal stimulation in the tail-flick test evokes immediate withdrawal/guarding responses whereas formalin-induced pain, even phase I, lasts for some minutes, and thus some degree of sensitization may occur, this giving to σ_1R antagonists the opportunity to exert an effect. Finally, in contrast to our data showing that i.t. S1RA inhibited the flinching (in both phases) but not the

lifting/licking (at any phase) in rats, i.t. pretreatment with BD-1047 has been described to reduce the formalin-induced paw licking behaviour in phase II, but not in phase I, of the formalin test in mice (Kim *et al.* 2006). Species differences (mice *vs.* rat), methodological differences (e.g., lumbar puncture *vs.* catheter for i.t. administration) and/or differences in the selectivity profile of administered drugs (BD-1047 *vs.* S1RA) could account for this difference.

At the mechanistic level, blocking of spinal σ_1R by i.t. administration of σ_1R antagonists (BD-1047 or BMY-14802) has been described to reduce formalin-induced spinal Fos expression and both PKC- and PKA-dependent phosphorylation of the NMDA subunit NR1 (pNR1) (Kim *et al.* 2006). The same authors also found that i.t. σ_1R agonists enhanced, while BD-1047 reduced, the phosphorylation of the NMDA subunit NR1 (pNR1) in the DH of the spinal cord (Kim *et al.* 2006, 2008), which was correlated to the effect on NMDA-induced pain behaviour (Kim *et al.* 2008). Altogether, results in the behavioural tests could be explained by the ability of σ_1R to modulate, at the postsynaptic level, NMDA-mediated responses in the spinal cord. Further studies showed that the role played by spinal cord σ_1R in the formalin test can also be applied to chronic pain conditions (Roh *et al.* 2008). In the chronic constriction injury model of neuropathic pain, the i.t. administration of the σ_1R antagonist BD-1047 attenuated the development of mechanical allodynia, but not thermal hyperalgesia, concurrent with an inhibition of nerve injury-induced NR1 expression and phosphorylation (Roh *et al.* 2008). In addition, it is known that NMDA and σ_1 receptors are functionally coupled and that σ_1R agonists raises cytosolic Ca^{2+} concentration by potentiating Ca^{2+} entry (via NMDA-induced Ca^{2+} influx) and Ca^{2+} mobilization from endoplasmic stores (via PLC and IP_3-induced Ca^{2+} mobilization). This Ca^{2+} augment activates Ca^{2+}-dependent second messengers including PKC and other CaMK's that ultimately account for the plastic changes underlying spinal sensitisation and pain hypersensitivity (Hua *et al.* 1999; Fang *et al.* 2002; Brenner *et al.* 2004; Kawasaki *et al.* 2004). The Ca^{2+} augment can also initiate the intracellular ERK activation in neurons of the superficial DH, which is followed by a widespread sequential induction in spinal microglia and astrocytes (Ma and Quirion 2005; Zhuang *et al.* 2005). Activated glia produce inflammatory mediators that sensitize DH neurons. Interestingly, increased ERK activation has been shown in spinal cords of WT but not σ_1R-KO mice exposed to sciatic nerve injury (de la Puente *et al.* 2009). Neuronal NOS (nNOS) also plays an important role in modulating synaptic transmission in both CNS and PNS (Meller and Gebhart 1993). Moreover, it has been

shown that NO produced by nNOS in spinal cord participates in the early induction (Levy and Zochodne 2004) and/or maintenance of neuropathic pain (Xu *et al.* 2007). Interestingly, Ca^{2+}-dependent second messengers cascades involving an increase in nNOS activity (nNOS possesses several putative sites for phosphorylation by Ca^{2+}/calmodulin-dependent kinases) are associated with an NO–induced increase in PKC-dependent phosphorylation of NR1, which has been suggested to mediate spinal σ_1R-induced sensitisation (Roh *et al.* 2011). In addition, recent studies suggest that the σ_1R plays an important role in the activation of p38 MAPK signalling pathway and in the modulation of the NADPH oxidase 2 and through this cascades contributes to the induction of the neuropathic pain (Moon *et al.* 2013; Choi *et al.* 2013).

In summary, the results described above support a modulatory role for σ_1R in spinal sensitisation, acting as postsynaptic regulators of NMDAR and Ca^{2+}-dependent cascades. The blockade of σ_1R might reduce injury-induced intracellular Ca^{2+}-dependent activity, which would result in a reduction of central sensitisation and pain hypersensitivity phenomena (See Fig. 14 for a schematic summary of putative mechanisms involving the effect of σ_1R antagonism).

Apart from the capacity of σ_1R to modulate postsynaptically NMDA-mediated responses in the spinal cord, we know based on neurochemical studies that S1RA is able to act presynaptically to attenuate the enhancement of DH Glu release secondary to intraplantar formalin injection. This can also be a key mechanism to inhibit NMDAR sensitization (phosphorylation) and Ca^{2+}-dependent cascades, but further studies are needed to actually know if the inhibition of Glu release in the DH exerted by systemically administered S1RA is due to an inhibition at the central nociceptor endings in the DH or if it occurs at the peripheral nociceptor endings (inhibition of ascending pathway) or supraspinally (activation of the descending inhibitory pathway) (Fig. 13). This latter possibility fits well with the increased NA levels and the involvement of spinal α_2-AR described in the previous section (see section *5.3* in this thesis).

On the other hand, i.t. S1RA failed to enhance systemic morphine antinociception. Although the involvement of spinal σ_1R on the modulation of opioid antinociception is poorly assessed in literature, our results are consistent with a previous study wherein the i.t. administration of the σ_1R agonist (+)pentazocine did not modify the antinociceptive effect of i.t. morphine (Mei and Pasternak 2002).

5.4.2 Supraspinal involvement of σ_1R

We next assessed the supraspinal involvement of σ_1R. In the formalin-induced pain model, i.c.v. S1RA attenuated both the flinching behaviour in phase II and the lifting/licking behaviours in phase I and II (*Article 3*). This is the first report describing a role for supraspinal σ_1R in this model. In line with our results, i.c.v. BD-1047 has been shown to exert an antinociceptive effect on the capsaicin-induced headache model (Kwon *et al.* 2009).

The observation that i.t. S1RA did not modify the lifting/licking time following formalin administration whereas i.c.v. S1RA was able to reduce it agrees with the need for supraspinal integration of the lifting/licking behaviour and a supraspinal action of σ_1R antagonists, as previously commented. Accordingly, σ_1R antagonists seem to modulate spinal reflex outputs (i.e., flinching) at the spinal cord level whereas they modulate supraspinally the responses requiring supraspinal integration (i.e., lifting/licking). Phase II (but not phase I) flinching behaviours were also attenuated by i.c.v. S1RA, which suggests that, although being primarily processed at the spinal cord level, descending σ_1R-mediated supraspinal modulation is possible, perhaps because supraspinal sensitization occur in phase II (but not in phase I) and this gives to σ_1R antagonists the opportunity to exert an effect.

On the other hand, i.c.v. S1RA enhanced systemic morphine antinociception in the tail-flick test (*Article 4*), which is consistent with the enhancement of systemic morphine effects by i.c.v. σ_1R antisense treatment or the diminished opioid antinociception found after supraspinal administration of (+)pentazocine in mice (Mei and Pasternak 2002). Apart from studying μ-opioid modulation, Pasternak's group also confirmed a diminution or enhancement on δ, κ_1, and κ_2 opioid antinociception in CD-1 mice by i.c.v. (+)pentazocine or antisense treatment, respectively, with a trend for the κ drugs to be more sensitive to (+)pentazocine. These observations confirm the importance of supraspinal σ_1R as a modulatory system influencing the analgesic activity of different opioid drugs.

The particular supraspinal site where σ_1R exerts its modulatory effect on opioid antinociception just starts to be clarified. PAG, LC and RVM, areas with σ_1R expression (Walker *et al.* 1992), have been identified as potent morphine-sensitive sites (Rossi *et al.* 1993, 1994). Morphine antinociception was found when microinjected in all three regions and it was lowered by co-administration of low doses of (+)-pentazocine in the

three regions (although the PAG was far less sensitive than the others) implying a highly sensitive σ_1 system, but only the RVM seems to exert a tonic σ_1 activity based upon the ability of the σ_1R antagonist haloperidol and the antisense treatment to enhance morphine actions (Mei and Pasternak 2007). Our microinjection studies revealed that S1RA administered into the RVM fails to modify the tail-flick latency when given alone and also fails to enhance the effects of morphine (*Article 4*). Thus, our results (absence of effects of S1RA when given into the RVM) strongly suggest that the σ_1R system in this brainstem region (RVM) does not modulate systemic morphine antinociception. In contrast to the study of Mei and Pasternak (2007), in which morphine was microinjected together with the σ_1R ligand, in our experiment morphine was systemically administered. This and other methodological differences (e.g., use of different σ_1R ligands) could explain the apparently discrepant results, that otherwise seem difficult to reconcile.

5.4.3 Peripheral involvement of σ_1R

We next characterized the effects of peripheral σ_1R blockade both in the modulation of peripheral opioid thermal antinociception (*Article 4*) and in the formalin-evoked pain (*Article 3*).

In the formalin-induced pain model we assessed the effects of peripheral σ_1R antagonism through i.pl. administration of S1RA and the results showed that S1RA attenuates the lifting/licking (but not flinching) behaviours evoked by formalin administration in phase I and II, indicating that peripheral σ_1R is also involved in this type of pain. These results are in accordance with a report showing that i.pl. (+)-pentazocine elicited nociceptive flexor responses (Ueda *et al.* 2001). We have no explanation for the differential effect on lifting/licking *vs.* flinching behaviours, unless primary afferent fibres that perceive and convey the nociceptive information evoking both behavioural responses are different.

Because only very scarce literature are available reporting a direct administration of compounds in the tail to study local effects (Kolesnikov *et al.* 1996; Dogrul *et al.* 2007), we dealt with another approach for assessing peripheral modulation of opioid antinociception. Loperamide is an opioid that, in contraposition to morphine, it does not cross the blood-brain barrier in appreciable amounts when systemically administered (Heykants *et al.* 1974; Schinkel *et al.* 1996). Systemic loperamide did not exert

antinociceptive effects in the tail-flick test, as previously described (Wüster and Herz 1978; Niemegeers *et al.* 1979; Menéndez *et al.* 2005; Sevostianova *et al.* 2005) and consistent with the view that analgesic effects of opioids on acute pain are primarily mediated through receptors located in the CNS (Yaksh and Rudy 1978; McNally 1999). However, loperamide produced an antinociceptive effect when combined with S1RA, suggesting that the peripheral σ_1R plays an important role on opioid thermal antinociception. In addition, these effects were reversed by systemic naloxone-methiodide, an opioid antagonist having weak penetration to the brain (Russell *et al.* 1982). These results are in accordance with a recent work that reported an important role for peripheral σ_1R antagonism on morphine-induced mechanical antinociception (Sánchez-Fernández *et al.* 2013a). Altogether, these data indicate that the tonic inhibition of morphine analgesia by σ_1R is also present at the periphery and is involved in both thermal and mechanical nociceptive pain (*Article 4*, Sánchez-Fernández *et al.* 2013a). It seems thus clear that the σ_1R exerts an inhibitory role precluding opioid-induced peripheral antinociception and that σ_1R antagonism "releases the brake" enabling opioids to exert clear cut antinociceptive effects by acting at the periphery.

After studying the local effects of S1RA, our results suggest that the behavioural effect observed after a systemic administration was the sum/interaction of simultaneous local effects (supraspinal, spinal and peripheral) in the formalin model and in opioid combination studies (Table 7).

Table 7. Summary of the effects of local σ_1R antagonist (S1RA) treatment on flinches and lifting+licking formalin-induced pain behaviours and on the modulation of opioid-induced antinociception in the tail-flick test. ✓: effect; ✗: no effect.

Treatment	Formalin model		Opioid-induced antinociception. Tail-flick test
	Flinches	Lifting + Licking	
Systemic	✓	✓	✓
Spinal	✓	✗	✗
Supraspinal	✓	✓	✓
Peripheral	✗	✓	✓

6. CONCLUSIONS

The conclusions of this thesis can be summarized as follows:

1. Selective σ_1R antagonists *per se* fail to exert antinociceptive effects but enhance the antinociceptive potency of several opioids in the acute thermal model of tail-flick in mice and rats.

2. The absence of σ_1R in σ_1R-KO mice does not modify the sensitivity to noxious heat stimulation and to opioid antinociception in our experimental conditions (tail-flick in mice). Therefore, σ_1R-KO mice become a valuable *in vivo* genetic tool to evaluate the unambiguous involvement of σ_1R in the modulation of opioid antinociception by drugs.

3. S1RA fails to potentiate morphine antinociception in the tail-flick test in σ_1R-KO mice suggesting that σ_1R actually mediates the enhancing effect of S1RA.

4. S1RA is able to restore morphine antinociception in tolerant mice.

5. The modulatory effect of σ_1R on opioid antinociception can be dissociated from other opioid-induced effects: S1RA reverses the reward effects of morphine and it does not modify the development of analgesic tolerance, physical dependence, constipation and mydriasis produced by morphine.

6. Antagonism at σ_1R *per se* elicits a dose-related diminution of formalin-induced pain behaviours in phase I (acute pain) and phase II (sensitization pain) in rats.

7. The concentric microdialysis technique (successfully set-up and validated as a part of this thesis) allows studying neurochemical modulation induced by pain and analgesics in the ipsilateral spinal cord of freely-moving rats. In particular, it enables the *in vivo* concomitant assessment of behavioural nociception and intra-dorsal horn microdialysis sampling in conscious animals.

8. Neurochemical studies using the concentric microdialysis approach in the ipsilateral dorsal horn revealed that systemic administration of a selective σ_1R antagonist results in inhibition of formalin-evoked Glu release, no modification of GABA levels, and enhancement of NA levels. This increased spinal NA activates spinal α_2-AR producing the attenuation of the formalin-induced pain behaviour as intrathecal pre-treatment with idazoxan, but not naloxone, prevents the systemic S1RA antinociceptive effect. These data clearly point to a regulation by σ_1R antagonism whereby inhibitory NAergic, but not GABAergic, signalling is increased and excitatory Glu signalling is inhibited in the DH of the spinal cord.

9. Neurochemical studies using the concentric microdialysis approach in the ipsilateral dorsal horn also revealed that σ_1R antagonism in combination with morphine does not modify spinal NA levels. Thus, the antinociceptive enhancement effects observed in the tail-flick test by the co-administration of σ_1R antagonists and opioids seem not to be mediated by increased NA modulation.

10. There are different sites of action for selective σ_1R blockade to produce antinociception. The modulation of opioid-induced thermal antinociception by selective σ_1R antagonists involves supraspinal and peripheral, but not spinal, σ_1R actions. In contrast, selective σ_1R antagonism *per se* exerts supraspinal, spinal and peripheral antinociceptive effects in the formalin-induced pain model, with only the supraspinal site(s) accounting for both spinal (i.e., flinches) and supraspinal (i.e., lifting and licking) integrated pain responses.

11. **The use of selective σ_1R antagonists represents a promising pharmacological strategy as opioid adjuvants and in monotherapy for the treatment of diverse types of pain.**

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