Characterization of the 5-HT$_7$ receptor as a new therapeutic target for the treatment of pain

Àlex Brenchat Barberà

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This Thesis adds better knowledge to the role of 5-HT$_7$ receptors in the control of pain and point to a new potential use of 5-HT$_7$ receptor agonists as promising drugs for the treatment of neuropathic pain.

ESTEVE

UNIVERSITAT DE BARCELONA

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Doctoral Thesis 2012

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Departament de Bioquímica i Biologia Molecular

Facultat de Biologia

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Facultat de Biologia, Universitat de Barcelona

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Caracterització del receptor 5-HT$_7$ com a diana terapèutica pel tractament del dolor

Memòria per optar al grau de
Doctor per la Universitat de Barcelona

Els resultats experimentals d'aquesta Tesi han estat obtinguts a
Laboratorios Dr. Esteve

Àlex Brenchat Barberà

Autor

Dr. José Miguel Vela Hernández
Director

Dra. Luz Romero Alonso
Codirectora

Dr. Antonio Zorzano Olarte
Tutor

Dedicat a la meva família i amics

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"The most beautiful thing we can experience is the mysterious. It is the source of all true art and all science. He to whom this emotion is a stranger, who can no longer pause to wonder and stand rapt in awe, is as good as dead: his eyes are closed."

"La cosa més bonica que podem experimentar és el misteri. És la font de tot veritable art i ciència. El qui no experimenta aquesta emoció, qui no es meravella ni s'entusiasma és com estar mort: té els ulls tancats."

Albert Einstein

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Abbreviations:

2-Br-LSD: 2-bromo-D-lysergic acid diethylamide
5-CT: 5-carboxamidotryptamine
5-HT: serotonin, 5-hydroxytryptamine
5-MeOT: 5-methoxytryptamine
8-OH-DPAT: 8-hydroxy-2-(di-n-propylamino)tetralin
AC: adenylyl cyclase
AI-AHR: antigen-induced airway hyperresponsiveness
ATP: adenosine triphosphate
BDNF: brain-derived neurotrophic factor
cAMP: 3'-5' cyclic adenosine monophosphate
CB: cannabinoid
CCI: chronic constriction injury
cDNA: complementary deoxyribonucleic acid
CFA: complete Freund's adjuvant
CGRP: calcitonin gene-related peptide
CNS: central nervous system
CRPS: complex regional pain syndrome
DH: dorsal horn
DRG: dorsal root ganglia
ED_{50}: effective dose 50
EFS: electrical field stimulation
ERK: extracellular signal-regulated kinase
GABA: gamma-amino butyric acid
i.t.: intrathecal
KO: knockout
M3G: morphine-3-glucuronide
MAPK: mitogen-activated protein kinase
MIA: monoiodoacetate
mRNA: messenger ribonucleic acid
NET: norepinephrine transporter
NMDA: N-methyl-d-aspartate
NSAIDs: nonsteroidal anti-inflammatory drugs
PCP: 1-(1-phenylcyclohexyl)piperidine, phencyclidine
PET: positron emission tomography
PGE2: Prostaglandin E2
PKA: protein kinase A
PKC: protein kinase C
PLC: phospholipase C
PPI: prepulse inhibition
PSNL: partial sciatic nerve ligation
REM: rapid eye movement
RVM: rostroventromedial medulla
SCI: spinal cord injury
SCN: suprachiasmatic nucleus
SERT: serotonin reuptake transporter
SNI: spared nerve injury
SNL: spinal nerve ligation
SSRIs: selective serotonin reuptake inhibitors
TGES: trigeminal ganglion electrical stimulation
TRP: transient receptor potential
TRPV: transient receptor potential vanilloid

I. SUMMARY

The work showed in this Thesis has been part of the "5-HT_7 and neuropathic pain" project in the pharmaceutical company Esteve. Thus, the aim of this Thesis was in line with the goal of the 5-HT_7 project at Esteve, focused on drug discovery of 5-HT_7 receptor ligands for the treatment of neuropathic pain.

Taking the advantage of a genetic approach (5-HT_7 receptor knockout mice) and pharmacological tools (5-HT_7 receptor ligands) we investigated at the preclinical level the role of 5-HT_7 receptors in nociception and the therapeutic interest of 5-HT_7 receptor ligands on pain treatment. The 5-HT_7 receptor ligands used were SB-258719 and SB-269970 as 5-HT_7 receptor antagonists, and AS-19, MSD-5a, E-55888 and E-57431 as 5-HT_7 receptor agonists. E-55888 and E-57431 developed by Esteve were described for the first time and their binding profile and functionality (cAMP formation) were examined. *In vivo* behavioral studies were performed in mice and rats subjected to nociceptive, inflammatory, neurogenic or neuropathic pain conditions.

Our results showed that 5-HT_7 receptors *per se* were not involved in the nociceptive response to a normally noxius stimulus, although when co-activated together with opioid receptors potentiated the opioidergic analgesic response in nociceptive pain conditions. Indeed, 5-HT_7 receptor knockout and wild-type mice showed similar sensitivity to a noxious heat stimulus, and systemic administration of the 5-HT_7 receptor agonist E-55888 or the 5-HT_7 receptor antagonist SB-258719 showed no effects on acute nociceptive pain using the tail flick test in mice. However, the 5-HT_7 receptor agonist E-55888 enhanced the morphine-induced analgesia in this test and this potentiation was significantly reversed by the 5-HT_7 receptor antagonist SB-258719.

On the other hand, we studied the role of 5-HT_7 receptors in pain conditions involving central sensitization. We showed that the 5-HT_7 receptor agonists AS-19, E-55888 and E-57431 inhibited capsaicin-induced mechanical hypersensitivity, nerve injury-induced mechanical and thermal hypersensitivity and reduced the phase II formalin-induced nociception. In contrast, a promotion of mechanical hypersensitivity after administration of the 5-HT_7 receptor antagonists SB-258719 and SB-269970 was observed. This reduction of hypersensitivity by agonists and promotion of hypersensitivity by antagonists was reversed by antagonists and agonists, respectively. It is important to note that effectiveness of the treatment with 5-HT_7 receptor agonists was not masked by non-specific motor effects, as no

motor incoordination was found in the rota-rod test at the doses used and no tolerance to the effect was evidenced following repeated systemic administrations.

The antinociceptive effects exerted by systemic 5-HT_7 receptor agonists seemed to be mediated by 5-HT_7 receptors localized in the spinal cord. We found that intrathecal administration of the 5-HT_7 receptor agonist E-57431 inhibited mechanical hypersensitivity secondary to capsaicin injection and nerve injury-induced mechanical hypersensitivity. In contrast, a pronociceptive effect was observed after local intraplantar injection of the selective 5-HT_7 receptor agonist E-57431 in the capsaicin model. Thus, the antinociceptive role mediated by central 5-HT_7 receptors seems to predominate over their pronociceptive role at the periphery, resulting in an overall analgesic effect when 5-HT_7 receptor agonists are administered by a systemic route. In line with these spinal antinociceptive effects, we found an increased immunoreactivity of 5-HT_7 receptors in the ipsilateral dorsal horn of the spinal cord in sciatic nerve-injured mice. This increased 5-HT_7 receptor expression in the dorsal horn induced by nerve injury could represent a physiological, compensatory, protective spinal mechanism relevant to the control of nociception in neuropathic pain conditions.

We observed that 5-HT_7 receptors co-localized with GABAergic neurons in the ipsilateral dorsal horn of the spinal cord. Therefore, we suggested that an indirect action through activation of 5-HT_7 receptors localized on inhibitory interneurons may be responsible of the antinociceptive effects observed after administration of 5-HT_7 receptor agonists.

Finally, using 5-HT_7 receptor knockout mice, we demonstrated that the 5-HT_7 receptor agonists AS-19, E-55888 and E-57431 exerted *in vivo* target specific effects on pain control. We observed that systemic administration of these 5-HT_7 receptor agonists reduced phase II formalin-induced nociception in wild-type but not in 5-HT_7 receptor knockout mice.

Taken together, these data add a piece of knowledge to the role played by 5-HT_7 receptors in the control of pain and point to a new potential use of 5-HT_7 receptor agonists as promising drugs for the treatment of neuropathic pain.

II. INTRODUCTION

2.1. Overview of pain

According to the International Association for the Study of Pain (IASP), pain is defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (IASP Pain terms, 1986). The concept of pain, and therefore its management, needs to consider the multidimensional characteristics of pain by taking into account nociception, pain perception, suffering and pain behavior (Loeser and Melzak, 1999).

Pain is a complex, intricate neurochemical process involving neurotransmitter and other molecules acting in both peripheral and central pain signaling pathways. Serotonergic, noradrenergic and, to a lesser extent, dopaminergic networks comprize major components of inhibitory or facilitatory modulation of pain depending on the type of neuron targeted and the receptor activated (Fields and Basbaum, 1994; Millan, 1997; Suzuki et al., 2004).

Acute pain has an important physiological protective function, acting as an alarm system that notifies the body of the presence of some dangerous, noxious stimulus in the environment. When tissue becomes damaged, a region of localized hypersensitivity surrounding the injured part can appear. This hypersensitivity minimizes movement of, or contact with, the injury until healing has occurred. However, in some cases, the so-called chronic pain occurs, which does not seem to have any beneficial function but results from cellular disturbances that provoke maladaptive changes in the neurons of the sensory system. This can cause spontaneous, persistent pain and severe hypersensitivity.

2.1.1. Pain pathways and mechanisms

Pain impulses travel from the periphery to the spinal cord through primary sensory nociceptors called first order neurons, which have their neuronal soma in the dorsal ganglia and synapse with dorsal horn neurons (second order neuron) in the spinal cord, which in turn send projections that cross to the contralateral side of the spinal cord and ascend to supraspinal areas (thalamic, limbic and cortical structures) (see figure 1). The grey matter of the spinal cord has ten laminae or layers based on the presence of different types of neurons and the place where fibers synapse (Rexed, 1952).

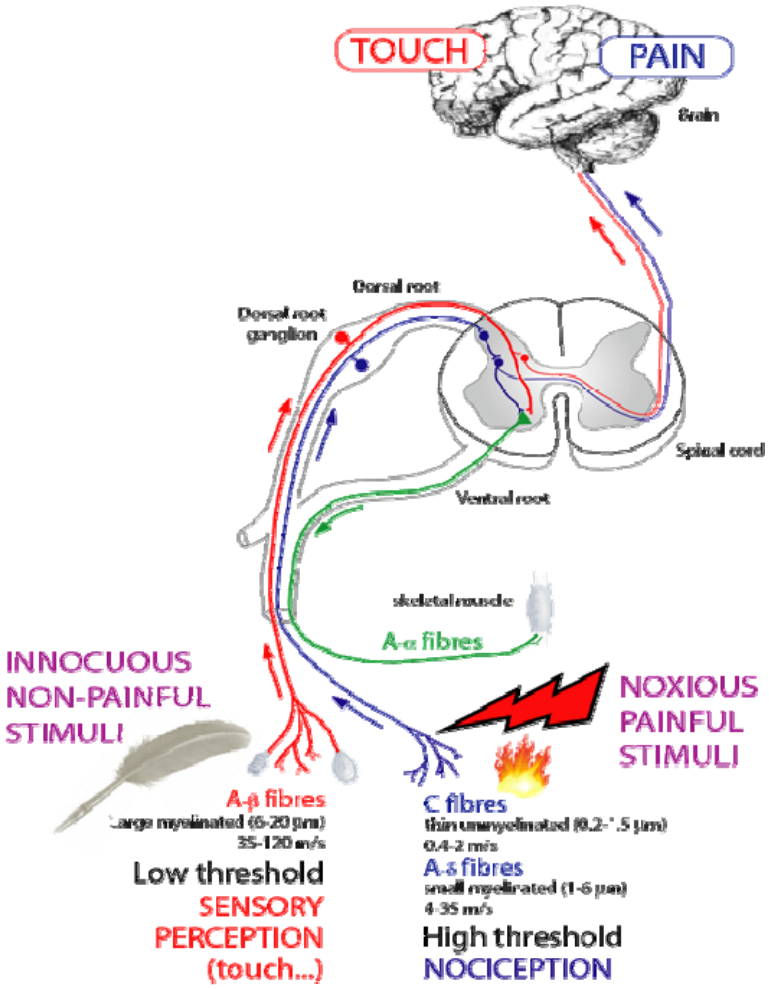


Figure 1. Graphic representation of different types of primary sensory afferents responsible to transmit peripheral information to the brain. Two main types of fibers responsible to transmit peripheral information to the brain can be distinguished. Aβ fibers are large myelinated fibers (6-20 µm) of low threshold that transmit innocuous non-painful stimuli at 35-120 m/s. They are responsible of sensory perception (touch). Nociceptive fibers include C fibers, thin unmyelinated (0.2-1.5 µm), and Aδ fibers, small myelinated (1-6 µm), that transmit noxious painful stimuli at 0.4-2 m/s and 4-35 m/s, respectively. Both are fibers of high threshold. Aα fibers have the largest diameter, the fastest conduction velocity, and they provide innervations of motor function to the skeletal muscles.

Primary sensory afferents are stimulated by biological, electrical, thermal, mechanical and chemical stimuli. They are extremely heterogeneous, differing in the neurotransmitters they contain, the receptors and ion channels they express, their speed of conduction, their response properties to stimuli, and their capacity to be sensitized during inflammation, injury, and disease. Three general types of fibers are described (Treede et al., 1992; Belmonte and Cervero, 1996) (see figure 1):

- **Aβ fibers** that transmit nonpainful stimuli and reduce pain in the dorsal horn when mechanoreceptors are stimulated. For example, if touch receptors (Aβ fibers) are stimulated, they dominate and block pain impulses. This ability to inhibit pain is the reason people immediately grab and massage the injured region. The touch blocks the transmission and duration of pain impulses. This capacity has implications for the use of touch and massage for some patients in pain.

- **Aα fibers** that provide innervations of motor function to the skeletal muscles. They have the largest diameter and the fastest conduction velocity.

- **Aδ fibers** that have lightly myelinated axons, conduct action potentials rapidly, and have medium- to large-diameter cell bodies. They produce sharp well-defined pain, called "fast pain" or "first pain", typically stimulated by a cut, an electrical shock, or a physical blow. Transmission through these fibers is so fast that the body's reflexes can actually respond faster than the pain stimulus, resulting in a retraction of the affected body part even before the person perceives pain.

- **C fibers** that have unmyelinated axons, conduct action potentials slowly, and have small-diameter cell bodies. They transmit dull burning or aching sensations, known as "second pain". These fibers are the ones that produce constant pain.

Pain perception occurs when stimuli are transmitted to the spinal cord and then to central areas of the brain where the painful information is processed. In addition to these ascending pathways, descending pathways projecting from supraspinal areas synapse to the spinal cord to modulate painful transmission (Millan, 2002). Changes in the functional properties of neurons in pain pathways are sufficient to reduce pain threshold, increase the magnitude and duration of responses to noxious input, and permit normally innocuous inputs to generate pain sensations. This enhancement in the function of neurons and circuits in nociceptive pathways caused by increased membrane excitability, synaptic efficacy and reduction of inhibition is called sensitization.

Pain is not then simply a reflection of peripheral inputs or pathology but is also a dynamic reflection of central neuronal plasticity. Sensitization of the nociceptive system allows the response to stimuli outside of the injury area and to low-threshold afferents that previously did not activate the nociceptive pain (Eide, 2000). Many patients with chronic pain do also exhibit hypersensitivity encountered, for example, while dressing.

Acute pain can cause peripheral sensitization (increase of sensitivity of nociceptive primary afferent neurons) and central sensitization (hyperexcitability of nociceptive neurons in the central nervous system). The processes of sensitization are thought to be the basis of spontaneous pain, allodynia, and hyperalgesia (see figure 2). The prevalence of continuous and spontaneous non-evoked pain is almost universal and mediated by pathophysiological mechanisms different from those that mediate hypersensitivity states (Backonja and Stacey, 2004).

Peripheral sensitization is produced by the action of inflammatory mediators such as bradykinin, prostaglandins, neuropeptides, and cytokines which activate corresponding receptors in nerve fibers. In addition, the expression of receptors can be downregulated or upregulated in pain conditions. The development of hyperexcitability of spinal cord (and supraspinal) neurons is called central sensitization and is produced by various transmitter/receptor systems that constitute and modulate synaptic activation of the neurons. In this case, the key transmitter is glutamate that activates N-methyl-d-aspartate (NMDA) and non-NMDA receptors on spinal cord neurons. Blockade of these receptors prevents and reduces central sensitization. In addition, excitatory neuropeptides (substance P and calcitonin gene-related peptide) and other mediators with complex actions facilitate central sensitization (Willis, 2001; Ji, 2004; Seybold, 2009).

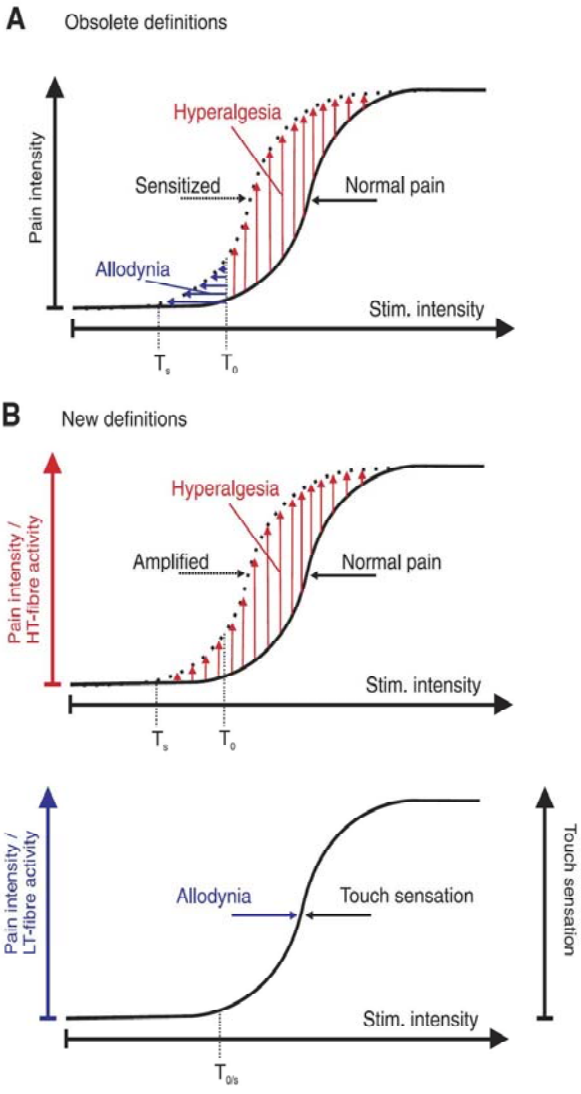


Figure 2. Changes proposed in 2008 by the International Association for the Study of Pain (IASP) task force in defining "hyperalgesia" and "allodynia." In A, the obsolete definitions are illustrated: pain in response to previously nonpainful stimuli was defined as "allodynia" (blue area in the stimulus-response function). T_0 refers to the normal pain threshold, and T_S refers to the pain threshold after sensitization. Enhanced responses to normally painful stimuli were called "hyperalgesia" (red area). A single mechanism, for example, the sensitization of nociceptive nerve endings (e.g., during a sunburn) leading to a shift in the stimulus-response function to lower stimulus intensities would always cause allodynia and hyperalgesia in combination but never in isolation, making this distinction meaningless. In B, the new definitions are illustrated. All forms of pain amplification including lowering in thresholds are now summarized under the umbrella term hyperalgesia (red ordinate and red area in top graph). Only if pain is cleary induced by low-threshold fibers should the term allodynia be used (blue ordinate in bottom graph). From Sandkühler, 2009.

2.1.2. Pain syndromes

Pain is not a unitary phenomenon, and its underlying biology can be dissociated along a number of dimensions. Acute pain can evolve into chronic pain because neuronal modification of gene expression may occur after injury, producing behavioral and histological changes. For example, a phenomenon called wind-up leads to intensified stimulation of nerve fibers that is referred to as non-nociceptive pain (Castro et al., 2011). Among pain syndromes we can distinguish at least four types based on their etiologies (Costigan et al., 2009):

- **Nociceptive pain** that is an alarm mediated by high-threshold unmyelinated C or thinly myelinated Aδ primary sensory neurons that feed into nociceptive pathways of the central nervous system (Woolf and Ma, 2007). These nociceptor neurons express specialized transducer ion channel receptors, mainly transient receptor potential (TRP) channels, tuned to respond to intense thermal or mechanical stimuli as well as exogenous and endogenous chemical mediators (Dhaka et al., 2006). Nociceptive pain has a protective function with a sensation so unpleasant that it cannot be ignored. It occurs in response to noxious stimuli and continues only in the maintained presence of noxious stimuli to alert us to external stimuli, such as pinprick or excessive heat, and internal stimuli, such as myocardial ischemia in patients with coronary artery disease. Loss of nociception, as in hereditary disorders associated with congenital insensitivity to pain (Indo, 2001; Cox et al., 2006), leads to repeated injury and unconscious self mutilation, illustrating the highly adaptive function of nociceptive pain.

- **Inflammatory pain** that occurs in response to tissue injury and the subsequent inflammatory response to permit healing and repair of the injured body part. In this pain conditions, the sensory nervous system undergoes a profound change in its responsiveness, showing hypersensitivity within the inflamed area and in contiguous noninflamed areas as a result of plasticity in peripheral nociceptors and central pain pathways (Woolf and Salter, 2000; Huang et al., 2006). With the pain system sensitized, it no longer acts just as a detector for noxious stimuli because can be activated also by low-threshold innocuous inputs. Typically, inflammatory pain disappears after resolution of the initial tissue injury. However, in chronic disorders such as rheumatoid arthritis the pain persists for as long as inflammation is active (Michaud et al., 2007).

The increase in pain sensitivity that follows injury is mediated by inflammatory mediators released at the site of injury. The number of inflammatory pain mediators and modulators includes not only a variety of small molecules such as bradykinin, prostanoids, adenosine triphosphate (ATP), protons, and nitric oxide, but also numerous cytokines, chemokines and growth factors. These mediators have different sources (immune cells, glial cells and neurones) and have diverse mechanisms and sites of action, including the activation and sensitization of nociceptive terminals; the regulation of primary nociceptive phenotype; and, in spinal cord, the pre-synaptic control of nociceptor transmitter release and the post-synaptic control of neuronal excitability. One of the most critical issues, of course, is to identify the relative importance of all these different mediators and mechanisms in particular pain states.

- **Neuropathic pain** that is intimately associated with the reaction of the nervous system to neural damage. Peripheral neuropathic pain results from lesions to the peripheral nervous system caused by mechanical trauma, metabolic diseases, neurotoxic chemicals, infection, or tumor invasion and involves multiple pathophysiological changes (Woolf and Mannion, 1999). Central neuropathic pain most commonly results from spinal cord injury, stroke, or multiple sclerosis (Ducreux et al., 2006). The primary disease and the neural damage it causes are only the initiators of a cascade of changes that lead to and sustain neuropathic pain. Although treatment targeted at the primary pathology is obviously essential, understanding the mechanisms responsible for the maladaptive plasticity offers specific therapeutic opportunities to prevent the development of neuropathic hypersensitivity and normalize function in established neuropathic pain. Its clinical manifestation is characterized by spontaneous ongoing or shooting pain and evoked amplified pain responses (Baron et al., 2010) and can be produced by a variety of nerve lesions, such as diabetic painful polyneuropathy, trigeminal neuralgia, chemotherapy-induced neuropathic pain and spinal cord injury (SCI).

- **Dysfunctional pain** that is caused by a malfunction of the somatosensory apparatus itself, and this malfunction can be considered a disease in its own right. Dysfunctional pain occurs in situations in which there is no identifiable noxious stimulus nor any detectable inflammation or damage to the nervous system. It is unclear in most cases what causes the manifestation or persistence of dysfunctional pain. In conditions such as fibromyalgia, irritable bowel syndrome, and interstitial cystitis, the pain appears to result from an autonomous amplification of nociceptive signals inside the central nervous system (Staud and

Rodriguez, 2006) with a disturbed balance of excitation and inhibition in central circuits and altered sensory processing that can be detected by functional imaging. Dysfunctional pain syndromes share some features of neuropathic pain: temporal summation with a progressive buildup in pain in response to repeated stimuli (windup), spatial diffuseness, and reduced pain thresholds (Staud et al., 2007).

2.1.3. Analgesic drugs developed

Research leading to the development of new analgesics and directed at various molecular targets related to pain mechanisms produced thousands of new publications (Kissin, 2010). However, those efforts have not yet yielded new analgesics with sufficient effectiveness to change the share of publications on opioids or nonsteroidal antiinflammatory drugs. Morphine and aspirin, introduced for the treatment of pain more than a century ago, continue to dominate biomedical publications despite their limited effectiveness in many areas (e.g., neuropathic pain) and multiple serious adverse effects.

Merely the diversity of molecular targets indicates that our understanding of clinical pain mechanisms is still limited; this is probably the main reason for the limited success in the development of new analgesics. The demonstrated paucity of novel analgesics is difficult to explain. Pain mechanisms that are not yet discovered or mechanisms that are already known but not appropriately used for drug development could be the root of the problem. However, three factors contributing to the apparent drought of novel analgesics can be suggested: (1) insufficient mechanism-based approach to clinical pain syndromes, (2) inadequate predictive validity of animal models for pain in humans, and (3) absence of the comparative benefit requirement for the approval of a new analgesic.

Inadequate predictive validity of animal models for pain in humans has been related to both adverse effects and lack of efficacy of drugs in humans that seemed to be safe and effective in animal models. This problem is so significant that some have called for abandonment of animal pain studies in favor of more extensive testing in humans (Langley et al., 2008).

Among drugs identified as analgesics, nonsteroidal anti-inflammatory drugs (NSAIDs), opioids, and triptans are the largest groups of drugs specifically developed as analgesics (see figure 3). In addition, anticonvulsants and antidepressants are the most numerous drugs

developed for indications other than pain but whose effectiveness in pain was later confirmed by a meta-analysis or by an Food and Drug Administration (FDA) review (Kissin, 2009).

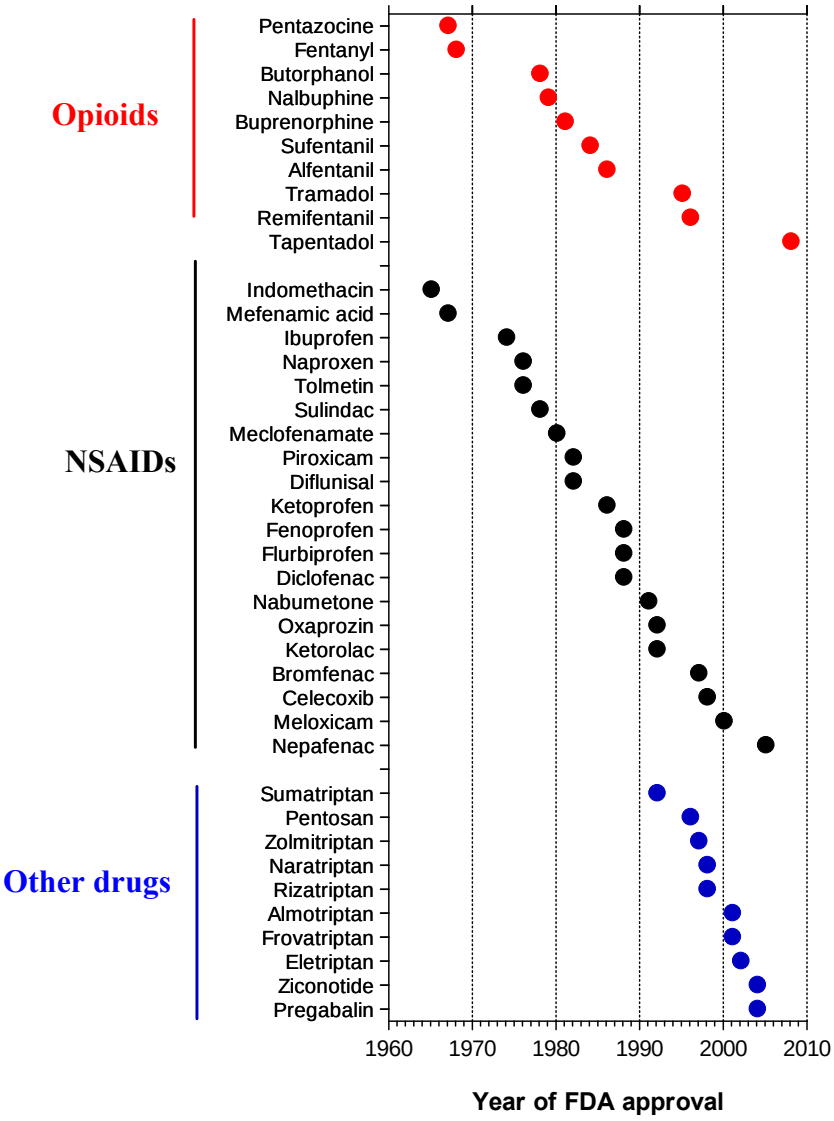


Figure 3. Analgesic drugs approved by the FDA. Drugs developed during the period 1960 to 2009 and presently in use for the treatment of pain. Among these 60 drugs, 40 were specifically developed as analgesics, and the remaining 20 were developed for non-pain indications, but their effectiveness in pain was later confirmed by a meta-analysis or by an FDA review. NSAIDs (20), opioids (10), and triptans (7) are the largest groups of analgesics. From Kissin, 2009.

Three drugs fit the category of drugs with a novel selective mechanism arising from a better understanding of the mechanism of an existing drug. These three analgesics, developed on the basis of a modified (but previously known) molecular target, are pentazocine, sumatriptan, and celecoxib. Although they demonstrate a lower degree of novelty than the drugs which have completely novel molecular targets, their impact on clinical practice seems to be very pronounced; their development led to the introduction of similar drugs acting on the same molecular target(s).

Analgesic drugs with completely new mechanisms of action directed at the novel molecular targets belong to four types. Three of these subgroups were identified some time ago in plants (chili peppers for capsaicin and cannabis for dronabinol) or snail venom (Conus magus for ziconotide), but novel molecular targets that determine their analgesic actions were discovered relatively recently: transient receptor potential vanilloid (TRPV1), an ion channel for capsaicin; cannabinoid (CB1 and CB2) receptors for dronabinol; and N-type voltage-sensitive calcium channel for ziconotide. The analgesic action of the N-methyl-d-aspartate antagonist ketamine (developed as a general anesthetic) was known for almost 50 years, but its therapeutic effect in postoperative pain was convincingly confirmed only recently.

2.1.4. Animal models of pain

In order to investigate pain, a battery of preclinical *in vivo* pain models has been developed to simulate clinical pain conditions with diverse etiology. Three entities have been described to define an animal pain model based on the experimental subject, assay developed and behaviors measured (Mogil, 2009) (see table 1).

Subject	Assay	Measure
Species + **Strain** + **Mutant?** + **Sex** + **Age** + **Husbandry** • Cage density • Diet • Social factors + **Testing procedures** • Arousal • Communication • Handling • Restraint	**Etiology** • Nociceptive (thermal, mechanical, chemical or electrical) • Inflammatory (algogen, sensitizing compound, inflammatory mediator, polyarthritic or monoarthritic) • Neuropathic (surgical or chemical) • Disease state (e.g. cancer, complex regional pain syndrome 1) + **Body part** • Cutaneous • Muscular • Orofacial • Visceral + **Time point post-injury**	**Reflex** • Heat or cold • Mechanical + **Spontaneous** • Autotomy • Directed behaviours (biting, flinching, guarding, licking, lifting and shaking) • Gait or posture + **Operant** • Learned escape • Place aversion • Reinforcement conflict + **Pain-affected complex behaviours** • Anxiety • Attention • Disability • Sociability • Sleep

Table 1. Entities that define an animal model of pain. Many variables describe an animal model, mainly those related to the subject (species, strain, genetic modification, sex, age, husbandry and testing procedures); assay developed (etiology, body part and time point post-injury); and behavior measured (reflex, spontaneous, operant and pain-affected complex behaviors). From Mogil, 2009.

It is well known that parameters of the experimental subject such as species, strain, genetic modification, sex and age importantly affect results in pain experiments. In addition, more subtle (and rarely considered) factors related to husbandry and testing procedures have been shown to affect results, sometimes dramatically (Mogil, 2009).

At least four separate etiologies of pain are commonly described: *nociceptive* in which a noxious stimulus is applied to a convenient body part (usually the hindpaws, tail or abdomen), leading to nocifensive withdrawal or to other simple behaviors that can be easily scored (see table 2); *inflammatory* that can be induced by injection of different substances (see table 3); *neuropathic* that is induced mainly by an injury of peripheral nerves (see figure 4); and *idiopathic pain* that attempts to more directly model prevalent clinical pain syndromes, often by inducing the disease, injury or physiologic state itself.

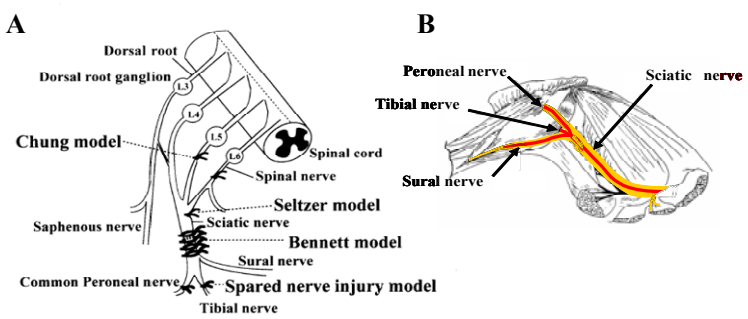


Figure 4. Illustration of different models of neuropathic pain (A). The sciatic nerve bifurcates into the tibial and common peroneal nerves. Prior to entering the leg, the tibial nerve branches to the medial sural cutaneous nerve (B). Adapted from reference Ueda and Inoue, 2001.

Nociceptive and inflammatory pain models are usually short-term whereas neuropathic and idiopathic pain models often last months, years or even indefinitely. In addition, the pathophysiology of pain also seems to depend on the particular tissue affected, on whether it is cutaneous, deep (muscles or joints) or visceral. The behaviors typically measured in these animal models of pain are either spinal reflexes (such as withdrawal from experimenter-applied stimuli), spino-bulbospinal reflexes (such as jumping or abdominal stretching) or simple innate behaviors (such as vocalization, scratching, biting, licking and guarding). Mechanical and thermal hypersensitiviy are the most common evoked withdrawal responses measured to study the analgesic effect of drugs in neuropathic pain (see table 3).

Furthermore, it has been noted that chronic-pain syndromes often feature dramatic changes in overall quality of life and in a wide variety of complex behaviors, including attention, memory, depression, anxiety, motivation and sleep (Loeser, 2000). If animal models of pain are truly relevant, equivalent changes in these parameters might be expected in animals. In line with this concern, alternative measures have been proposed, such as operant measures of pain (learned escape from electric footshock in shuttle boxes, self-administration, conditioned place preference to an analgesic in the presence of pain, and conditioned place aversion) and

pain-affected complex behaviors (anhedonia, anxiety, depression, appetite suppression, attentional deficits, autonomic dysregulation, cognitive deficits, disability or functional impairment, hypolocomotion, memory impairment, negative affect, social interaction disturbances and sleep disruption).

Table 2. Methods to assess pain. Modified from Sandkühler, 2009

Modality	Test Name	Test Method	Outcome Parameter
Mechanical	von Frey filaments[1]	Application of nonnoxious calibrated static hairs on skin	Force threshold to elicit hindpaw or face withdrawal
	Randal Sellito[2]	Application of linearly increasing mechanical force in noxious range on skin	Force threshold to elicit hindpaw withdrawal from noxious stimulus
	Dynamic allodynia[3]	Innocuous brushing, stroking of skin	Time latency to elicit hindpaw withdrawal or nociceptive behaviors
Heat	Tail flick or tail immersion[4]	Application of radiant heat on tail or immersion of tail in hot water	Time latency to elicit tail withdrawal
	Plantar Hargreave's[5]	Application of radiant heat on skin	Time latency to elicit hindpaw or tail withdrawal
	Hot plate[6]	Animal placed on heated metal plate	Time latency to elicit forepaw and hindpaw licking and escape behavior
Cold	Acetone[7]	Application of acetone on skin	Duration/intensity of nociceptive behaviors (hindpaw withdrawal and licking)
	Cold plate or cold water[8]	Animal placed on cooled metal plate	Time latency to elicit forepaw and hindpaw licking and escape behaviors
Electrical	Electrical shocks[9]	Electrical current application to tail, paw, viscera or dental pulp	Withdrawal thresholds, vocalization, escape latency

Data from: [1]Chaplan et al., 1994. [2]Randall and Selitto. [3]Field et al., 1999. [4]D'Amour and Smith, 1941. [5]Hargreaves et al., 1988. [6]Menéndez et al., 2002. [7]Vissers and Meert, 2005. [8]Jasmin et al., 1998; Attal et al., 1990. [9]Bonnet and Peterson, 1975.

Rats and mice are the main used species in such animals models of pain usually involving direct trauma (crush, transection, ligation, heat, cold, electrical stimulation) or application of irritants (bee venom, chemotherapy agents, capsaicin, carrageenan, formalin and other chemical irritants). See table 3 for a summary of animal models of pain with their clinical relevance in a human disease.

Table 3: Animal models of pain. Modified from Sandkühler, 2009

Model Name (Most Commonly Used)	Behavioral Phenotype	Method of Induction	Human Relevance/Disease
Carrageenan[1]	M, H	Injection of inflammatory agents into hindpaw	Inflammation and neurogenic pain
Formalin[2]	F, L		
Capsaicin[3]	M, H, F		
Bee venom[4]	M, H, F, L		
Carrageenan-induced monoarthritis[5]	M, H, G, S	Injection of carrageenan into joint	Arthritis, rheumatoid arthritis, osteoarthritis
Complete Freund's adjuvant (CFA)-induced polyarthritis[6]	M, G, S	Injection of CFA into tail or hindpaw	
Monoiodoacetate (MIA)-induced osteoarthritis[7]	M, G, S	Injection of MIA into joint	
Incision model[8]	M	Surgery	Postoperative pain
Ovariohysterectomy[9]	M		
Ultraviolet model[10]	M, H, C	Ultraviolet B (290–320 nm) dermal irradiation	Sunburn, burn injury
Burn or thermal injury model[11]	M, H	Prolonged noxious heat application on skin	
Chronic postischemia pain[12]	M, C	Temporary hindpaw ischemia and reperfusion or vascular occlusion	Ischemia-reperfusion injury, complex regional pain syndrome (CRPS), compartment syndrome, peripheral ischemic disease
Chronic constriction injury (CCI)[13]	M, H, C, G, WB	Constriction, ligation, or transection to injure various peripheral nerves (spinal, sciatic, saphenous) or facial nerves (trigeminal, mental)	Neuropathic pain, CRPS, nerve entrapment
Spinal nerve ligation (SNL)[14]	M, H, C, L		
Partial sciatic nerve ligation (PSNL)[15]	M, H, C, G, L		
Spared nerve injury (SNI)[16]	M, H, C, G, WB		
Sciatic nerve crush[17]	M		
Cryoneurolysis[18]	M, A, hyperesthesia		
Phototoxicity[19]	M, H, C		
Distal nerve injury[20]	M		
Complete nerve transection[21]	A		
Infraorbital nerve injury[22]	M, H	Injury of infraorbital nerve (CCI, ischemic injury)	Trigeminal neuralgia
Trigeminal ganglion compression[23]	M	Injury of trigeminal ganglia	
Temporomandibular joint or orofacial pain[24]	M, face grooming, scratching	Acute injection of inflammatory agent (CFA, carrageenan) in temporomandibular joint or face	Temporomandibular persistent pain

continue next page

Table 3. Animal models of pain. Modified from Sandkühler, 2009.

Model Name	Behavioral Phenotype	Method of Induction	Human Relevance/Disease
Diabetic neuropathy[25]	M	Administration of streptozotocin to induce diabetes	
Postherpetic neuralgia[26]	M, H	Inoculation with herpex simplex virus type I	Neuropathy secondary to disease
Experimental autoimmune neuritis or acute inflammatory demyelinating polyradiculo-neuropathy[27]	M, H	Immunization with peripheral myelin P2 peptide	
Experimental osteolytic sarcoma, squamous cell carcinoma or melanoma[28]	M, H	Intramedullary, intraplantar, or intragingival injection of cancer cells	Cancer pain
Inflammatory bowel syndrome[29]	M, W	Injection of irritants (acetic acid, capsaicin, mustard oil, zymosan) into hollow organs or visceral mechanical distention	Visceral pain
Peripheral acidosis[30]	M	Injection of acid in gastrocnemius muscle	Muscle pain
Sickness syndrome[31]	M, H	Systemic, intrathecal, or central lipopolysaccharide/inflammatory mediator administration	Fever, central nervous system inflammatory diseases
Spinal cord injury[32]	M, H, C	Ischemic or traumatic contusion, compression, or transection of spinal cord	Spinal cord injury neuropathic pain
Sciatic inflammatory neuritis[33]	M	Acute injection or perineuronal administration of inflammatory agents directly on nerves (zymosan or CFA)	Neuritis, neuropathic pain
Migraine[34]	M, H, S	Various manipulations: neurovascular, electrical, genetic	Migraine
"Name of agent"-induced neuropathic pain[35]	M, H, C	Systemic administration of clinically used chemotherapeutic agents (vincristine, paclitaxel, cisplatin) or antiretroviral agents	Neuropathic pain induced by drugs

Abbreviations: *M* mechanical hypersensitiviy; *H* heat hypersensitivity; *C* cold hypersensitivity; *L* licking/lifting; *F* flinching; *G* Guarding; *S* Spontaneous pain; *W* writhing; *WB* weight bearing; *A* autotomy.

Data from: [1]Meller et al., 1994. [2]Abbott et al., 1995. [3]Gilchrist et al., 1996 ; Joshi et al., 2006. [4]Lariviere and Melzack, 1996. [5]Gabriel et al., 2007. [6]Newbould, 1965. [7]Pomonis et al., 2005. [8]Brennan et al., 1996. [9]Gonzalez et al., 2000. [10]Davies et al., 2005. [11]Nozaki-Taguchi and Yaksh, 1998. [12]Seo et al., 2008; Coderre et al., 2004. [13]Bennet, 1993. [14]Kim and Chung, 1992. [15]Seltzer et al., 1990. [16]Decosterd and Woolf, 2000. [17]Decosterd et al., 2002. [18]DeLeo et al., 1994. [19]Kupers et al., 1998. [20]Siegel et al., 2007. [21]Wall et al., 1979. [22]Imamura et al., 1997. [23]Ahn et al., 2009. [24]Imbe et al., 2001. [25]Courteix et al., 1993. [26]Dalziel et al., 2004. [27]Moalem-Taylor et al., 2007. [28]Wacnik et al., 2006. [29]Laird et al., 2001a. [30]Sluka et al., 2001. [31]Mason, 1993. [32]Yezierski, 2000. [33]Chacur et al., 2001. [34]Bergerot et al., 2006. [35]Polomano et al., 2001. [36]Joseph et al., 2004.

2.2. The 5-HT$_7$ receptor

The 5-HT$_7$ receptor was first identified from brain complementary deoxyribonucleic acid (cDNA) libraries screened to find new sequences showing homology to known 5-HT receptors. Since the identification of the 5-HT$_7$ receptor in the early 1990's, much effort has been made to investigate the nature and biological function of this new member of a large family of G-protein coupled serotonin receptors. The 5-HT$_7$ receptor has been cloned from different genomes: human (Bard et al., 1993), rat (Shen et al., 1993), mouse (Plassat et al., 1993), pig (Bhalla et al., 2002), guinea pig (Tsou et al., 1994), rabbit (Pootanakit and Brunken, 2000), and frog (Nelson et al., 1995).

2.2.1. General aspects of serotonergic system

The neurotransmitter serotonin, 5-hydroxytryptamine, 5-HT (see figure 5), participates in many functions of vertebrates, insects, and plants. In mammals, serotonergic activity occurs in both the central nervous system (CNS) and in the periphery. The serotonergic system has been described to be implicated in many diseases particularly depression, anxiety, schizophrenia, obsessive compulsive disorder, panic disorder, migraine, hypertension, eating disorders, vomit and irritable bowel syndrome.

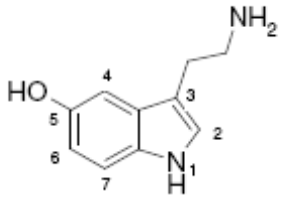


Figure 5. Chemical structure of serotonin

This neurotransmitter was originally discovered as a chemical component in the blood (serum) which was known to cause contraction (tonus) of blood vessels and consequently named serotonin (Rapport et al., 1948a, 1948b). The compound was recognised in 1964 to be a neurotransmitter (Dahlström and Fuxe, 1964). This knowledge was followed by extensive efforts to develop new therapeutic agents that could modify serotonergic functions.

The 5-HT receptor family has long been a target of intense research in both academia and industry. Current efforts are aimed at identifying more potent and selective ligands for the different receptor subtypes. It is anticipated that such selective receptor ligands will provide

the tools to advance the definition of functional effects and can lead to enhanced drug treatments with fewer side effects for a variety of disorders.

Serotonin is involved in a wide variety of physiological processes by binding to multiple 5-HT receptors grouped now into seven major families (5-HT_1-5-HT_7). Application of molecular cloning techniques has revealed the existence of 13 different genes, each encoding a distinct G protein-coupled 5-HT receptor subtype (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-ht_{1e}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT_4, 5-ht_{5a}, 5-ht_{5b}, 5-HT_6 and 5-HT_7). These 5-HT receptors are coupled positively or negatively to adenylate cyclase, presumably via G-protein, leading to an increase or decrease, respectively, in 3'-5'cyclic adenosine monophosphate (cAMP) production in response to stimulation (see figure 6A). In contrast, the 5-HT_3 receptor, a ligand-gated ion channel, has a very different molecular structure and signaling mechanism from the other 5-HT receptor subtypes, which are all coupled to G protein (Hoyer et al., 1994, 2002).

The Serotonin Club Receptor Nomenclature Committee of the IUPHAR (International Union of Pharmacology) originally founded in 1984 was assigned the task of providing a classification system based on operational (drug related), structural (primary amino acid sequence) and transductional (receptor coupling) information (Humphrey et al., 1993). A summarizing graphical representation of the current classification of 5-HT receptors, according to the criteria of the IUPHAR is illustrated in figure 6B.

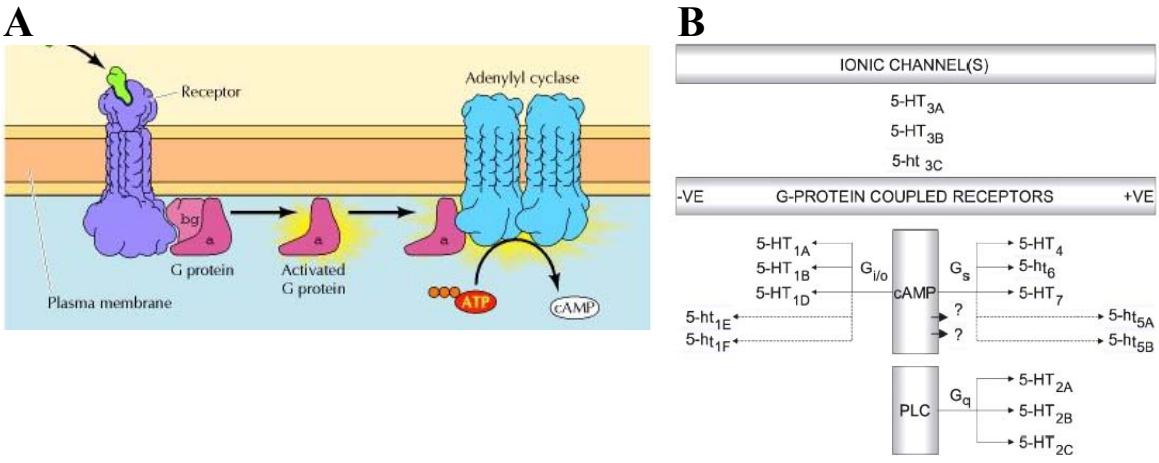


Figure 6. A. Binding of a ligand promotes the interaction of the receptor with a G protein. The activated G protein α subunit then dissociates from the receptor and stimulates adenylyl cyclase, which catalyzes the conversion of ATP to cAMP. Modified from The Cell: A Molecular Approach. 2nd edition. Geoffrey M Cooper. **B.** Graphical representation of the current classification of 5-HT receptors. Abbreviations: 3'-5' cyclic adenosine monophosphate (cAMP); phospholipase C (PLC); negative (-ve); positive (+ve). From Hoyer et al., 2002.

2.2.2. Molecular characteristics of the 5-HT$_7$ receptor

The human 5-HT$_7$ receptor gene is located on chromosome 10 (10q21–q24) (Gelernter et al., 1995) and has two introns in the coding region (Erdmann et al., 1996; Heidmann et al., 1997). The full length mammalian 5-HT$_7$ receptor consists of a protein of 445-479 amino acids with seven putative transmembrane spanning regions (see figure 7). A transcribed human 5-HT$_7$ receptor pseudogene has also been identified that possesses over 90% homology with the other known 5-HT$_7$ receptor sequences (Olsen et al., 1997; Olsen and Schechter, 1999). Although the primary structure (amino acid sequence) of the receptor exhibits a high degree of interspecies homology (95%), the similarity with other serotonin receptor subtypes appears to be considerably lower <50%.

Alternative splicing of the 5-HT$_7$ receptor gene in rat and human gives rise to different isoforms, namely 5-HT$_7$(a), (b), (c), (e) in rat and 5-HT$_7$(a), (b), (d) in human that differ primarily in the length of their intracellular carboxyl terminals (see figure 7). The relative abundance of all 5-HT$_7$ receptor isoforms is different among humans and rats, possibly indicating different functional properties and physiological roles for each of these isoforms. Comparative studies suggest no differences in the pharmacological profile or functional coupling to adenylyl cyclase among human 5-HT$_7$ receptor isoforms (Krobert et al, 2001).

5-HT$_7$ receptors are coupled positively to adenylate cyclase, presumably via Gs, leading to an increase in cAMP production in response to stimulation (Bard et al., 1993). Additional studies have revealed that stimulation of the 5-HT$_7$ receptor not only cause activation of adenylyl cyclase 5 (AC$_5$) through coupling with Gαs, but also AC$_1$ and AC$_8$ (Baker et al., 1998). These neural specific Ca^{2+}/calmodulin-stimulated isoforms of adenylyl cyclase (AC$_1$ and AC$_8$) are insensitive to Gαs, and are expressed in areas of the brain were 5-HT$_7$ receptors are localized (Xia and Storm, 1997). Thus, it is hypothesized that 5-HT$_7$ receptors activation might regulate intracellular cAMP in certain areas of the brain by mobilizing intracellular Ca^{2+}.

The C-terminal region of the third intracellular loop have two specific charged amino acid residues highly conserved among all isoforms of the 5-HT$_7$ receptor, Glu325 and Lys327 that play a critical role in the interaction with the Gαs-protein (Obosi et al., 1997). It has also been demonstrated that intespecies difference in binding affinities observed for the compounds at human and rat 5-HT$_7$ receptors is due to the nature of the residue Phe(7.38) of the human 5-HT$_7$ receptor [Cys(7.38) in rat]. These studies suggest that Phe(7.38) in the human receptor is

integrated in the hydrophobic pocket in the central part of the binding site [Phe(6.51)-Phe(6.52)] and allows a tighter binding of the ligands when compared with the rat receptor (Varin et al., 2009).

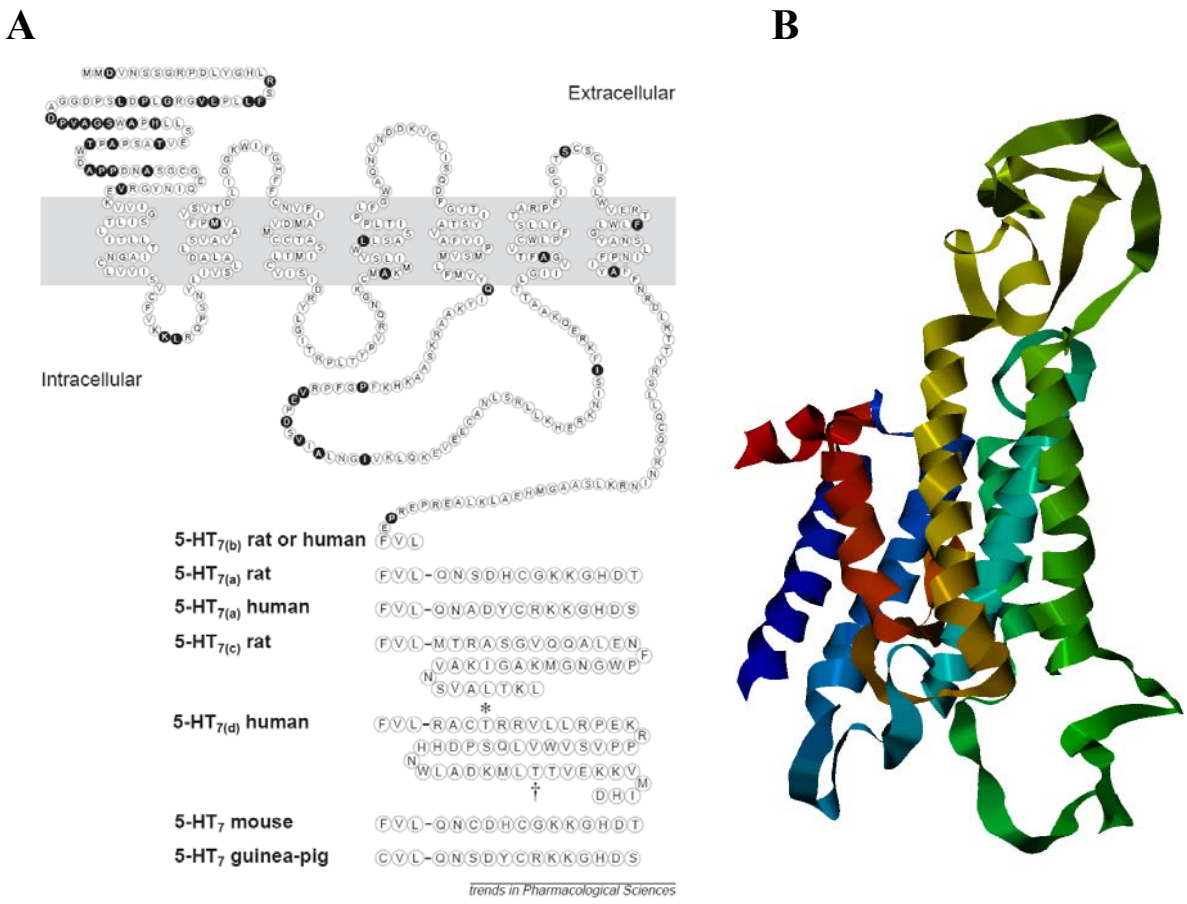


Figure 7. A. Snake-plot of 5-HT$_7$ receptor isoforms. Full sequence with the 7 trans-membrane domains and C-termini of either rat or human 5-HT$_{7(b)}$ in white dots and black fonts. Other C-termini are illustrated starting from corresponding C-terminus of 5-HT$_{7(a)}$. Black dots and white fonts represent amino acids that exhibit differences among mammals. the deduced protein sequence for the human 5-HT$_7$(d) isoform. The protein kinase C site is indicated by an asterisk and the casein kinase II site is indicated by a cross. Adapted from reference Heidmann et al., 1997. **B.** Structure of the 5-HT$_7$ receptor modelled from residue 85 to 399, based on template 2r4rA, with a sequence identity of 44% and a X-ray resolution of 3.40. Model created in 2008-11-05. From The Protein Model Portal.

Variation in the length of their C-termini, and number of consensus sites for phosphorylation by cAMP-dependent protein kinases PKA and PKC raise the possibility that the 5-HT$_7$ receptor isoforms could display differences in their desensitisation or trafficking properties (Heidmann et al., 1997). Furthermore, some 5-HT$_7$ receptor isoforms have been reported to contain consensus sequences for PDZ-domain containing proteins raising the possibility to couple to alternative signalling pathways (Hamblin et al., 1998). Only the 5-HT$_7$(d) human

isoform has two additional phosphorylation consensus sites in the predicted intracellular C-terminal protein sequence, one for PKC and one for casein kinase II (Heidmann et al., 1997).

5-HT_7 receptors have also been reported to mediate the activation of the extracellular signal-regulated kinases (ERK1 and ERK2) suggesting a possible role in the control of the mitogen-activated protein kinase (MAPK) pathway which mediates the effects of brain derived neurotrophic factor, brain-derived neurotrophic factor (BDNF), on synaptic function and neural plasticity (Errico et al., 2001; D'Sa and Duman, 2002).

Recently, it has been demonstrated that 5-HT_7 receptors undergo post-translational modification by the palmitic acid, which is covalently attached to the protein through a thioester-type bond. 5-HT_7 receptors are dynamically palmitoylated in an agonist-dependent manner and subjected to repeated cycles of palmitoylation/depalmitoylation. However, non-palmitoylated 5-HT_7 receptors are indistinguishable from the wild-type for their ability to interact with Gαs and G(12)-proteins after agonist stimulation. Thus, 5-HT_7 receptor dynamic palmitoylation is suggested to be important only for the fine tuning of receptor-mediated signaling (Kvachnina et al., 2009). In addition, the endogenous lipid oleamide has been reported to act as a non-selective allosteric modulator of 5-HT_7 receptors (Hedlund et al., 1999; Thomas et al., 1999).

Constitutive activity of 5-HT_7 receptors has been reported by several groups *in vitro* as increased concentrations of 5-HT antagonists (inverse agonists) reduce the basal adenylyl cyclase activity of cells expressing the human 5-HT_7 receptors (Krobert and Levy, 2002). This means that even in the absence of an agonist, these receptors are able to activate G proteins and mutations in different structural domains of the receptor can enhance or reduce this agonist independent activity. Cysteine residues 404 and 438/441 located in the C-terminal receptor domain are the main palmitoylation sites. Mutation of the proximal palmitoylation site Cys404-Ser (either alone or in combination with Cys438/441-Ser) significantly increased the agonist-independent, G(s)-mediated constitutive 5-HT_7 receptor activity, while the activation of Gα(12)-protein was not affected (Kvachnina et al., 2009).

Desensitization and down-regulation following agonist exposure are common among G protein-coupled receptors, but there is some discrepancy at this regard for 5-HT_7 receptors. Down-regulation has been described in some studies (e.g., in the hypothalamus after fluoxetine treatment for 21 days) (Sleight et al., 1995). However, a recent study has demonstrated a desensitization of the 5-HT_7 receptor after a long-term activation of 5-HT_7

receptors without reducing (down-regulation) the amount of 5-HT_7 receptors in the membrane. It was demonstrated that activation of 5-HT_7 receptors inhibited the 5-HT transporter (SERT) reducing the 5-HT uptake, but these effects on SERT disappeared after long-term activation of 5-HT_7 receptors (Iceta et al., 2009). Other studies have speculated, that the restoration of 5-HT neuronal firing upon chronic antidepressant treatment with selective serotonin reuptake inhibitors (SSRIs), may be attributed to desensitization of 5-HT_7 receptors (Bosker et al., 2009), and that desensitization is evoked by both 5-HT_7 receptor agonists and antagonists without being accompanied by down-regulation of the receptor (Krobert et al., 2006). On the other hand, significantly increased 5-HT_7 receptor messenger ribonucleic acid (mRNA) expression has been reported in raphe nuclei, hippocampus and prefrontal cortex after 5-HT_7 receptor activation (Pérez-García et al., 2006). It has also been described up-regulation of the 5-HT_7 receptor in pathological situations (Pierce et al., 1996, 1997).

Regarding the 5-HT_7 receptor distribution, the mRNA encoding the 5-HT_7 receptors is highly expressed within the Central Nervous System (see figure 8): cortex, septum, cerebellum, striatum, thalamus, hypothalamus, olfactory complex, trigeminal ganglia, mesencephalon, and the hippocampus, while generally lower levels of expression are detected in areas such as basal ganglia, midbrain, hindbrain and amygdala (Gustafson et al., 1996; Neumaier et al., 2001). Notably, there are some discrepancies among research groups regarding the presence of 5-HT_7 receptors in the suprachiasmatic nucleus (Moyer and Kennaway, 1999).

However, low levels have been reported in peripheral tissues: lung, kidney, liver, pancreas, placenta, spleen, testis, ovary, retina, heart, coronary, pulmonary and uterine arteries, superior vena cava, saphenous vein, and various regions of the gastro-intestinal tract, including the stomach, colon and ileum (Krobert et al., 2001).

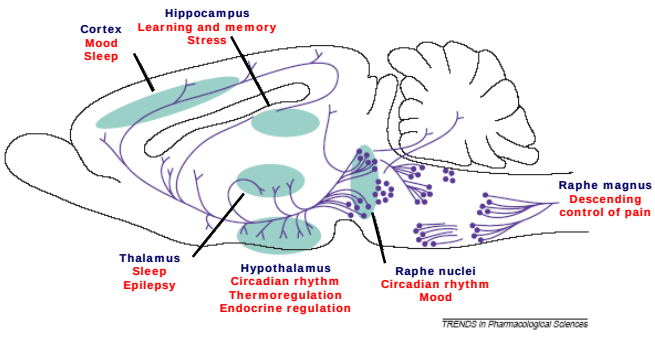


Figure 8. A sagittal view of the rodent brain, showing the 5-HT-producing neurons of the brain stem with their ascending and descending projections (purple). Regions that are relatively rich in 5-HT_7 receptor expression (green) and their putative correlation with 5-HT_7 receptor-mediated functions are indicated. Modified from Hedlund and Sutcliffe, 2004

2.2.3. Pharmacological tools for the 5-HT$_7$ receptor

Several compounds known to be ligands for other 5-HT receptor subtypes were also found to display high affinity for recombinant 5-HT$_7$ receptors from various species (human, rat, mouse, guinea-pig), as shown in table 4. All four species homologues of the 5-HT$_7$ receptor have similar affinities for these ligands. One of them, 8-OH-DPAT, displays relatively high affinity for 5-HT$_7$ receptors, although it was considered, prior to 1993, a selective agonist for the 5-HT$_{1A}$ receptor. When comparing afinity values obtained from human cloned receptor with those from rat cloned receptor some remarkable differences in affinity can be noted. In most cases, Ki values from rat cloned receptor are higher than those from human cloned receptors.

Table 4. Ki values (nM) of selected ligands at recombinant 5-HT$_7$ receptor from human, rat, mouse, and guinea-pig expressed in Cos-7 cells using [^{3}H]5-HT as radioligand.

Ligand	Human[1]	Rat[2]	Mouse[3]	Guinea pig[4]
5-CT	1.0	0.2	1.0	0.4
Methiothepin	4.0	0.4	6.3	10.0
5-MeOT	5.0	0.6	6.3	1.0
Metergoline	6.3	6.3	31.6	-
5-HT	7.9	1.6	5.0	1.0
Mesulergine	20.0	20.0	25.1	-
2-Br-LSD	31.6	-	10.0	-
Methysergide	79.4	12.6	12.6	39.8
Spiperone	100.0	-	63.1	100.0
Cyproheptadine	125.9	31.6	-	-
Tryptamine	158.5	15.8	-	-
8-OH-DPAT	501.2	31.6	251.2	50.1
Sumatriptan	1000.0	251.2	1995.3	251.2
Ketanserin	1258.9	251.2	398.1	-

Abbreviations: *5-CT* 5-carboxamidotryptamine; *5-MeOT* 5-methoxytryptamine; *5-HT* serotonin, 5-hydroxytryptamine; *2-Br-LSD* 2-bromo-D-lysergic acid diethylamide; *8-OH-DPAT* 8-hydroxy-2-(di-n-propylamino)tetralin.

Data are from [1]Bard et al., 1993; [2]Shen et al., 1993; [3]Plassat et al., 1993; [4]Tsou et al., 1994.

Many 5-HT_7 receptor ligands have been widely used for *in vitro* and *in vivo* experiments to study the role of 5-HT_7 receptors. However, some of these compounds are not useful pharmacological tools because of the lack of 5-HT_7 receptor selectivity (see table 5).

Table 5. Affinities (Ki; nM) of several 5-HT receptor agonists and antagonists for various 5-HT receptor subtypes.

	5-HT_{1A}	5-HT_{1B}	5-HT_{1D}	5-HT_{2A}	5-HT_{2B}	5-HT_{2C}	5-HT_4	5-HT_6	5-HT_7
Agonists									
5-HT	4.0	2.5	3.2	1258.9	10.0	4.0	199.5	50.1	2.0
5-CT	0.3	5.0	2.5	>10000	125.9	631.0	3162.3	251.2	0.3
5-MeOT	10.0	398.1	4.0	3162.3	15.8	25.1	100.0	20.0	1.6
8-OH-DPAT	2.0	>10000	1258.9	10000.0	1995.3	6309.6	-	>10000	31.6
Antagonists									
Methiothepin	79.4	50.1	501.2	1.6	0.6	25.1	>10000	2.0	0.4
Lisuride	0.8	199.5	31.6	5.0	-	20.0	-	5.0	0.8
Risperidone	251.2	2511.9	10.0	0.2	31.6	63.1	-	398.1	1.3
LY215840	39.8	1000.0	631.0	20.0	2.0	4.0	-	-	15.8
Metergoline	7.9	39.8	0.8	1.0	-	0.6	>10000	31.6	63.1
Mesulergine	631.0	12589.3	6309.6	25.1	3.2	1.6	>10000	1584.9	20.0
Methysergide	25.1	1584.9	4.0	2.5	1.6	2.5	>10000	398.1	12.6
Ritanserin	794.3	1584.9	398.1	1.6	5.0	1.3	-	39.8	20.0
Spiperone	63.1	5011.9	5011.9	1.6	3162.3	1258.9	>10000	-	20.0
Cyproheptadine	316.2	5011.9	-	3.2	-	12.6	-	125.9	50.1
Mianserin	1000.0	6309.6	398.1	7.9	50.1	10.0	-	50.1	63.1
Clozapine	125.9	631.0	398.1	3.2	7.9	12.6	-	20.0	63.1

Abbreviations: *5-HT* serotonin, 5-hydroxytryptamine; *5-CT* 5-carboxamidotryptamine; *5-MeOT* 5-methoxytryptamine; *8-OH-DPAT* 8-hydroxy-2-(di-n-propylamino)tetralin.

Data for 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1D} receptors are from Hoyer, 1988; Van Wijngaarden et al., 1990 and Schotte et al., 1996 for pig, rat and calf brain membranes or human recombinant receptors. The data for 5-HT_6 receptors are from Monsma et al., 1993 and Roth et al., 1994 for rat receptors. The data for 5-HT_7 receptors are from Ruat et al., 1993; Shen et al., 1993 and Roth et al., 1994 for rat receptors. Data for 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors were taken from Baxter et al., 1995; Schotte et al., 1996; Wijngaarden et al., 1990; Wainscott et al., 1996, 1998; and Leysen, 1985. Data for 5-HT_4 receptors were collected from Hoyer et al., 1994 and Dumuis et al., 1988.

The important progress achieved in the development of antagonists and agonists with high affinity and selectivity for the 5-HT_7 receptor provided more appropriate tools to really assess the physiological role of 5-HT_7 receptors.

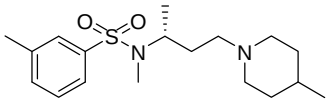
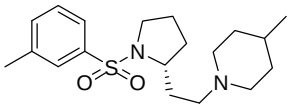
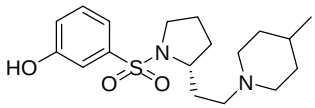
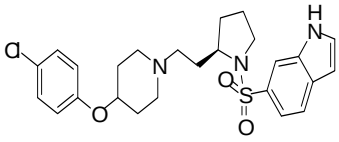
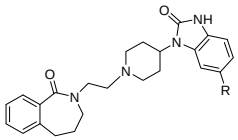
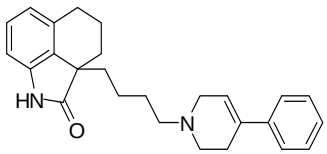
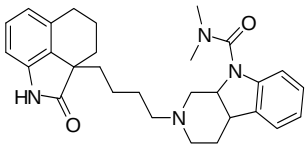
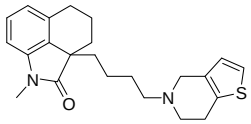
The first 5-HT_7 receptor antagonist identified and used to characterize 5-HT_7 receptor-mediated responses was LY-215840 (Cohen, 1992). However, LY-215840 lacks 5-HT_7 receptor selectivity (see table 5).

The aryl sulphonamide selective 5-HT_7 receptor antagonist, SB-258719, displays a moderately high affinity (Ki = 31.6 nM) for the human 5-HT_7(a) receptor and 100-fold selectivity versus a range of other 5-HT and non-5-HT receptors (see table 6) (Forbes et al., 1998). Subsequently, DR-4004 (Kikuchi et al., 1999), was also reported as a 5-HT_7 receptor antagonist selective against a number of other 5-HT receptor subtypes, although it has a limited utility as pharmacological tool because of its high affinity for dopamine D2 and alpha1 adrenoreceptors (Kogan et al., 2002).

Analogues of SB-258719 with higher 5-HT_7 receptor affinity were synthesized, including SB-258741 and SB-269970 (see table 6) (Lovell et al., 2000). SB-269970 (Ki = 1.2 nM) is a potent 5-HT_7 receptor antagonist displaying 100-fold selectivity versus other 5-HT receptor subtypes apart from the 5-ht_{5A} receptor (50-fold). Structure-activity studies around SB-269970-A led to the identification of SB-656104, displaying a 3-4-fold longer half life *in vivo* following intraperitoneal administration compared to SB-269970 (Forbes et al., 2002; Thomas et al., 2003). Subsequently, additional 5-HT_7 receptor-selective antagonists were reported including SB-691673 (Forbes et al., 2003), DR-4365 and DR-4446 (Kikuchi et al., 2002a, b), being the latter used as a potential positron emission tomography (PET) ligand ([^{11}C]-DR-4446) (Zhang et al., 2002).

The most widely used pharmacological tools are the 5-HT_7 receptor antagonists SB-269970 (Hagan et al., 2000) and DR-4004 (Kikuchi et al., 1999). Although generally considered selective, they also interact with other receptors (Kogan et al., 2002; Bonaventure et al., 2004).

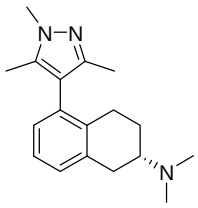
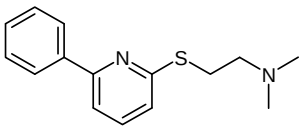
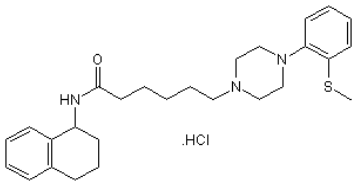
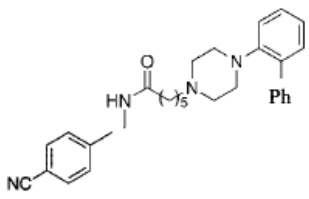
Table 6. 5-HT_7 receptor antagonists. Modified from Thomas and Hagan, 2004.

5-HT_7 receptor antagonists		
Compound	***Structure***	***Selectivity (Ki)***
SB-258719[1] *GlaxoSmithKline*		5-HT_7 = 31.6 nM 5-HTRs 100-fold
SB-258741[2] *GlaxoSmithKline*		5-HT_7 = 3.2 nM 5-HT_{5A} 50-fold 5-HTRs 100-fold
SB-269970[3] *GlaxoSmithKline*		5-HT_7 = 1.2 nM 5-HT_{5A} 50-fold 5-HTRs 100-fold
SB-656104[4] *GlaxoSmithKline*		5-HT_7 = 2.0 nM 5-HT_{1D} 10-fold 5-HT_{2A} 30-fold 5-HTRs 100-fold
SB-691673[5] *GlaxoSmithKline*		5-HT_7 = 2.0 5-HTRs 100-fold
DR-4004[6] *Meiji Seika*		5-HT_7 = 1.99 - 50.1 nM α_1-adrenoceptor = 39.8 nM α_2-adrenoceptor = 501.2 nM Dopamine D_2 = 39.8 nM Histamine H_1 = 125.9 nM
DR-4365[7] *Meiji Seika*		5-HT_7 = 3.2 nM 5-$HT_{1B,1D,2A,2C,3,4,6}$100-fold 5-HT_{1A} 30-fold
DR-4446[8] *Meiji Seika*		5-HT_7 = 10.0 nM 5-$HT_{1B,1D,2A,2C,3,4,6}$100-fold 5-HT_{1A} 30-fold

Data from [1]Thomas et al., 1998; Thomas et al., 1999; Forbes et al., 1998. [2]Lovell et al., 2000. [3]Lovell et al., 2000; Hagan et al., 2000. [4]Smithkline Beecham, 2002; Forbes et al., 2002; Thomas et al., 2003. [5]Forbes et al., 2003. [6]Meiji Seika Kaisha, 2002; Kikuchi et al., 1999; Kogan et al., 2002; Kikuchi et al., 2003. [7]Meiji Seika Kaisha, 1999a; Kikuchi et al., 2002; [8]Meiji Seika Kaisha, 1999b; Kikuchi et al., 2002; Zhang et al., 2002.

Regarding to selective 5-HT_7 receptor agonists (see table 7), the most frequently used ligand has been AS-19 (Sanin et al., 2003), but a recent binding profile showed that it has affinity also for 5-HT_{1A} receptor and α_{2a} adrenoreceptors (Bosker et al., 2009). It should be noted that 8-OH-DPAT was previously considered a selective agonist for 5-HT_{1A} receptors, and the knowledge that it also activated 5-HT_7 receptors prompted the revaluation of some previous findings. The finding that some actions of 8-OH-DPAT were actually mediated by the 5-HT_7 receptor is supported by the fact that they were inhibited by the selective antagonists SB-269970 and DR-4004 (Ehlen et al., 2001; Sprouse et al., 2004).

Table 7. 5-HT_7 receptor agonists.

5-HT_7 receptor agonists		
Compound	***Structure***	***Selectivity (Ki)***
AS-19[1] *Lilly Research Laboratories*		5-HT_7 = 4.6 nM 5-HT_{1A} = 44.0 – 110.0 nM α_{2a} = 486.0-850.0 nM α_{1a} = 180.0-219.0 nM
MSD-5a[2] *Merck Sharp & Dohme*		5-HT_7 = 0.6 nM 5-HT_{1A} 29-fold 5-HT_{2A} 533-fold Dopamine D_2 750-fold
LP-44[3] *Università degli Studi di Bari*		5-HT_7 = 0.2 nM 5-HT_{1A} 239-fold 5-HT_{2A} 1482-fold Dopamine D_2 33-fold
LP-211[4] *Università degli Studi di Bari*		5-HT_7 = 15.0 nM 5-HT_{2B} = 67.0 nM 5-HT_{2C} = 91.0 nM 5-HT_{5A} = 178.0 nM 5-HT_{1B} = 215.0 nM 5-HT_{1A} = 379.0 nM 5-HT_{1D} = 394 nM

Data from [1]Sanin et al., 2003; Bosker et al., 2009. [2]Thomson et al., 2004. [3]Leopoldo et al., 2004; Monti et al., 2008. [4]Leopoldo et al., 2008; Hedlund et al., 2010.

MSD-5a is also a 5-HT_7 receptor agonist showing high affinity for 5-HT_7 receptors (Ki = 0.6 nM), but its selectivity against 5-HT_{1A} (Ki = 16 nM) and other receptors is not high (Thomson et al., 2004). It was described as a potent partial agonist at 5-HT_7 receptors, giving at maximum 80% of the response evoked by a full agonist such as 5-carboxamidotryptamine.

Perrone and coworkers at Bari University, Italy, have studied the structure-activity relationships of a number of ligands characterized by an 1-arylpiperazine as the basic moiety. The main issue addressed with this framework was selectivity over 5-HT_{1A} receptors. That was indeed achieved in various ligands such as LP-44 and LP-211 (Leopoldo et al., 2004; Monti et al., 2008; Leopoldo et al., 2008; Hedlund et al., 2010). LP-211 displayed potent 5-HT_7 receptor agonist activity while maintaining relatively low affinity for 5-HT_{1A} and dopamine D_2 receptors (Leopoldo et al., 2008).

The discovery of new selective 5-HT receptor agonists or antagonists used as pharmacological tools would significantly enhance the understanding of 5-HT_7 receptor function. Furthermore, several laboratories have independently created constitutive knockout mouse strains lacking the 5-HT_7 receptor that would help to elucidate the role of 5-HT_7 receptors (Hedlund et al., 2003; Guscott et al., 2005; Witkin et al., 2007).

2.2.4. 5-HT_7 receptor function in normal and pathological processes

Several compounds tested in preclinical and clinical studies display moderate to high affinity for the 5-HT_7 receptor (Roth et al., 1994), and some of them are currently used as therapeutics (see figure 9A). Despite most of these drugs display high affinity for other 5-HT receptor subtypes, or even for non-serotonergic receptors (eg, dopamine, histamine and/or α-adrenergic receptors), some of their pharmacological and/or therapeutic effects are suggested to be due, at least partially, to 5-HT_7 receptors.

Interesting findings have been made on different diseases; however, discrepant results have been obtained in different behavioral studies probably because of the lack of really selective pharmacological tools. These inconsistencies have occurred mainly for anxiety, pain, schizophrenia, psychiatric and neurological disorders. In contrast, very consistent findings have been obtained for depression or sleep with the observation that inhibition of the 5-HT_7 receptor synergistically potentiates the effect of clinically used antidepressants.

It is not surprising that, based on the biological proof of concept generated using the potent, selective antagonist SB-269970 and 5-HT$_7$ receptor knockout mice, discovery programs of pharmaceutical companies have focused on the identification of newer 5-HT$_7$ receptor antagonists and selective agonists, and consequently a number of scientific articles and patents have been published (see figure 9B).

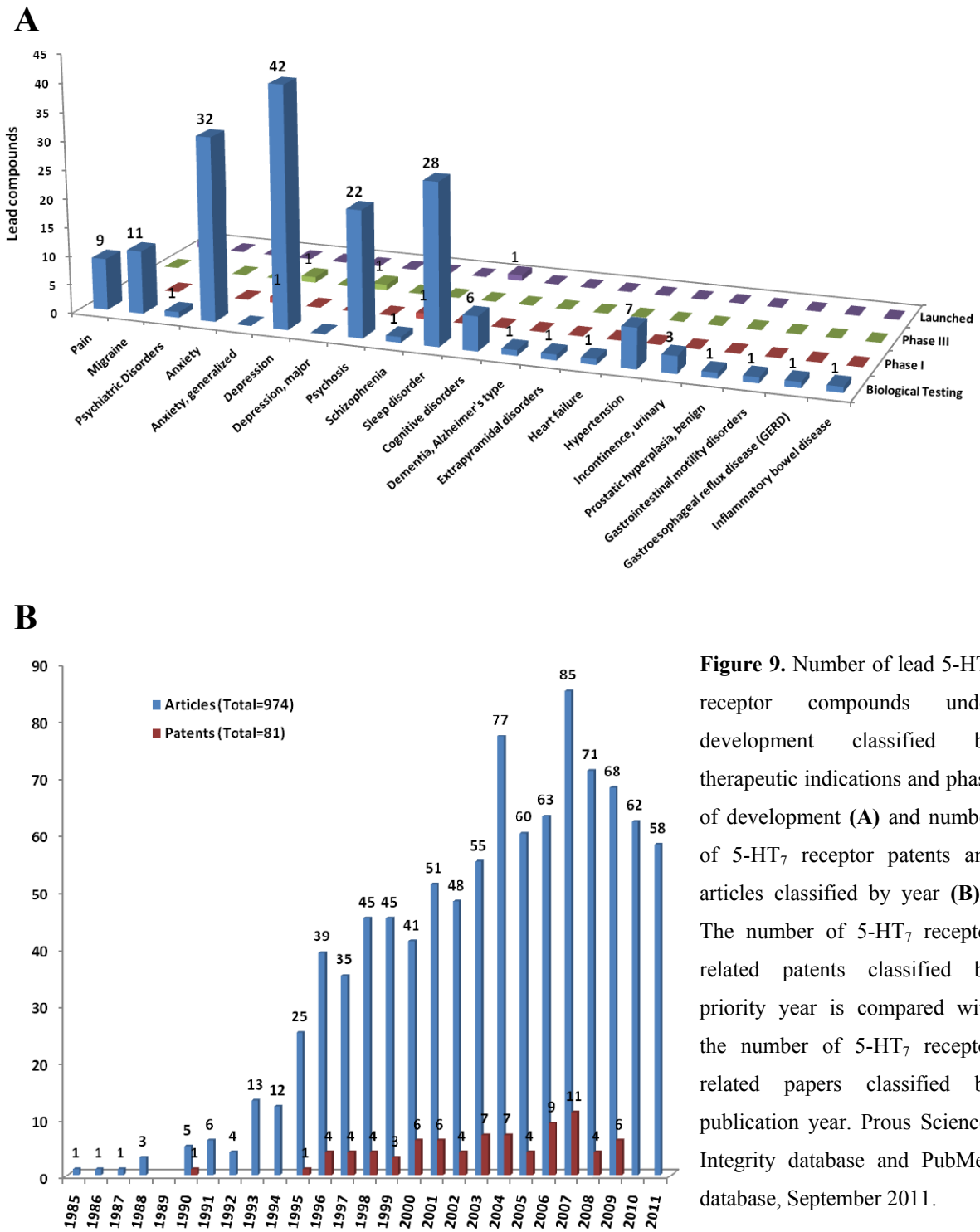


Figure 9. Number of lead 5-HT$_7$ receptor compounds under development classified by therapeutic indications and phase of development **(A)** and number of 5-HT$_7$ receptor patents and articles classified by year **(B)** . The number of 5-HT$_7$ receptor related patents classified by priority year is compared with the number of 5-HT$_7$ receptor related papers classified by publication year. Prous Science-Integrity database and PubMed database, September 2011.

The involvement of 5-HT_7 receptors in human disorders has been described using pharmacological tools and/or mice lacking functional 5-HT_7 receptors in animal behavioral models to reproduce, at least in part, human diseases. Most studies using 5-HT_7 receptor knockout mice have been focused on disorders related to depression, schizophrenia, sleep, learning and locomotion (Roberts et al., 2004; Guscott et al., 2005; Hedlund et al., 2005; Galici et al., 2008; Liu et al., 2009; Shelton et al., 2009). A considerable number of studies have been performed to evaluate the physiological role of the 5-HT_7 receptor in different human disorders (see table 8):

- **Thermoregulation**

The first evidence that the 5-HT_7 receptor is involved in body temperature homeostasis was the reported hypothermic effect of 5-CT on body temperature in guinea pigs, which could be blocked by SB-269970 (Hagan et al., 2000). Such an antagonistic effect has also been observed for SB-656104, another selective 5-HT_7 receptor antagonist (Thomas et al., 2003). It was not possible to block the hypothermic effect of 5-CT in mice with the 5-HT_{1A} receptor antagonist WAY-100635 or the 5-$HT_{1B/D}$ antagonist GR127935 (Guscott et al., 2003). In contrast, the hypothermic effect of 8-OH-DPAT in rats could be inhibited by a 5-HT_{1A} antagonist (WAY-100135), but only partially by SB-269970 (Hedlund et al., 2004). The importance of the 5-HT_7 receptor was further emphasized when it was observed that 5-HT and 5-CT failed to induce hypothermia in 5-HT_7 receptor knockout mice (Guscott et al., 2003; Hedlund et al., 2003). When including 8-OH-DPAT in the analysis in combination with selective antagonists and knockout mice to discriminate between 5-HT_{1A} and 5-HT_7 receptors it was revealed that both receptor subtypes are involved in 5-HT-mediated hypothermia (Hedlund et al., 2004). These findings provide an explanation for the results obtained with antagonists and also why 8-OH-DPAT fails to induce hypothermia in 5-HT_{1A} receptor knockout mice (Heisler et al., 1998). The 5-HT_7 receptor agonist LP-211 has also been found to induce hypothermia in mice. This hypothermic effect could be inhibited by SB-269970, but not WAY-100135, and was absent in 5-HT_7 receptor knockout animals (Hedlund et al., 2010). Interestingly, the 5-HT_7 receptor seems to be most important at low agonist concentrations, thus contributing to the fine-tuning of temperature homeostasis, whereas the 5-HT_{1A} receptor comes into play at higher agonist concentrations possibly providing a defense against hyperthermia (Hedlund et al., 2004).

- **Locomotion**

As mentioned above, the 5-HT_7 receptor has been found to be involved in spinal locomotion already in early development (Madriaga et al., 2004). A series of studies have attempted to characterize this involvement in more detail, again using both pharmacological tools and knockout mice.

Furthermore, it has been demonstrated that 5-HT_7 receptor antagonists block locomotion in cat, rat and mouse preparations, but that they have little effect in mice lacking 5-HT_7 receptors. In 5-HT_7 receptor knockout mice 5-HT induced rhythmic activity, but coordination among flexor and extensor motor nuclei and left and right sides of the spinal cord was disrupted. In adult 5-$HT_7R^{+/+}$ mice, 5-HT_7 receptor antagonists impair locomotion, producing patterns of activity resembling those induced by 5-HT in 5-$HT_7R^{-/-}$ mice (Jordan et al., 2008; Liu et al., 2009). Moreover, 5-$HT_7R^{-/-}$ mice displayed greater maximal extension at the hip and ankle joints than 5- $HT_7R^{+/+}$ mice during voluntary locomotion (Liu et al., 2009). Further evidence that the 5-HT_7 receptor is involved in spinal central pattern generator was obtained in a study examining electrically stimulated locomotion (Dunbar et al., 2010).

Paraplegic mice pretreated with the selective 5-HT_{1A} receptor antagonists WAY-100135 or WAY-100635 displayed significantly less 8-OH-DPAT-induced movement. A similar reduction of 8-OH-DPAT-induced movements was found in animals pretreated with SB-269970. Moreover, a near complete blockade of 8-OH-DPAT-induced movement was observed in 5-$HT_7R^{+/+}$ mice pretreated with 5-HT_{1A} and 5-HT_7 receptor antagonists, and in 5-$HT_7R^{-/-}$ mice pretreated with 5-HT_{1A} receptor antagonists (Landry et al., 2006).

The overall conclusion of these studies was that 8-OH-DPAT potently induces locomotor-like movement in the previously paralyzed hindlimbs of low-thoracic-transected mice and that both 5-HT_{1A} and 5-HT_7 receptors are involved in spinal locomotor rhythmogenesis *in vivo*. In a related field it has been found that the 5-HT_7 receptor is also involved in modulating phrenic nerve motor output, a finding that might also be of relevance for spinal cord injury (Macfarlane and Mitchell, 2009).

- **Circadian rhythm**

It has been shown that the effects related with circadian rhythm control of 8-OH-DPAT, DR-4004 and other non-selective 5-HT_7 receptor ligands on the suprachiasmatic nucleus (SCN) are mediated by the 5-HT_7 receptor (Lovenberg et al., 1993; Ying and Rusak, 1997; Ehlen et al., 2001; Yu et al., 2001; Antle et al., 2003; Glass et al., 2003).

- **Sleep**

A role of 5-HT_7 receptors in sleep disorders has been suggested as both 5-HT_7 receptor antagonists SB-656104 and SB-269970 produced a qualitatively similar effect on REM sleep parameters increasing the latency and decreasing the amount of time spent in REM sleep (Hagan et al., 2000; Thomas et al., 2003). Another study has shown that mice lacking the 5-HT_7 receptor exhibit the same reduction in time spent in REM sleep (Hedlund et al., 2005).

In addition, three antidepressants of the SSRI class (citalopram, fluoxetine, and paroxetine), but not a tricyclic antidepressant (desipramine), are able to potentiate the effects in REM sleep seen in knockout mice 5-$HT_7R^{-/-}$ mice (Shelton et al., 2009). Combination of SB-269970 and citalopram resulted in higher latency and lower amount of REM sleep (Bonaventure et al., 2007).

Furthermore, it is interesting to note that atypical antipsychotics such as clozapine and risperidone, with high affinity for the 5-HT_7 receptor, show the greatest improvements on sleep parameters (Roth et al., 1994; Cohrs, 2008).

- **Cardiovascular diseases**

Reported data suggest that hypotension to 5-HT and 5-CT is mainly mediated by 5-HT_7 receptors (Centurion et al., 2004). Furthermore, central 5-HT_7 receptors play an important facilitatory role in the reflex activation of vagal outflow to the heart evoked during the cardiopulmonary reflex, baroreflexes and the chemoreflex, as well as other autonomic changes caused by these reflexes (Kellet et al., 2005). The fact that cardiovascular responses to all reflexes tested were attenuated by the antagonism of 5-HT_7 receptors suggests that 5-HT_7 receptors in the brainstem facilitate the processing of the autonomic responses to cardiovascular reflex activation, and that a 5-HT-containing pathway to the brainstem provides a normalizing input during challenges produced by cardiovascular reflex activation which seems to be mediated by 5-HT_7 receptors (Damaso et al., 2007).

- **Respiratory disease**

The 5-HT_7 receptor antagonist SB-269970 abolished the antigen-induced airway hyperresponsiveness. Experiments in tracheal rings showed that pre-incubation with the 5-HT_7 receptor agonist LP-44 induced a significant increase of the cholinergic contractile response to the electrical field stimulation (Segura et al., 2010).

- **Learning and Memory**

A number of studies have demonstrated a possible role for the 5-HT_7 receptor in learning and memory, however further studies are clearly required using 5-HT_7 receptor-selective ligands to clarify the potential role of the 5-HT_7 receptor in cognitive processes.

5-HT_7 receptor agonists have been suggested to be therapeutically useful in the treatment of learning and memory deficits because electrophysiological data have shown increased neuronal excitability in hippocampal CA regions (Bacon et al., 2000; Gill et al., 2002) after 5-HT_7 receptors activation. Furthermore, 5-HT_7 receptor knockout mice have been reported to show both a selective impairment in contextual fear conditioning and a decrease in synaptic plasticity in the hippocampal CA1 region (Bourson et al., 1997). Discrepant results have also been reported, as 5-HT_7 receptor blockade can both attenuate the memory enhancing effects of the 5-$HT_{1A/7}$ agonist 8-OH-DPAT and exhibit memory enhancing properties in animal models of learning and memory (Meneses and Terron, 2001; Meneses, 2002).

- **Substance abuse**

A phenomenon that is closely linked with substance abuse is novelty-seeking behavior. It has been recently hypothesized that the 5-HT_7 receptor influences such behavior, however the relevance for the 5-HT_7 receptor in this disorder remains to be fully determined (Ballaz et al., 2007a, b).

There has been found correlation between the level of 5-HT_7 receptor mRNA expression in several brain regions and the degree of novelty-seeking behavior. Notably, there was higher expression in the hippocampus in the low responding rats (Ballaz et al., 2007a). The 5-HT_7 receptor antagonist SB-269970 reduced the exploration of a new object (Ballaz et al., 2007b). However, mice lacking the 5-HT_7 receptor did not differ from wild-type mice in novel object recognition, but exhibited reduced novel location recognition (Sarkisyan and Hedlund, 2009). It has also been found that the reduced impulse behavior of adult rats treated with methylphenidate during their adolescence is counteracted by SB-269970 (Leo et al., 2009). In addition, methylphenidate has been shown to upregulate 5-HT_7 receptor mRNA expression in the striatum and nucleus accumbens (Adriani et al., 2006; Leo et al., 2009).

- **Anxiety**

The selective 5-HT_7 receptor antagonist SB-269970 has been observed to induce anxiolytic effects in different anxiety models, inhibiting the activity of ascending dorsal raphe 5-HT

neurones (Wesolowska et al., 2006a, b). However, mice lacking the 5-HT_7 receptor (5-$HT_7R^{-/-}$) did not show differences compared to 5-$HT_7R^{+/+}$ mice in two anxiety models (Roberts et al., 2004; Guscott et al., 2005).

Further studies in animal models of anxiety using 5-HT_7 receptor-selective ligands are necessary in order to confirm a possible correlation between the 5-HT_7 receptor and anxiety.

- **Psychiatric disorders**

The 5-HT_7 receptor has been suggested to be one potential site at which antipsychotics such as clozapine, risperidone and amisulpride, which exhibit moderate to high potency as 5-HT_7 receptor antagonists (Roth et al., 1994; Thomas et al., 1998), might exert their therapeutic effects in schizophrenia (Vysokanov, 1998; Zhukovskaya et al., 2000; Abbas et al., 2009). However, no correlation was found regarding single nucleotide polymorphisms in the 5-HT_7 receptor gene in relation to the clinical efficacy of risperidone, an antipsychotic with high affinity for the 5-HT_7 receptor (Roth et al., 1994; Wei et al., 2009).

In vivo studies have not provided support for a role for the 5-HT_7 receptor in schizophrenia, as the overall profile of activity of the selective 5-HT_7 receptor antagonist SB-258741 did not resemble that of clinically proven antipsychotics (Pouzet et al., 2002). Somewhat conflicting results were obtained in studies using 5-HT_7 receptor knockout mice and the selective antagonist SB-269970 (Guscott et al., 2005; Galici et al., 2008; Semenova et al., 2008).

In a human study, it has been shown that 5-HT_7 receptor mRNA is down-regulated in the dorsolateral prefrontal cortex, but not the hippocampus, of schizophrenics (East et al., 2002). However, a role for the 5-HT_7 receptor in the aetiology or treatment of schizophrenia has not yet been established.

On the other hand, as the pharmacological treatment of choice for obsessive-compulsive disorder are antidepressants, specifically SSRIs, the 5-HT_7 receptor blockade or inactivation with its antidepressant-like effects has been suggested to be relevant for this disorder (Heyman et al., 2006). It has been demonstrated, using a fairly well-established model for obsessive-compulsive disorder, that pharmacological blockade of the 5-HT_7 receptors with SB-269970 or genetic inactivation of the receptor results in a reduction of stereotypic behavior characteristic of this disorder (Hedlund and Sutcliffe, 2007).

- **Depression**

Taken together findings in circadian rhythms and sleep studies, there is a consistent body of evidence that implicates the 5-HT_7 receptor in depression. Chronic antidepressant administration down-regulates 5-HT_7 receptors in rat hypothalamus (Sleight et al., 1995; Gobbi et al., 1996; Mullins et al., 1999). In addition, pharmacological blockade of the 5-HT_7 receptor or inactivation of the receptor gene leads to an antidepressant-like behavioral profile (Guscott et al., 2005; Wesolowska et al., 2006a, 2006b, 2007). The selective 5-HT_7 receptor antagonist SB-269970 has been shown to exert a synergistic interaction with antidepressants (Bonaventure et al., 2007; Wesolowska et al., 2007; Wesolowska and Kowalska, 2008). Interestingly, higher doses of SB-269970 result to be active alone in behavioral models of depression (Hedlund et al., 2005; Bonaventure et al., 2007).

As glucocorticoid-induced atrophy has been suggested to represent a contributory factor in relation to the development of unipolar depression, and 5-HT_7 receptors play a role in the regulation of hippocampal glucocorticoid receptor expression (Laplante et al., 2002), it has been suggested that 5-HT_7 receptors may be implicated in depression. In this respect, adrenalectomy increases the level of 5-HT_7 receptor mRNA in rat hippocampus (Le Corre et al., 1997; Yau et al., 1997).

Interestingly, it has been demonstrated using 5-HT_7 receptor knockout mice that the antidepressant effects of amisulpride, an atypical antipsychotic with high affinity for the 5-HT_7 receptor, are mediated by 5-HT_7 receptors (Abbas et al., 2009). The same is suggested to occur for the atypical antipsychotic aripiprazole (Lawler et al., 1999).

- **Epilepsy**

A role for the 5-HT_7 receptor in epilepsy has also been proposed based on the correlation of the relative affinity of a number of 5-HT_7 receptor antagonists, including mianserin, mesulergine and ritanserin, with their ability to protect against audiogenic seizures in mice (Bourson et al., 1997). Furthermore, the selective 5-HT_7 receptor antagonist SB-269970 reduced the potentiated spontaneous epileptic activity of the 5-$HT_{1A/7}$ receptor agonist 8-OH-DPAT (Graf et al., 2004). In another study investigating both electrically and chemically induced tonic–clonic seizures it was found that the seizure threshold was lower in mice lacking the 5-HT_7 receptor (Witkin et al., 2007).

It has also been suggested that 5-HT_7 receptors are involved in the regulation of tonic–clonic seizure-induced antinociception (Freitas et al., 2009). However, many studies are needed to sort out the role of the 5-HT_7 receptor as being either pro- or anticonvulsant.

- **Migraine**

5-HT_7 receptors have been suggested to play a role in the pathophysiology of migraine modulating the excitability of rat intralaminar and midline thalamic neurones and mediate the activation of trigeminal motorneurones as well as primary afferent neurones (Chapin and Andrade, 2001; Inoue et al., 2002; Meuser et al., 2002). It has been proposed the possibility that 5-HT_7 receptor-selective antagonists may be useful in the prophylactic treatment of migraine by preventing the craniovascular vasodilatation, hyperalgesia and neurogenic inflammation resulting from 5-HT_7 receptor activation. Consistent with this possibility, the clinically active dose for a number of non-selective migraine prophylactic agents has been reported to correlate with their 5-HT_7 receptor antagonistic activity (Terron, 2002). In line with this hypothesis, 5-HT_7 receptor mRNA is located in vascular tissues implicated in migraine including intra- and extra-cranial blood vessels and meningeal tissues (Ullmer et al., 1995; Schmuck et al., 1996). However, additional clinical studies are still required in order to determine the utility of 5-HT_7 receptor selective antagonists for the treatment of migraine.

Table 8. Role of 5-HT_7 receptors in animal models. Modified from Hedlund, 2009.

	Model	*Method*	*Effect*
ANXIETY	Dark-light transfer[1]	KO	No change
	Conflict drinking, Four plate and Plus maze[2]	SB-269970	Anxiolytic like
	Plus maze[3]	KO	No change
CARDIO VASCULAR	Vagosympathectomized rats[4]	SB-269970	Blockade of an induced hipotension
	Cardiovascular reflexes in anaesthetized rats[5]	SB-269970, SB-656104	Blockade of vagal bradycardia evoked by the activation of chemo-, baro-, cardiopulmonary reflexes.
	Cardiovascular reflexes in awake rats[6]	SB-269970	Blockade vagal bradycardia evoked by the activation of chemo-, baro-, cardiopulmonary reflexes.

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Table 8. Role of 5-HT_7 receptors in animal models. Modified from Hedlund, 2009.

	Model	*Method*	*Effect*
CIRCADIAN RHYTHM	Raphe electrical stimulation[7]	DR-4004	Attenuation the raphe-electrically stimulated SCN 5-HT release
	Wheel-running[8]	DR-4004	Inhibition of the phase shifting induced by 8-OH-DPAT
	Suprachiasmatic nucleus slices[9]	SB-269970	Inhibition of the phase shifting induced by 8-OH-DPAT
	Spontaneous activity cycle[10]	KO	Elimination of a nonphotic response to 8-OH-DPAT in mice lacking the 5-HT_7 receptor
	Spontaneous activity cycle [11]	LP-211	Induction of a phase advancement
DEPRESSION	Forced swim test[12]	KO, SB-269970, SB-258719, amisulpride	Antidepressant like
	Forced swim test[13]	SB-269970 + antidepressants	Antidepressant like and synergistic interaction
	Tail suspension test[14]	KO, SB-269970, amisulpride	Antidepressant like
	Tail suspension test[15]	SB-269970 + citalopram	Antidepressant like and synergistic interaction
EPILEPSY	Audiogenic seizures[16]	Non-selective 5-HT_7 receptor antagonists	Reduction of epileptic activity
	Absence epilepsia[17]	SB-269970	Reduction of epileptic activity
	Chemically and electrically induced seizure[18]	KO	Lower seizure thershold
	Spontaneously spike-wave discharges[19]	SB-258719	Reduction of epileptic activity

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Table 8. Role of 5-HT$_7$ receptors in animal models. Modified from Hedlund, 2009.

	Model	*Method*	*Effect*
LEARNING AND MEMORY	Autoshaping learning task[20]	AS-19	Facilitation of memory consolidation. Impairment of short-term memory but improvement of long-term memory
	Autoshaping learning task[21]	SB-269970	No effect. Reversion of AS-19 effects
	Autoshaping learning task[22]	SB-269970 and DR-4004	Decrease in the improvement of perfomance by 8-OH-DPAT. Reversion of amnesia induced by scopolamine and dizocilpine
	Novel object test, Barnes maze[23]	SB-269970, KO	Reduction in novel location exploration. Impairment in memory compilation
	Step-through passive avoidance task[24]	SB-269970	No effect alone, but intensified impairments caused by 8-OH-DPAT
	Radial arm maze task[25]	SB-269970	Improvement of reference memory without any effect in working memory
	Barnes maze; fear conditioning; motor, cued and operant conditioning[26]	KO	Only impairment in contextual fear conditioning. No change in other tests
	Reversal learning task[27]	SB-269970	Improvement of the PCP-induced cognitive dysfuntion
LOCOMOTION	Locomotor activity, Rotarod[28]	KO	No changes
	Haloperidol-induced catalepsy[29]	SB-269970	Partly reversion of 5-HT-anticataleptic effect
	Fictive locomotor activity[30]	SB-269970, KO	Reversibly blockade or modulation of the locomotor-like electrophysiological recordings pattern
	Locomotor-like movement in paraplegic mice[31]	SB-269970, KO	Reduction of 8-OH-DPAT-induced movements with SB-269970 pretreatment
	Phrenic long-term facilitation[32]	SB-269970	Attenuation of the phrenic tonic activity
MIGRAINE	Meningeal artery dilation[33]	SB-269970	Inhibition of dilation
	Electrical stimulation of the trigeminal ganglion (TGES)[34]	SB-269970, AS-19	SB-269970 inhibited the release of CGRP evoked by TGES. No effect of AS-19 but reversed the SB-269970-induced inhibitory effect

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Table 8. Role of 5-HT_7 receptors in animal models. Modified from Hedlund, 2009.

	Model	*Method*	*Effect*
PAIN	Formalin[35]	SB-269970	Peripheral antinociception. No spinal effect but reversed the pronociceptive effect of intrathecal 5-CT.
	Tail flick[36]	KO	No change
	Tail flick[37]	SB-269970	Spinal inhibition of opioid analgesia
	Tail shock[38]	SB-269970	Intrathalamic blockade of antinociceptive effects after intrathalamic injection of 8-OH-DPAT
	Paw flick[39]	SB-269970	Spinal inhibition of opioid analgesia
RESPIRATORY DISEASES	AI-AHR[40]	SB-269970	Inhibition of the AI-AHR
	Electrical field stimulation (EFS)[41]	LP-44	Increase in the cholinergic contractile response to the EFS
NEUROPSYCHIATRIC DISORDERS	PPI[42]	KO, SB-269970	No change
	Amphetamine-disrupted PPI[43]	KO, SB-258741	No change
	Amphetamine-disrupted PPI[44]	SB-269970	Antipsychotic like
	Phencyclidine-disrupted PPI[45]	KO, SB-258741	Antipsychotic like
	Ketamine-disrupted PPI[46]	SB-269970	No change
	Marble burying[47]	KO, SB-269970	Reduction of the stereotypic behavior of obsessive-compulsive disorder
SLEEP	Electroencephalogram[48]	KO, SB-269970, SB-656104-A	Less time in REM sleep, increased REM latency
	Electroencephalogram[49]	KO + antidepressants	Less time in REM sleep, increased REM latency
	Electroencephalogram[50]	LP-44, SB-269970	Reduction of REM and of number of REM periods

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Table 8. Role of 5-HT_7 receptors in animal models. Modified from Hedlund, 2009.

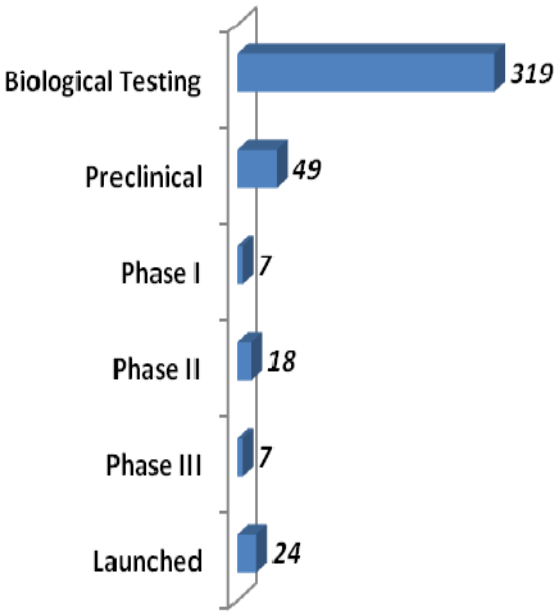
	Model	*Method*	*Effect*
SUBSTANCE ABUSE	Novel object discrimination[51]	SB-269970	Lower novelty seeking
	Delayed reinforcement[52]	SB-269970	Counteraction of methylphenidate-reduced impulsivity
THERMO-REGULATION	Rectal temperature[53]	SB-269970	Reversion of the hypothermic effect of 8-OH-DPAT
	Rectal temperature[54]	SB-258719 SB-269970 SB-656104-A	Reversion of the hypothermic effect of 5-CT
	Rectal temperature[55]	KO	5-CT and 5-HT reduced rectal temperature in wild-type but not in knockout mice
	Rectal temperature[56]	LP-211, SB-269970, KO	Hypothermic effect of LP-211 reversed by SB-269970. No effect in KO mice.

Abbreviations: *KO* knockout; *PPI* prepulse inhibition; *REM* rapid eye movement; *CGRP* calcitonin gene-related peptide; *SCN* suprachiasmatic nucleus; *AI-AHR* antigen-induced airway hyperresponsiveness; *PCP* 1-(1-phenylcyclohexyl)piperidine, phencyclidine

Data from: [1]Roberts et al., 2004. [2]Wesolowska et al., 2006a, b. [3]Guscott et al., 2005. [4]Centurion et al., 2004. [5]Kellett et al.,, 2005. [6]Damaso et al., 2007. [7]Glass et al., 2003. [8]Ehlen et al., 2001. [9]Sprouse et al., 2004. [10]Gardani and Biello, 2008; [11]Adriani et al., 2011. [12]Guscott et al., 2005; Hedlund et al., 2005; Abbas et al., 2009. [13]Wesolowska et al., 2007; Wesolowska and Kowalska 2008. [14]Hedlund et al., 2005; Wesolowska et al., 2006a; Bonaventure et al., 2007; Abbas et al., 2009. [15]Bonaventure et al., 2007. [16]Bourson et al., 1997. [17]Graf et al., 2004. [18]Witkin et al., 2007. [19]Graf et al., 2004. [20]Perez-Garcia and Meneses, 2009; Meneses et al., 2008. [21]Perez-Garcia and Meneses, 2009; Perez-Garcia et al., 2006; Meneses et al., 2008. [22]Meneses, 2004. [23]Sarkisyan and Hedlund, 2009. [24]Eriksson et al., 2008. [25]Gasbarri et al., 2008. [26]Roberts et al., 2004. [27]McLean et al., 2009. [28]Roberts et al., 2004. [29]Wei and Chen, 2009. [30]Madriaga et al., 2004; Jordan et al., 2008; Liu et al., 2009; Dunbar et al., 2010. [31]Landry et al., 2006. [32]Macfarlane and Mitchell, 2009. [33]Terrón and Martínez-García 2007; Martínez-García et al., 2009. [34]Wang, et al., 2010. [35]Rocha-González et al., 2005. [36]Roberts et al., 2004. [37]Dogrul and Seyrek, 2006. [38]Harte et al., 2005. [39]Dogrul et al., 2009. [40]Segura et al., 2010. [41]Segura et al., 2010. [42]Guscott et al., 2005; Semenova et al., 2008; Galici et al., 2008. [43]Pouzet et al., 2002; Semenova et al., 2008. [44]Galici et al., 2008. [45]Pouzet et al., 2002; Semenova et al., 2008. [46]Hedlund and Sutcliffe, 2007. [47]Galici et al., 2008. [48]Hagan et al., 2000; Hedlund et al., 2005; Bonaventure et al., 2007; Thomas et al., 2003. [49]Shelton et al., 2009. [50]Monti et al., 2008. [51]Ballaz et al., 2009. [52]Leo et al., 2009. [53]Hedlund et al., 2004. [54]Guscott et al., 2003. Thomas et al., 2003. [55]Hagan et al., 2000, Guscott et al., 2003, Hedlund et al., 2003. [56]Hedlund et al., 2010.

2.3. Role of the 5-HT$_7$ receptor on pain

Serotonin has been described to exert algesic or analgesic effects depending on the site of action and the receptor subtype it acts on (Eide and Hole, 1993; Millan, 2002). Much effort has been directed towards understanding the role of individual classes of 5-HT receptors in nociception (see figure 10) (Millan, 2002; Jeong et al., 2004; Lopez-Garcia, 2006), particularly 5-HT$_{1A}$, 5-HT$_{1B/1D}$, 5-HT$_{2A}$, 5-HT$_{2C}$ and 5-HT$_3$ receptors (Eide and Hole, 1993; Oyama et al., 1996; Obata et al., 2000; Kjorsvik et al., 2001; Kayser et al., 2002; Bardin and Colpaert, 2004; Colpaert, 2006; Kayser et al., 2007). 5-HT$_7$ receptors could participate, in concert with other 5-HT receptors (Kayser et al., 2002; Scott et al., 2006), in the endogenous control of pain. However, so far, the role played by the 5-HT$_7$ receptor in nociception has not been thoroughly investigated.



SERT/NET inhibitors (AD-337 – Phase II, Midalcipran, Bicifadine – Phase III)
Non-selective SERT/NET inhibitors (VANH-36, MCI-225 – Phase II)
5-HT$_{1A}$ receptor agonists (F-13640 – Phase II)
5-HT$_{1B}$ receptor agonists (Anpirtoline – Phase I)
5-HT$_{1D}$ receptor agonists (ALX-0646 – Phase I, IS-159 – Phase II, Alniditan, Avitriptan – Phase III)
5-HT$_{1F}$ receptor agonists (LY-33437, LY-573144 – Phase II)
5-HT$_{1B/1D}$ receptor agonists (BMS-181885 – Phase I, Donitriptan – Phase II)
Non-selective 5-HT$_2$ receptor antagonists (Loxapine – Phase II, Dotarizine, Amperozide – Phase III)
5-HT$_{2A}$ receptor antagonists (Sergolexole – Phase II)
5-HT$_{2B}$ receptor antagonists (MT-500, BF-1 – Phase I)
5-HT$_3$ receptor antagonists (Bemesetron, Ricasetron – Phase II)

SERT/NET inhibitors (Amitriptyline, Venlafaxine, Duloxetine, *O*-Desmethylvenlafaxine)
5-HT$_{1B/1D}$ receptor agonists (Sumatriptan, Naratriptan, Zolmitriptan, Almotriptan, Frovatriptan, Rizatriptan, Eletriptan)
μ-opioid + SERT/NET inhibitor (Tramadol)

Figure 10. Lead compounds with affinity for a 5-HT receptor subtype under development for a pain indication classified based on the phase of development. Prous Science-Integrity database, September 2011.

At the peripheral level, 5-HT, acting in combination with inflammatory mediators, may ectopically excite and sensitize afferent nerve fibers, thus contributing to peripheral sensitization and hyperalgesia (Beck and Handwerker, 1974; Obata et al., 2000; Sommer,

2004). The role of the central 5-HT system in nociception, particularly the 5-HT descending pathway, has also been the subject of considerable research (Yoshimura and Furue, 2006). However, descending 5-HTergic pathways projecting into the dorsal horn not only suppress (descending inhibition) but may also potentiate (descending facilitation) nociceptive messages depending on the 5-HT receptor involved and its localization (Oyama et al., 1996; Millan, 2002; Suzuki et al., 2004; Dogrul et al., 2009). In addition to spinal mechanisms, an antinociceptive effect of 5-HT mediated through ascending components involving thalamic nuclei has been reported (Harte et al., 2005; Xiao et al., 2005).

The 5-HT_7 receptor is expressed in pain-related areas of the nervous system, particularly the thalamus (Stowe and Barnes, 1998; Neumaier et al., 2001; Martin-Cora and Pazos, 2004), the dorsal horn of the spinal cord (Meuser et al., 2002; Doly et al., 2005) and the dorsal root ganglia (DRG) (Pierce et al., 1996, 1997; Wu et al., 2001; Liu et al., 2005). Pierce et al. (1996, 1997) detected the presence of 5-HT_7 receptor messenger RNA in rat and human dorsal root ganglia and increased levels of 5-HT_7 receptor messenger RNA have been reported in rat DRG after bee venom- and complete Freund's adjuvant-induced inflammation (Wu et al., 2001; Liu et al., 2005). In addition, by immunohistochemistry, Doly et al. (2005) described the expression of 5-HT_7 receptors at the lumbar level in small and medium-sized DRG cells as well as in their central projections to the superficial laminae of the dorsal horn, which is consistent with a predominant role in nociception. 5-HT_7 receptor appeared predominantly on ''nociceptor-like'' small diameter neurons of the rat lumbar DRG. The majority of stained cell bodies were less than 30 μM in diameter, which corresponds to C-fiber neurons (Meuser et al., 2002) (see figure 11).

At the spinal cord, the immunoreactivity of the 5-HT_7 receptor antibody complex was localized in the superficial layers of the spinal cord dorsal horn, almost exclusively distributed in lamina I and II, where C- and A-delta-fiber type primary afferent nociceptors terminate. Substantially less immunoreactivity appeared in deeper layers, with little background staining elsewhere and mild to moderate staining of some motor neurons in the ventral horn. Electron microscopic examination of the dorsal horn revealed three main localizations: (1) postsynaptic localization on peptidergic cell bodies and in numerous dendrites, (2) presynaptic localization on unmyelinated and thin myelinated peptidergic fibers, and (3) on astrocytes.

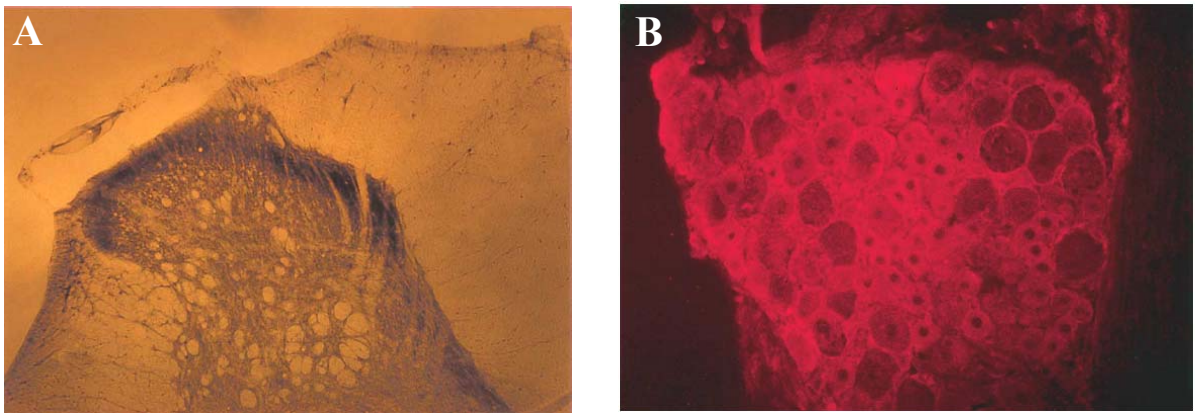


Figure 11. Immunoreactivity of the 5-HT_7 receptor localized in the superficial layers of the spinal cord dorsal horn **(A)** and immunoreactivity of the 5-HT_7 receptor localized in the rat lumbar dorsal root ganglion **(B).** From Meuser et al., 2002.

At the supraspinal level, the distribution of 5-HT_7 receptors in some cortical areas and several nuclei in the thalamus, midbrain, pons and medulla is also compatible with a role in pain control. However, the role played by the 5-HT_7 receptor in nociception has been poorly investigated, mainly because of the lack of selective 5-HT_7 receptor ligands. Indeed, the 5-HT_7 receptor has a ligand recognition profile resembling that of the 5-HT_{1A} receptor (Lovenberg et al., 1993; Sleight et al., 1995; Wilcox et al., 2001; Perrone et al., 2003), and some among the antinociceptive actions attributed to nonselective 5-HT_{1A} receptor agonists could, in fact, be mediated by 5-HT_7 receptors. It is, for instance, unclear how hyperalgesia can be promoted through activation of 5-HT_{1A} receptors by a direct action on primary afferents (Taiwo et al., 1992) when 5-HT_{1A} receptors couple negatively to adenylyl cyclase (Albert et al., 1990). In support of this, 5-HT in DRG is known to increase cAMP levels via activation of 5-HT_7 receptors, which shifts the voltage dependence of hyperpolarization-activated cation currents to more depolarized potentials and increases neuronal excitability (Cardenas et al., 1999). This led to the suggestion that 5-HT_7 receptors may modulate excitability, neurotransmitter release and firing patterns in certain subpopulations of sensory neurons (Cardenas et al., 1999).

The method of spinal cord dorsal horn c-Fos immunoreactivity has been shown to be a reliable marker for neuronal activity following noxious stimulation in the rat (Hunt et al., 1987; Bullitt, 1990). Recently, Meuser and coworkers (2002) showed that intra-articular injection of the mixed 5-HT_{1A}/5-HT_7 receptor agonist, 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT), induced c-Fos expression in the dorsal horn of the rat spinal cord and this effect was prevented by intra-articular administration of the non-selective

5-HT_7 receptor antagonist methiothepin (Meuser et al., 2002). This observation supports a role of the 5-HT_7 receptor in nociception at the periphery and suggests that 5-HT_7 receptor antagonists may have therapeutic usefulness in the treatment of inflammatory pain states.

The peripheral role of 5-HT_7 receptors in nociception was also investigated by Rocha-Gonzalez et al. (2005) in rats by means of the formalin test, in which formalin is injected into the paw to induce flinching behaviors. Microinjection of formalin was preceded by local administration of SB-269970 and/or 5-HT and 5-CT into the paw. Local administration of SB-269970 into the paw significantly reduced 1% formalin-induced flinching, while local 5-HT or 5-CT increased dose-dependently 0.5% formalin-induced nociceptive behavior. Furthermore, SB-269970 could locally counteract the pronociceptive effect of 5-CT. So, it was concluded that activation of the 5-HT_7 receptor in the periphery induces nociceptive behaviors and contributes to formalin-induced nociception. In agreement with this, the presence of 5-HT_7 receptors in myelinated and unmyelinated axons of the digital nerves in rat hindpaw was demonstrated by immunohistochemistry.

When spinal nociception is considered, the interpretation of 5-HT_7 receptor function becomes more complex. Data in the literature suggest that spinal activation of 5-HT_7 receptors is ultimately critical in the expression of opiate-induced nociception, likely through activation of descending inhibition. In this way, it had been demonstrated that the antinociceptive effect of morphine administered either systemically or into the rostroventromedial medulla (RVM) is blocked by intrathecal administration of the 5-HT_7 receptor antagonist SB-269970 (Dogrul and Seyrek, 2006; Dogrul et al., 2009). Similar conclusions were drawn in a study assessing the effects of systemic administration of tramadol and its major active metabolite O-desmethyltramadol (M1) to noxius stimulus (radiant heat tail-flick) and postoperative pain (plantar incision test). Intrathecal injection of SB-269970 and SB-258719 blocked both tramadol- and M1-induced antinociceptive and antihyperalgesic effect. These studies indicate that activation of spinal 5-HT_7 receptors plays a key role in the antinociceptive pathway activated by both morphine and tramadol.

On the other hand, Rocha-Gonzalez et al. (2005) suggested a pronociceptive role of spinal 5-HT_7 receptors in the formalin test. Despite administration of SB-269970 into the spinal cord did not affect nociceptive behavior in response to formalin, low doses of intrathecal (i.t.) 5-CT increased formalin-induced flinching, which was reduced by SB-269970. Thus, the results suggest that 5-HT_7 receptor activation in the spinal cord potentiates formalin-induced

nociception in the presence of low levels 5-CT. It should be noted here that high levels of i.t. administered 5-CT, in contrast to low 5-CT levels, decreased formalin-induced nociceptive behavior, which was partially inhibited by the 5-HT_{1A} receptor antagonist, WAY-100635. Unfortunately, the effect of SB-269970 was not mentioned here, so it is unclear if this antinociception is also mediated by spinal 5-HT_7 receptors. Therefore, it could be possible that spinal 5-HT_7 receptors exhibit biphasic activity, promoting and inhibiting nociception in the presence of low and high levels of 5-HT, respectively.

Besides peripheral and spinal 5-HT_7 receptors, supraspinal 5-HT_7 receptors have also been implicated in the modulation of nociception (Stowe and Barnes, 1998; Neumaier et al., 2001; Martin-Cora et al., 2004). Intracerebroventricular administration of the 5-HT_1/5-HT_2/5-HT_7 receptor antagonist, methiothepin blocked the antinociceptive effect of the non-steroidal antiinflammatory S-(+)-ketoprofen, as assessed by the pain-induced functional impairment model in the rat (Diaz-Reval et al., 2004). In another study, 8-OH-DPAT was shown to exert antinociceptive effects when injected into the medial thalamus, specifically the nucleus parafascicularis and the central lateral thalamic nucleus (Harte et al., 2005). In rats exposed to an electric shock to the tail, intrathalamic micro-injection of SB-269970 was shown to block the antinociceptive effect of 8-OH-DPAT which raised the threshold of the electrical tailshock intensity to trigger vocalizations. This action could also be counteracted by the selective 5-HT_{1A} antagonist WAY-100635. Interestingly, 5-HT_7 receptor activation in the thalamus only had an antinociceptive effect on vocalizations, which are pain behaviors organized at the medullar and forebrain level of the neuraxis, but did not suppress the spinal motor reflexes, which are triggered at the spinal level (Harte et al., 2005).

Taken together, available data appear to suggest differing roles of the 5-HT_7 receptor in peripheral versus central pain mediation. It was thus possible that 5-HT_7 receptors could elicit a spectrum of pro and antinociceptive actions depending on the localization of the receptor and the stimulus quality and modality. Indeed, different regions in the periphery and central nervous system have been suggested to be involved in modulation of nociception by serotonin, and a role for 5-HT_7 receptors in the control of pain has been outlined. Future studies will in all likelihood help to clarify fully the relationship between the 5-HT_7 receptor and pain.

2.3.1. Patents surrounding 5-HT$_7$ receptor ligands on pain

Patents of 5-HT$_7$ receptor ligands are mainly focused on anxiety (22), depression (21), psychosis (24), sleep disorders (15), cognitive disorders (12) and pain (11) (see figure 12A).

The intellectual property of these patents mostly belongs to the pharmaceutical company Esteve (12 patents), followed by other companies such as Astellas Pharma (6), Janssen (5), Medivation (5) (see figure 12B).

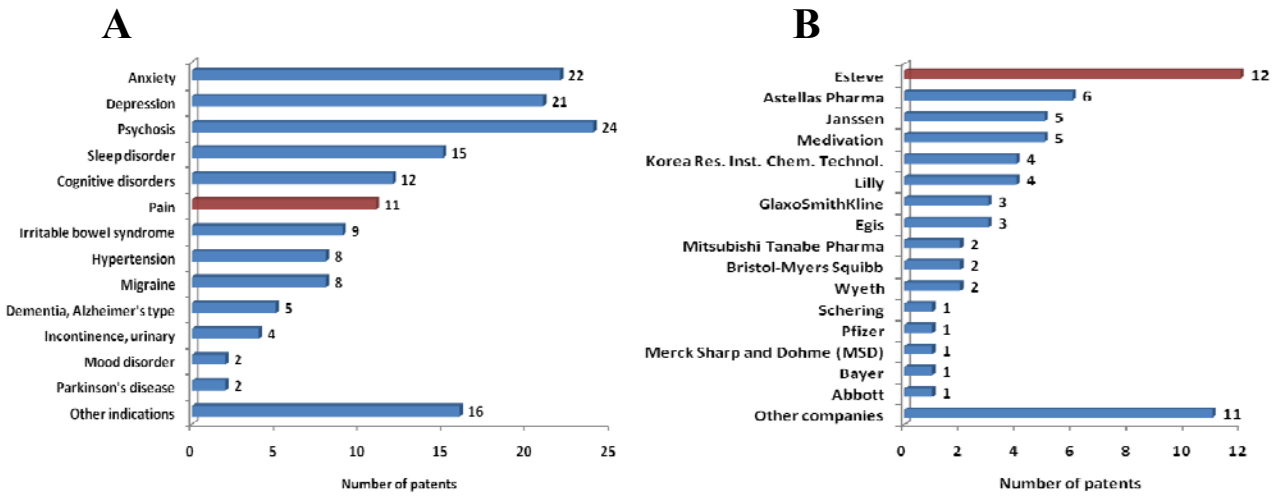


Figure 12. Number of patents of lead 5-HT$_7$ receptor compounds under development classified by therapeutic indication **(A)** or by company **(B)**. Prous Science-Integrity database, September 2011.

Regarding the therapeutic pain indication, the intellectual property of most 5-HT$_7$ receptor compounds patented for analgesia is owned by the pharmaceutical company Esteve (9), followed by Astellas pharma (3), Lilly (3), Janssen (1) and GlaxoSmithKline (1) (see figure 13A).

Most patented lead compounds under development for pain are 5-HT$_7$ receptor antagonists (9) (see figure 13B). There are other lead patented compounds under development for pain with affinity also for other receptors (5-HT$_{2B}$, 5-HT$_6$ and opioid).

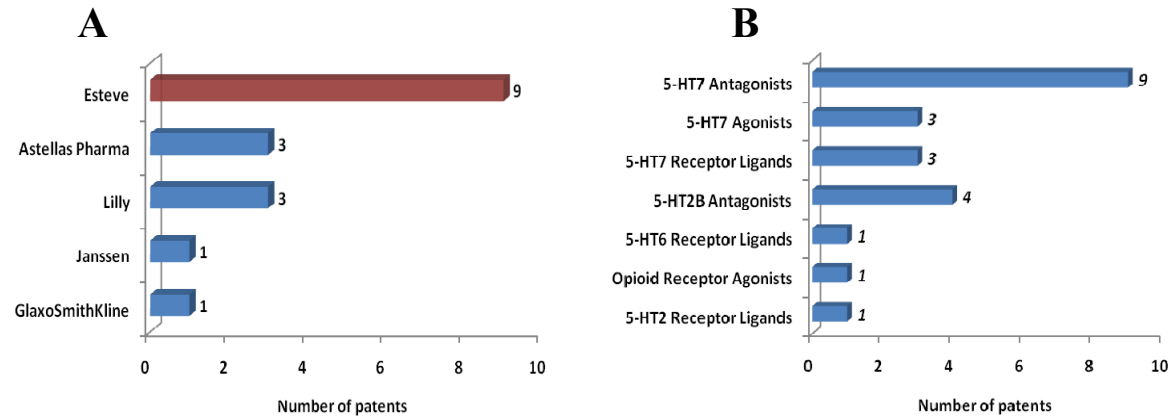


Figure 13. Number of patents of lead 5-HT$_7$ receptor compounds under development for pain indication, classified by company **(A)** or by their functionality and selectivity **(B)**. Prous Science-Integrity database, September 2011.

The first pain-related patent involving the 5-HT$_7$ receptor appeared in 1999, 9 years after the first 5-HT$_7$ receptor-related patent. An increase in pain-related 5-HT$_7$ receptor patents was experimented in 2007, with seven patents (see figure 14).

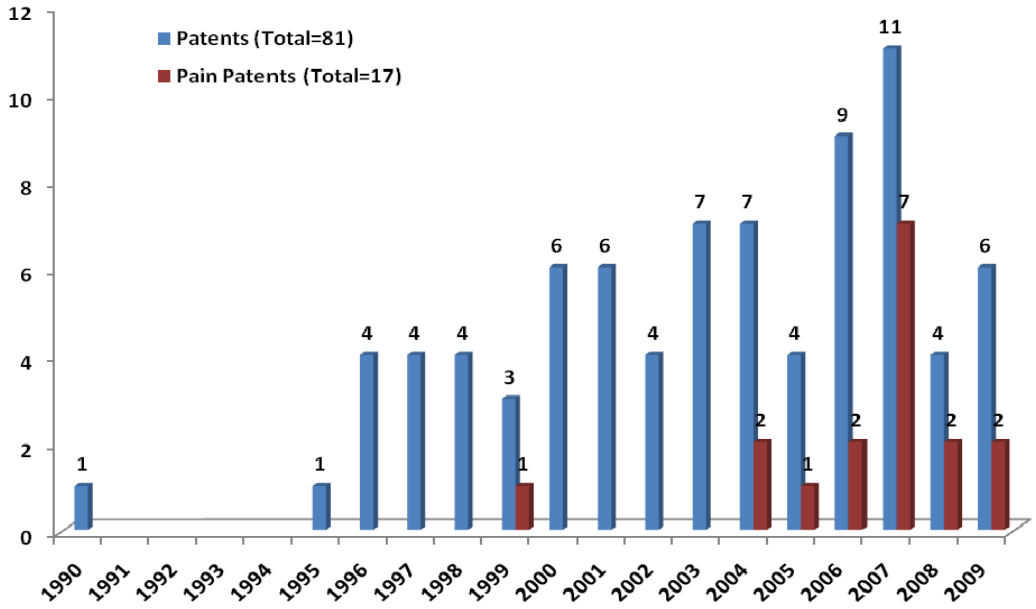


Figure 14. Comparison of patents involving 5-HT$_7$ receptor for pain indication versus all 5-HT$_7$ receptor patents. Prous Science-Integrity database, September 2011.

III. HYPOTHESIS

Serotonin has been recognized as one of the main neurotransmitters participating in pain transmission, processing and control. The presence of 5-HT_7 receptors in pain-related areas of the nervous system, particularly the thalamus, the dorsal horn of the spinal cord, and the dorsal root ganglia suggested a role of 5-HT_7 receptors in nociception (Meuser et al., 2002; Doly et al., 2005). However, data supporting a role for 5-HT_7 receptors in nociception were scarce and came mostly from studies using non-selective ligands. It was particularly remarkable the absence of data in chronic pain (i.e., neuropathic pain).

The only two studies addressing the peripheral role of 5-HT_7 receptors in nociception suggested a pronociceptive role of 5-HT_7 receptors in the periphery (Meuser et al., 2002; Rocha-González et al., 2005). However, contradictory results had been reported regarding the spinal contribution of 5-HT_7 receptors in nociception (Rocha-González et al., 2005; Dogrul and Seyrek, 2006). Supraspinal areas, particularly the thalamus, had also been suggested to be involved in modulation of nociception by serotonin, and a role for 5-HT_7 receptors had been outlined (Díaz-Reval et al., 2004; Harte et al., 2005).

On the other hand, 5-HT_7 receptors have a ligand recognition profile resembling that of 5-HT_{1A} receptors. Accordingly, the antinociceptive effects attributed to 5-HT_{1A} receptors could be mediated, in part, by 5-HT_7 receptors (Harte et al., 2005).

Based on previous findings and in line with the objectives of the 5-HT_7 receptor project at Esteve, focused on the discovery of 5-HT_7 receptor ligands for the treatment of neuropathic pain, in the studies contained herein we **examined the possibility that selective 5-HT_7 receptor agonists or antagonists could be interesting drugs for the treatment of pain.**

IV. OBJECTIVES

In order to investigate the role of 5-HT_7 receptors in nociception and the therapeutic interest of new selective 5-HT_7 receptor ligands for the treatment of pain, the following objectives were intended:

1.- To study the role of 5-HT_7 receptors in nociceptive pain conditions and their interaction with the opioidergic system. For this purpose, the effects of systemic 5-HT_7 receptor agonists and antagonists per se or co-administered with morphine were evaluated in response to a noxious thermal stimulus using the tail-flick test in mice.

2.- To investigate the role of 5-HT_7 receptors in pain conditions involving central sensitization. For this purpose, the effects on mechanical hypersensitivity and thermal hyperalgesia of 5-HT_7 receptor agonists and antagonists administered systemically were evaluated in neurogenic (capsaicin test), inflammatory (formalin test) and neuropathic (nerve injury) pain models in mice.

3.- To explore the site and mechanism of action involved in the control of pain of 5-HT_7 receptors. For this purpose, 5-HT_7 receptor agonists were administered peripherally into the paw and intrathecally into the spinal cord in neurogenic and neuropathic pain conditions in rats. In addition, the expression of 5-HT_7 receptors in the spinal cord and its co-localization with GABAergic neurons were also investigated by immunohistochemistry.

4.- To determine the *in vivo* specificity of 5-HT_7 receptor agonists for the 5-HT_7 receptor. For this purpose, the effects of systemic 5-HT_7 receptor agonists on the phase II of the formalin test were evaluated in 5-HT_7 receptor knockout and wild-type mice.

V. SUMMARY OF METHODS

We took advantage of genetic (5-HT_7 receptor knockout mice) and pharmacological (5-HT_7 receptor ligands) approaches to examine the role of 5-HT_7 receptors in the control of nociception. Experiments were performed using mice or rats subjected to direct nerve injury, exposure to acute noxious thermal stimuli, or the application of irritants (capsaicin or formalin) to cause pain. Nociceptive pain was measured in naive animals exposed to acute noxious thermal stimuli using hot plate, tail flick and tail immersion tests (see figure 15A-C). Pain-related behaviors (mechanical allodynia/hypersensitivity by means of automatic or manual von Frey test, and thermal hyperalgesia by means of plantar/Hergreaves test) were evaluated on neurogenic (capsaicin injection into the paw) and neuropathic pain (partial sciatic nerve ligation in mice and spared nerve injury in rats) conditions (see figure 15D-F). The licking/biting time was recorded after formalin injection to evaluate phase I (0 - 10 min) and phase II (15 - 45 min).

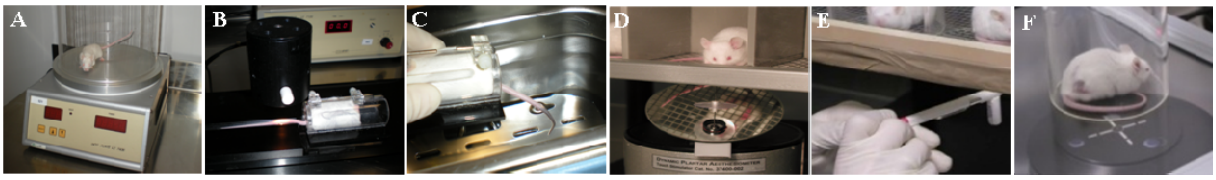


Figure 15. Nociceptive behavioral tests using different apparatus: hot plate **(A)**, tail flick **(B)** and tail immersion **(C)**, automatic von Frey **(D)**, manual von Frey **(E)** and Plantar test **(F)**.

Drugs were administered systemically (oral, intraperitoneal or subcutaneous), peripherally into the paw or intrathecally into the spinal cord; and tested following different experimental approaches (see Results section). Two new selective and potent 5-HT_7 receptor agonists, E-55888 and E-57431, developed by Esteve, were herein described by the first time. Other reported 5-HT_7 receptor ligands were also used (SB-258719 or SB-269970 as 5-HT_7 receptor antagonists, and AS-19 or MSD-5a as 5-HT_7 receptor agonists). The binding profile and cAMP measurements were examined for compounds tested *in vivo*. In addition, to investigate target-specific effects of the 5-HT_7 receptor agonists tested on pain, their effects on the formalin test were examined in 5-HT_7 receptor knockout and wild-type mice.

Statistical analysis was made to test differences among several groups using ANOVA, post-hoc and Student's t test. Data were presented as mean responses in the corresponding unit ± SEM or as mean percentage of analgesia respect to the corresponding control group. The level of significance was set at $p < 0.05$. Data analysis and graphing were done using GraphPad Prism software (version 4.0; GraphPad Software, Inc., USA).

VI. RESULTS

6.1. 5-HT$_7$ receptors in acute nociception and opiate-induced analgesia

Potentiation of morphine analgesia by adjuvant activation of 5-HT$_7$ receptors. *J* Pharmacol *Sci 2011; 116:388-391*

Alex Brenchat, Miriam Ejarque, Daniel Zamanillo, José Miguel Vela, Luz Romero

Potenciació de l'efecte analgèsic de la morfina degut a l'activació adjuvant dels receptors 5-HT$_7$

En aquest estudi vàrem observar que l'activació dels receptors 5-HT$_7$ potenciava els efectes antinociceptius de la morfina enfront un estímul tèrmic nociu utilitzant el test del tail-flick.

Malgrat l'agonista selectiu del receptor 5-HT$_7$ E-55888 no tenia efectes per si sol en el tail-flick, vàrem descobrir que era capaç de potenciar els efectes antinociceptius de la morfina en el tail-flick. A més, vam comprovar que la potenciació observada era deguda a efectes farmacodinàmics i no farmacocinètics ja que no vam observar diferències en la concentració de morfina i del seu metabòlit M3G, tant en plasma com en cervell quan la morfina era coadministrada amb l'agonista selectiu del receptor 5-HT$_7$ E-55888. Els resultats d'aquest treball ens van permetre demostrar que els agonistes selectius pel receptor 5-HT$_7$ podrien ser d'interès terapèutic en l'alleugeriment del dolor com a adjuvants d'altres analgèsics com els opioides.

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Short Communication

Potentiation of Morphine Analgesia by Adjuvant Activation of 5-HT$_7$ Receptors

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Abstract. Spinal blockade of 5-HT$_7$ receptors has been reported to inhibit the antinociceptive effect of opioids. In this study, we found that subcutaneous administration of the selective 5-HT$_7$ receptor agonist E-55888 (10 mg/kg) or the antagonist SB-258719 (5 mg/kg) exerted no effect on the tail-flick test in mice. However, E-55888, but not SB-258719, increased (2.6-fold) the analgesic potency of oral morphine. The potentiating effect exerted by E-55888 was prevented by SB-258719. A pharmacokinetic interaction was discarded as morphine plasma and brain concentrations were not significantly modified when co-administered with E-55888. These results reinforce the involvement of 5-HT$_7$ receptors in opioid analgesia and point to a potential use of 5-HT$_7$ receptor agonists as adjuvants of opioid analgesia.

Keywords: 5-HT$_7$ receptor, analgesia, drug combination

Available data in the literature suggest that spinal activation of 5-HT$_7$ receptors is ultimately critical in the expression of opiate-induced analgesia, likely through activation of descending inhibition. The antinociceptive effect of morphine administered either systemically or into the rostroventromedial medulla was blocked by intrathecal administration of the selective 5-HT$_7$ receptor antagonist SB-269970 (1, 2). Similarly, intrathecal injection of the 5-HT$_7$ receptor antagonists SB-269970 and SB-258719 has been recently shown to block the antinociceptive and antihyperalgesic effects of both tramadol and its opioid metabolite M1 (3). Accordingly, both tramadol- and M1-induced antinociceptive effects were found to be significantly diminished in 5-HT lesioned mice (3). Because selective 5-HT$_7$ receptor antagonists reversed the antinociceptive effects of morphine, it can be hypothesized that activation of 5-HT$_7$ receptors might enhance morphine-induced antinociception. To test this hypothesis, we evaluated the analgesic effect of morphine co-administered with the selective 5-HT$_7$ receptor agonist E-55888, the antagonist SB-258719, or both.

Experiments were performed in 6- to 8-week-old male, CD1 mice (Charles River, Spain). Animals were housed in groups of five, provided with food and water ad libitum, and kept in controlled laboratory conditions with the temperature maintained at 21 ± 1°C and light in 12-h cycles (on at 07:00 h and off at 19:00 h). Experiments were carried out in a sound-attenuated, air-regulated experimental room. All experimental procedures and animal husbandry were conducted according to ethical principles for the evaluation of pain in conscious animals (4) and to ethical guidelines of the European Communities Council Directive of 24 November 1986 (86/609/ECC). The experimental work received approval by the Local Ethical Committee.

Drugs used for treatments were as follows: E-55888 (dimethyl-{2-[3-(1,3,5-trimethyl-1*H*-pyrazol-4-yl)-phenyl]ethyl}amine dihydrochloride), developed by Esteve (5); SB-258719 (*N*,3-dimethyl-*N*-[1(*R*)-methyl-3-(4-methyl-1-piperidinyl)propyl]benzenesulfonamide hydrochloride) (6), synthesized in our laboratory for the purpose of this study; and morphine hydrochloride purchased from Alcaliber (Madrid, Spain). E-55888 is a potent selective 5-HT$_7$ receptor agonist whose affinity, functionality and selectivity profile has been previously described (5), and SB-258719 is a potent selective 5-HT$_7$ receptor antagonist used as a pharmacological tool (6, 7).

Drugs were dissolved in physiological saline and administered in a volume of 5 ml/kg through the subcutane-

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ous (s.c.) route or 10 ml/kg through the oral (p.o.) route. Doses of morphine (4 – 64 mg/kg, p.o.), the selective 5-HT$_7$ receptor antagonist SB-258719 (5 mg/kg, s.c.) and the selective 5-HT$_7$ receptor agonist E-55888 (10 mg/kg, s.c.) are referred to their salt forms. All experiments were performed under blind conditions in independent groups of mice.

The tail-flick test was performed as a pharmacodynamic behavioral endpoint to study antinociceptive effects of treatments. Animals were placed in a loose Plexiglas restrainer with its tail extending through a hole on a tail-flick apparatus (Panlab, Barcelona, Spain), as previously described (8). A photobeam was placed on the tail about 4 cm from the tip. The latency to the tail-flick response 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 3 s. A cut-off latency time of 10 s was imposed to avoid damage of tail tissues.

To elucidate a possible pharmacokinetic interaction between morphine and E-55888, plasma and brain levels of morphine and its metabolite morphine-3-glucuronide (M3G) were quantified and compared (morphine treated group vs. co-treated group receiving morphine plus E-55888). Cardiac blood was collected and the brain was removed 45 min after treatments in mice anesthetized with isoflurane. The blood was centrifuged and the serum and whole brain (without the cerebellum) were stored at −80°C until analysis. Morphine and its metabolite M3G were extracted from plasma (100 μl) and brain homogenate (0.4 mol/l perchloric acid containing 0.1% ascorbic acid and 0.1% EDTA, 0.3 – 0.4 g of tissue/500 μl) and quantified by reversed-phase high-performance liquid chromatography (HPLC) – electrospray ionization – tandem mass spectrometry LC – ESI – MS – MS, according to previously described procedures (9). Deuterated (d3) analogues of each analyte were used as internal standards (plasma: 0.125 ng/μl d3-morphine and 0.25 ng/μl of d3-M3G; brain homogenates: 0.0625 ng/μl of both deuterated compounds). Chromatographic separations were performed on a (5 μm, 2.1 mm i.d. × 150 mm) Atlantis dC18 column (Waters, Barcelona, Spain) and a Hydro-RP 100A (2.5 μm, 2 mm i.d. × 100 mm) column (Phenomenex, Torrance, CA, USA) for plasma (sample injection volume of 5 μl) and brain (sample injection volume of 20 μl), respectively. Sample analysis was performed on a triple quadrupole mass spectrometer (PE Sciex API 3000; Applied Biosystems, Madrid, Spain), operating in electrospray mode, with a standard nebulizer ion-spray source. The standard curves were highly linear ($R^2 > 0.9996$) over the concentration range of each analyte. The limit of quantification was 0.5 ng/ml, and the coefficient of variation was < 10%.

Tail latency responses were expressed in seconds ± S.E.M. or in percentage of analgesia calculated with respect to the corresponding control group without morphine (test latency − control group latency / cut-off latency − control group latency) × 100 ± S.E.M. Plasma levels were expressed in ng/ml and brain levels, in ng/g tissue.

Statistical analyses to test differences among several groups were made by ANOVA followed by the Bonferroni multiple comparison test. A non-paired Student's *t*-test was used to test differences between two groups. The level of significance was set at $P < 0.05$. In the dose–response study, ED_{50} (dose of morphine that produced half of its maximal effect) was calculated from the concentration–response curves using non-linear regression analysis. Data analysis and graphing were done using GraphPad Prism software (v4.0; GraphPad Software, Inc., San Diego, CA, USA).

Subcutaneous administration of the selective 5-HT$_7$ receptor antagonist SB-258719 (5 mg/kg, s.c.) or the selective 5-HT$_7$ receptor agonist E-55888 (10 mg/kg,

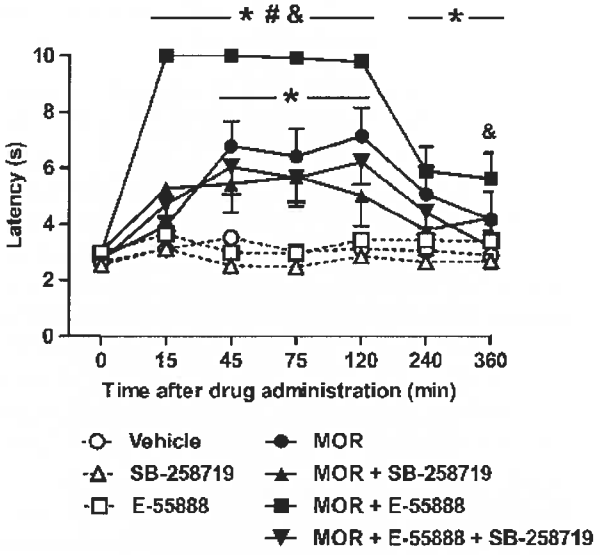

Fig. 1. Time-course potentiation of morphine-induced analgesia by the selective 5-HT$_7$ receptor agonist E-55888 in the tail-flick test. Morphine showed significant antinociceptive effects from 45 to 120 min after oral administration (32 mg/kg). The analgesic effect of oral morphine (32 mg/kg) was significantly potentiated from 15 to 120 min after co-administration with the selective 5-HT$_7$ receptor agonist E-55888 (10 mg/kg, s.c.). The potentiating effect of morphine-induced analgesia by E-55888 was significantly reversed by the selective 5-HT$_7$ receptor antagonist SB-258719 (5 mg/kg). $^*P < 0.05$ vs. vehicle-treated group; $^{\#}P < 0.05$ vs. morphine (MOR)-treated group; $^{\&}P < 0.05$ vs. MOR + E-55888 + SB-258719–treated group (ANOVA followed by Bonferroni multiple comparison test). Each point in the graph represents the tail-flick latency response expressed in seconds ± S.E.M. (n = 8 – 11 mice per group).

s.c.) did not exert significant effects on the tail-flick test in mice. However, the analgesic effect of oral morphine (32 mg/kg, p.o.) was dramatically potentiated from 15 to 120 min when co-administered with the selective 5-HT_7 receptor agonist E-55888. The selective 5-HT_7 antagonist SB-258719 was unable to significantly modify morphine analgesia, but reversed the potentiating effect exerted by E-55888, which confirmed the involvement of 5-HT_7 receptors (Fig. 1).

In order to quantify the degree of potentiation of morphine-induced analgesia by E-55888, dose–response curves were performed at a fixed time (45 min after drug administration). Morphine exerted a robust dose–response analgesic effect (ED_{50} = 31.64 mg/kg, p.o.) to a noxious heat stimulus using the tail-flick test (Fig. 2). The co-administration of oral morphine with the selective 5-HT_7 agonist E-55888 (10 mg/kg, s.c.) improved significantly the analgesic potency (ED_{50}) of morphine (2.6 times), from 31.64 to 12.22 mg/kg (Fig. 2). On the other hand, no significant differences in the concentration of morphine or its metabolite M3G in the plasma and brain were observed when morphine (32 mg/kg, p.o.) was co-administered with E-55888 (10 mg/kg, s.c.) (Fig. 3).

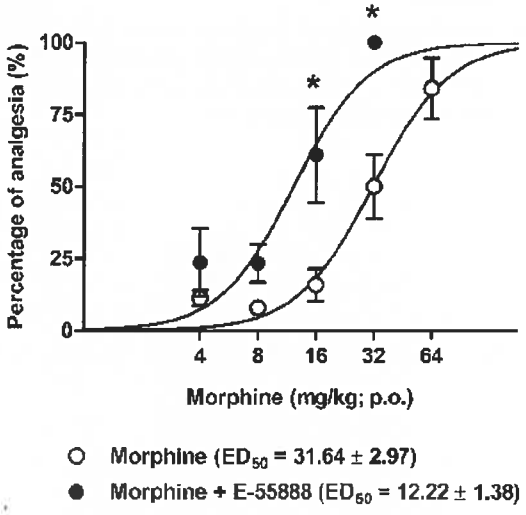

Fig. 2. Dose–response potentiation of morphine analgesia by the selective 5-HT_7 receptor agonist E-55888 in the tail-flick test. Co-administration of different doses of morphine (4 – 64 mg/kg, p.o.) and the selective 5-HT_7 receptor agonist E-55888 (10 mg/kg, s.c.) significantly improved (*$P < 0.05$, Student *t*-test) the analgesic potency (ED_{50}) of morphine (2.6 times), from 31.64 to 12.22 mg/kg. Analgesic efficacies of morphine at doses of 16 and 32 mg/kg were significantly improved (*$P < 0.05$, ANOVA followed by the Bonferroni multiple comparison test) when co-administered with E-55888. Each point in the graph represents the mean percentage of analgesia calculated respect to the corresponding control group without morphine (test latency – control group latency / cut-off latency – control group latency) × 100 ± S.E.M. (n = 8 – 11 mice per group).

Despite having previously described that systemic administration of 5-HT_7 receptor agonists induced antiallodynic and antihyperalgesic effects in pain conditions involving central sensitization (5, 10), we observed in this study that subcutaneous administration of the selective 5-HT_7 receptor agonist E-55888 (10 mg/kg) did not exert a significant effect on acute thermal nociception in the tail-flick test. However, a clear 5-HT_7 receptor–mediated potentiation of morphine analgesia in the tail-flick test was found when E-55888 was co-administered with morphine. Our results suggest no pharmacokinetic interaction between morphine and the selective 5-HT_7 receptor agonist E-55888 and support a pharmacodynamic interaction responsible for the potentiation of morphine antinociception. In fact, opiate-induced analgesia has been suggested to recruit 5-HT_7 receptors to contribute to the descending pain inhibitory pathway from the rostroventromedial medulla (1). Previous data reported a reversion of morphine analgesia by intrathecal administration of selective 5-HT_7 antagonists (1 – 3). However, in our experimental conditions, subcutaneous co-administration of the selective 5-HT_7 antagonist SB-258719 did not significantly reduce oral morphine-induced analgesia. This could be related to differences in the route of administration (intrathecal vs. systemic), the systemic route recruiting multiple putative sites within the pain trans-

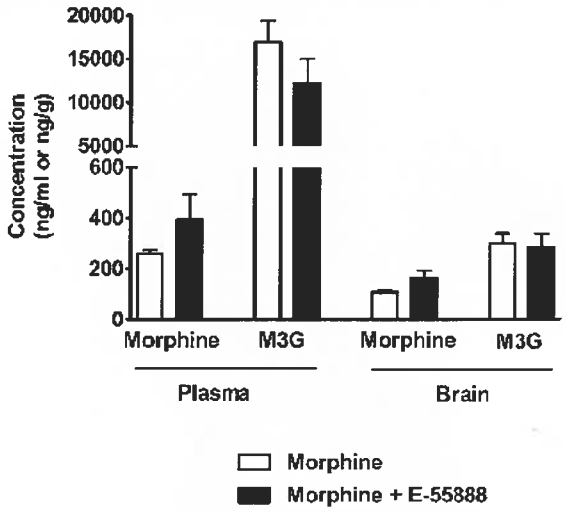

Fig. 3. Quantification of morphine and its metabolite M3G in plasma and brain in the morphine-treated group and the co-treated group of morphine with the selective 5-HT_7 receptor agonist E-55888. No significant differences ($P > 0.05$, Student *t*-test) in the concentration of morphine or its metabolite M3G in the plasma and the brain were observed when morphine (32 mg/kg, p.o.) was co-administered with the selective 5-HT_7 receptor agonist E-55888 (10 mg/kg, s.c.). Plasma levels are expressed in ng/ml and brain levels in ng/g tissue. Each bar is the mean ± S.E.M. (n = 6 mice per group).

mission pathway (periphery, spinally, and supraspinally), as well as to differences in the actual concentrations reached in the spinal cord.

Altogether, the present results reinforce the role played by 5-HT$_7$ receptors in mediating opioid analgesia. In addition, our results indicate that systemic administration of a selective 5-HT$_7$ receptor agonist *per se* is not enough to reproduce the antinociception exerted by opioids in acute thermal nociceptive models (i.e., tail-flick test), in line with the knowledge involving multiple molecular mechanisms, cellular targets, and pathways/locations in opioid analgesia.

This work points to a new potential use of 5-HT$_7$ receptor agonists for pain alleviation as adjuvants with other analgesics, particularly opioids (i.e., morphine). Nevertheless, this study is limited to acute thermal nociceptive pain conditions. Further studies in other different types of pain, including those involving central sensitization would be particularly interesting.

Acknowledgment

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6.2. 5-HT$_7$ receptors in neurogenic pain

5-HT$_7$ receptor activation inhibits mechanical hypersensitivity secondary to capsaicin sensitization in mice. *Pain 2009;141:239-247*

Alex Brenchat, Luz Romero, Mónica García, Marta Pujol, Javier Burgueño, Antoni Torrens, Michel Hamon, José Manuel Baeyens, Helmut Buschmann, Daniel Zamanillo, José Miguel Vela

L'activació del receptor 5-HT$_7$ inhibeix la hipersensibilitat mecànica secundària a la sensibilització per capsaicina en ratolins

Aquest treball tenia com objectiu estudiar el paper potencial del receptor 5-HT$_7$ en la nocicepció secundària a la injecció intraplantar de la capsaicina en els ratolins. La capsaicina provoca una hipersensibilitat de les neurones de l'asta dorsal (sensibilització central). Amb aquest propòsit, vàrem avaluar l'efecte en la hipersensibilitat mecànica induïda per la capsaicina de diferents lligands selectius (agonistes del receptor 5-HT$_7$: AS-19, MSD-5a, E-55888; antagonistes del receptor 5-HT$_7$: SB-258719, SB-269970; l'agonista del receptor 5-HT$_{1A}$: F-13640; i l'antagonista del receptor 5-HT$_{1A}$: WAY-100635). Es va avaluar també el perfil in vitro d'afinitat i d'eficàcia intrínseca en estimular el receptor 5-HT$_7$, dels agonistes del receptor 5-HT$_7$ utilitzats. E-55888 va resultar ser un agonista complet en canvi el AS-19 i el MSD-5a es van comportar com agonistes parcials, amb un efecte màxim corresponent al 77% i 61%, respectivament. Els nostres resultats in vivo van mostrar que l'administració sistèmica d'agonistes del receptor 5-HT$_7$ produïa un efecte antinociceptiu dosi-depenent que era revertit pels antagonistes del receptor 5-HT$_7$, però no per un antagonista 5-HT$_{1A}$. L'ordre d'eficàcia in vivo (E-55888>AS-19>MSD-5a) va resultar correspondre a les seves eficàcies in vitro com agonistes del receptor 5-HT$_7$. Contràriament als agonistes, l'administració dels antagonistes del receptor 5-HT$_7$ promovia la hipersensibilitat mecànica, reforçant la implicació del receptor 5-HT$_7$ en el control de la hipersensibilitat mecànica induïda per capsaicina. Els resultats del present treball ens van evidenciar el paper inhibidor de la serotonina en el control de la nocicepció a través de l'activació dels receptors 5-HT$_7$, i ens van suggerir l'interès terapèutic dels agonistes del receptor 5-HT$_7$ en el camp de l'analgèsia.

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5-HT_7 receptor activation inhibits mechanical hypersensitivity secondary to capsaicin sensitization in mice

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ABSTRACT

This work aimed to evaluate the potential role of the 5-HT_7 receptor in nociception secondary to a sensitizing stimulus in mice. For this purpose, the effects of relevant ligands (5-HT_7 receptor agonists: AS-19, MSD-5a, E-55888; 5-HT_7 receptor antagonists: SB-258719, SB-269970; 5-HT_{1A} receptor agonist: F-13640; 5-HT_{1A} receptor antagonist: WAY-100635) were assessed on capsaicin-induced mechanical hypersensitivity, a pain behavior involving hypersensitivity of dorsal horn neurons (central sensitization). For the 5-HT_7 receptor agonists used, binding profile and intrinsic efficacy to stimulate cAMP formation in HEK-293F cells expressing the human 5-HT_7 receptor were also evaluated. AS-19 and E-55888 were selective for 5-HT_7 receptors. E-55888 was a full agonist whereas AS-19 and MSD-5a behaved as partial agonists, with maximal effects corresponding to 77% and 61%, respectively, of the cAMP response evoked by the full agonist 5-HT. Our *in vivo* results revealed that systemic administration of 5-HT_7 receptor agonists exerted a clear-cut dose-dependent antinociceptive effect that was prevented by 5-HT_7 receptor antagonists, but not by the 5-HT_{1A} receptor antagonist. The order of efficacy (E-55888 > AS-19 > MSD-5a) matched their *in vitro* efficacy as 5-HT_7 receptor agonists. Contrary to agonists, a dose-dependent promotion of mechanical hypersensitivity was observed after administration of 5-HT_7 receptor antagonists, substantiating the involvement of the 5-HT_7 receptor in the control of capsaicin-induced mechanical hypersensitivity. These findings suggest that serotonin exerts an inhibitory role in the control of nociception through activation of 5-HT_7 receptors, and point to a new potential therapeutic use of 5-HT_7 receptor agonists in the field of analgesia.

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1. Introduction

Central sensitization is a term coined to describe the increased excitability of CNS nociceptive neurons triggered by persistent peripheral input [53]. This neoplastic change plays a role in clinically relevant states, such as inflammatory and neuropathic pain, which are characterized by abnormal pain perception and nociceptive hypersensitivity (i.e., hyperalgesia and allodynia). Many pain models revolve around the notion of sensitization as a mechanism underlying behavioral nociceptive hypersensitivity. One of them is the capsaicin model. Intradermal capsaicin injection results in a short period of acute pain (around 5 min duration) followed by a longer period of hyperalgesia and allodynia in both experimental animals [17] and humans [29]. Immediate capsaicin-induced pain involves the direct activation of peripheral nociceptors, whereas delayed hypersensitivity depends on the sensitization of spinal cord neurons [3,29,51] and involves the release of key neurotransmitters implicated in spinal sensitization, such as glutamate and substance P [20,28,46].

Many different neurotransmitter systems, ion channels and enzymes have been implicated in pain transmission, processing and control. Among them, serotonin (5-hydroxytryptamine [5-HT]) has been described to exert algesic or analgesic effects depending on the site of action and the receptor subtype it acts on [11,34]. At the peripheral level, 5-HT, acting in combination with inflammatory mediators, may ectopically excite and sensitize afferent nerve fibers, thus contributing to peripheral sensitization and hyperalgesia [4,36,47]. The role of the central 5-HT system in nociception, particularly the 5-HT descending inhibitory pathway, has also been the subject of considerable research [55]. However, descending pathways projecting into the dorsal horn not only suppress (descending inhibition) but may also potentiate (descending facilitation) nociceptive messages depending on the 5-HT receptor

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involved [34,37,49]. In addition, an antinociceptive effect of 5-HT mediated through an ascending component involving thalamic nuclei has been reported [19,54].

Much effort has been directed towards understanding the role of individual classes of 5-HT receptors in nociception [21,30,34]. 5-HT_{1A}, 5-$HT_{1B/1D}$, 5-HT_{2A}, 5-HT_{2C} and 5-HT_3 receptors have received most attention [2,7,11,25–27,36,37]. However, so far, the role played in nociception by the 5-HT_7 receptor has not been thoroughly investigated, mainly because of the lack of selective 5-HT_7 receptor ligands. Indeed, the 5-HT_7 receptor has a ligand recognition profile resembling that of the 5-HT_{1A} receptor [32,39,45,52], and some among the antinociceptive actions attributed to non-selective 5-HT_{1A} receptor agonists could, in fact, be mediated by 5-HT_7 receptors. The important progress achieved in the development of antagonists and agonists with high affinity and selectivity for the 5-HT_7 receptor now provides appropriate tools to really assess the potential role of this receptor in nociception and pain.

In the present study, we examined the potential contribution of the 5-HT_7 receptor subtype in modulating capsaicin-induced mechanical hypersensitivity in mice by employing recently developed ligands, including E-55888, which is a new highly selective and potent 5-HT_7 receptor agonist. The intrinsic efficacy as activators of human 5-HT_7 receptors and the selectivity of 5-HT_7 receptor agonists used in this study were also investigated.

2. Methods

2.1. Animals

Male CD1 mice (Harlan Ibérica, Barcelona) aged from 6 to 8 weeks old were used. Animals were housed in groups of five, provided with food and water *ad libitum* and kept under controlled laboratory conditions (temperature: 21 ± 1 °C; 12 h light/dark cycles with light on at 07:00 h). Experiments were carried out in a soundproof and air-regulated room. The number of mice ranged from 9 to 16 in each experimental group as 3–4 independent experiments using 3–4 mice per group were always performed. All experimental procedures and animal husbandry were conducted according to ethical principles for the evaluation of pain in conscious animals [56] and to ethical guidelines of the European Community on the Care and Use of Laboratory Animals (European Communities Council Directive of 24 November 1986, 86/609/ECC), and received approval by the Local Ethical Committee.

2.2. Drugs

Capsaicin (8-methyl-*N*-vanillyl 6-nonamide) was purchased from Sigma–Aldrich Co. (Spain) and dissolved in 1% DMSO (vehicle). Drugs used for treatments were E-55888 (dimethyl-{2-[3-(1,3,5-trimethyl-1H-pyrazol-4-yl)-phenyl]-ethyl}-amine dihydrochloride), developed by Laboratorios Esteve; AS-19 (dimethyl-[5-(1,3,5-trimethyl-1H-pyrazol-4-yl)-1,2,3,4-tetrahydro-naphthalen-2(*S*)-yl]-amine) [22,44]; MSD-5a (dimethyl-[2-(6-phenyl-pyridin-2-ylsulfanyl)-ethyl]-amine hydrochloride), corresponding to compound 5a in Thomson et al. [50]; SB-258719 (*N*,3-dimethyl-*N*-[1(*R*)-methyl-3-(4-methyl-1-piperidinyl)propyl]benzenesulfonamide) [13]; SB-269970 ((2*R*)-1-[(3-hydroxyphenyl)sulfonyl]-2-[2-(4-methyl-1-piperidinyl)ethyl]pyrrolidine hydrochloride) [31]; WAY-100635 (*N*-[2-[4-(2-methoxyphenyl)piperazin-1-yl]ethyl]-*N*-(2-pyridyl) cyclohexanecarboxamide trihydrochloride) [14]; and F-13640 (1-(3-chloro-4-fluorophenyl)-1-[4-fluoro-4-(5-methylpyridin-2ylmethylaminomethyl)piperidin-1-yl]methanone fumarate) [6]. AS-19 and SB-269970 were from Tocris Bioscience (United Kingdom), WAY-100635 from Sigma–Aldrich Co. and the other compounds used were synthesized at Laboratorios Esteve. AS-19 was dissolved in 1% DMSO and the other compounds were dissolved in physiological saline. Doses are expressed as the weight of free base. All the compounds and vehicles were administered in a volume of 5 ml/kg through the subcutaneous (s.c.) route. When two compounds were co-administered, they were injected s.c. in opposite flanks of the mice.

2.3. Binding profile and cAMP measurements

Binding affinities of E-55888 and AS-19 were determined through commercial radioligand binding assays by MDS Pharma and/or CEREP, as indicated, according to their standard assay protocols. Assays were done twice and concentrations were tested in duplicate. (For details regarding the specific receptors and experimental conditions see http://discovery.mdsps.com/Catalog/OnlineCatalog/Profiling/Assays/AssayList.aspx?id=5 and http://www.cerep.fr/Cerep/Users/pages/catalog/binding/catalog.asp).

For the determination of efficacy/potency of 5-HT_7 receptor agonists used in this study, cAMP measurements were performed using a system based on homogeneous time-resolved fluorescence (HTRF) [15] applied to human embryonic kidney (HEK)-293F cells that stably express the human 5-$HT_{7(a)}$ receptor, as previously described [43]. This assay is based on competition between cAMP produced by cells and cAMP-XL665 conjugate for binding onto monoclonal anti-cAMP-cryptate conjugate. The HTRF cAMP kit from CisBio (CisBio Int., France) was used according to the instructions of the manufacturer. Briefly, after overnight incubation in serum-free medium, cells were added to 96-well plates (20,000 cells/well) in Ham's F12 (Gibco, Invitrogen Co., Spain) incubation buffer (40 μl/well) containing 1 mM 3-isobutyl-1-methyl-xanthine (IBMX; Sigma–Aldrich Co.) and 20 μM pargyline (Sigma–Aldrich Co.). Then, 10 μl of different concentrations of the tested compound was added, and the plates were incubated for 30 min at 37 °C. The reaction was stopped by using a mixture of 25 μl of cryptate and 25 μl of XL-665 prepared in the lysis buffer supplied by the manufacturer. Plates were then incubated for an additional hour at room temperature, and cAMP contents were calculated from the 665 nm/620 nm ratio using a RubyStar Plate reader (BMG LabTech, Germany).

2.4. Capsaicin model

Sensitization by subplantar capsaicin injection results in hypersensitivity to both thermal and mechanical stimuli [17]. In this study, sensitization by subplantar capsaicin injection was used to assess the effect of several 5-HT_7 and 5-HT_{1A} receptor ligands on the response to a mechanical punctate stimulus.

Mice were injected with capsaicin (1 μg or 4 ng in 20 μl of 1% DMSO) or vehicle (1% DMSO) into the mid-plantar surface of the right hindpaw. Administration of 5-HT_7 and/or 5-HT_{1A} receptor ligands was performed 30 min before capsaicin injection, and withdrawal latencies to mechanical stimulus were determined 15 min after capsaicin injection.

2.4.1. Evaluation of mechanical hypersensitivity: von Frey test

Mechanical hypersensitivity was quantified by measuring the hindpaw withdrawal response latency to von Frey filament stimulation according to the protocol and evaluation procedure previously described [12]. Briefly, mice were placed into compartment enclosures in a test chamber with a framed metal mesh floor, and allowed to acclimate to their environment for 120 min before testing. The mechanical stimulus was applied onto the plantar surface of the right, capsaicin-injected, hindpaw from below the mesh floor by using an automated testing device (dynamic plantar aesthesiometer; Ugo Basile, Italy) that lifts a straight filament (0.5 mm in diameter). The filament exerted a fixed upward pressure (1 or 0.25 g) onto the plantar surface. When the animal with-

drew its hindpaw, the mechanical stimulus automatically stopped and the latency time was recorded. Latency was defined as the time from the onset of exposure to the filament to the cessation of the pressure when the sensor detected the paw withdrawal. A cut-off latency of 60 s was imposed. Paw withdrawal latencies were measured in triplicate for each animal.

2.4.2. Experimental approaches

When fixed at 1 g, the pressure exerted by the filament is insufficient to induce timely pain responses in normal control mice (in the absence of capsaicin), and thus prompt withdrawal responses found 15 min after sensitization with 1 μg capsaicin reflect mechanical hypersensitivity (see Section 3 and Fig. 2). This approach enabled us to evaluate the possible antinociceptive effect of 5-HT_7 and 5-HT_{1A} receptor ligands.

Alternatively, in some experiments, only 4 ng (instead of 1 μg) of capsaicin was injected into the paw and the pressure exerted by the filament was reduced to 0.25 g instead of to 1 g. Mice receiving this minor amount of capsaicin (250 times less than that used to induce hypersensitivity) did not display hypersensitivity when mechanically stimulated with a low upward pressure of 0.25 g (see Section 3 and Fig. 7). Dose–response curves of capsaicin applying increasing pressures against the plantar surface were tested using the electronic von Frey device to identify the maximal dose of capsaicin (4 ng) and pressure (0.25 g) that were unable to induce withdrawal responses (data not shown). This approach was used to test the possibility that treatments could exert a pronociceptive effect.

2.5. Data analysis

Data are presented as mean paw withdrawal latency responses in seconds ± SEM. Statistical analysis to test differences among several groups was made using an initial one-way analysis of variance (ANOVA) followed by the Newman-Keuls multiple comparison test. A non-paired Student's *t* test was used to test differences between two groups. The level of significance was set at $P < 0.05$. In the *in vitro* experiments looking for intrinsic efficacy (cAMP formation), the values of E_{max} (maximum enhancement of cAMP levels) and EC_{50} (concentration of 5-HT_7 receptor agonist that produced half of its maximal enhancement of cAMP levels) were calculated from the concentration–response curves using nonlinear regression analysis. Similarly, in the *in vivo* experiments, E_{max} (maximum effect) and ED_{50} (dose of 5-HT_7 receptor agonist that produced half of its maximal effect) values were calculated from the concentration–response curves using nonlinear regression analysis. Data analysis and graphing were done using GraphPad Prism software (version 4.0; GraphPad Software, Inc., USA).

3. Results

3.1. Selectivity, efficacy and potency of 5-HT_7 receptor agonists used in the study

In this study, pharmacological investigations aimed at addressing the involvement of the 5-HT_7 receptor in the control of hypersensitivity following capsaicin sensitization. Compounds used in this study cross the blood–brain barrier (data not shown), and information on the selectivity (binding profile), efficacy and potency of the pharmacological tools has been compiled in this section.

E-55888 is a new highly selective, potent 5-HT_7 receptor agonist with high efficacy. It showed high affinity for 5-HT_7 receptors (K_i = 2.5 nM) with significant affinity for 5-HT_{1A} (K_i = 700 nM) and no significant affinity (K_i > 1 μM or% inhibition at 10^{-6} M lower than 50%) for other 5-HT receptor subtypes and 170 additional targets including receptors, transporters and ion channels (*in vitro* binding screening packages by CEREP and MDS Pharma Services) (Table 1). When tested in a functional assay, E-55888 concentra-

Table 1
Binding profiles of the 5-HT_7 receptor agonists, E-55888, AS-19 and MSD-5a.

Receptor	Affinity [K_i (nM)]		
	E-55888	AS-19	MSD-5a
h5-HT_{1A}	700 (280×)	89.7 (149.5×)	16 (28.6×)
r5-HT_{1B}	n.s.	490 (816.6×)	–
b5-HT_{1D}	n.s.	6.6 (11×)	–
r5-HT_1 non-selective	–	48.1 (80.2×)	–
h5-HT_{2A}	n.s.	n.s	320 (533.3×)
h5-HT_{2B}	n.s.	n.s.	–
h5-HT_{2C}	n.s.	n.s.	–
r5-HT_2 non-selective	–	n.s.	–
h5-HT_3	n.s.	n.s.	–
h5-HT_{4e}	n.s.	–	–
gp5-HT_4	–	n.s.	–
h5-HT_{5A}	n.s.	98.5 (164.2×)	–
h5-HT_6	n.s.	n.s.	–
h5-HT_7	2.5	0.6	0.6
h5-HT transporter (SERT)	n.s.	n.s.	–
hD_2	n.s.	n.s.	450 (750×)
hσ_1	n.s.	657 (1095×)	–
rI_2, central	n.s.	282 (470×)	–
Other receptors	n.s.[#]	n.s.[##]	–

n.s., not significant (K_i > 1 μM or % inhibition at 10^{-6} M lower than 50%); –, data not available.
b, bovine; gp, guinea pig; h, human; ha, hamster; m, mouse; r, rat; rb, rabbit.
Data obtained from CEREP, MDS Pharma Services and Thomson et al. [50].
Data in brackets following K_i values represent the affinity ratio *vs.* 5-HT_7 receptors calculated as K_i for the tested receptor/K_i for 5-HT_7 receptor. It is expressed as number-fold higher (×) for 5-HT_7 than for the tested receptor.

[#] *In vitro pharmacology screening package including the following assays:* CEREP: A_1 (h), A_{2A} (h), A_3 (h), α_1 (non-selective) (r), α_2 (non-selective) (r), β_1 (h), β_2 (h), AT_1 (h), AT_2 (h), BZD (central) (r), BZD (peripheral) (r), BB (non-selective) (r), B_2 (h), CGRP (h), CB_1 (h), CCK_A (CCK_1) (h), CCK_B (CCK_2) (h), D_1 (h), D_3 (h), $D_{4.4}$ (h), D_5 (h), ET_A (h), ET_B (h), GABA (non-selective) (r), GAL1 (h), GAL2 (h), PDGF, CXCR2 (IL-8B) (h), TNF-α (h), CCR1 (h), H_1 (h), H_2 (h), MC_4 (h), MT_1 (h), M_1 (h), M_2 (h), M_3 (h), M_4 (h), M_5 (h), NK_1 (h), NK_2 (h), NK_3 (h), NPY_1 (h), NPY_2 (h), NT_1 (NTS1) (h), δ2 (DOP) (h), κ (KOP) (gp), μ (MOP) (agonist site) (h), ORL1 (NOP) (h) PACAP (PAC_1) (h), PCP (r), TXA_2/PGH_2 (TP) (h), P2X (r), P2Y (r), σ (non-selective) (r), sst (non-selective), VIP_1 ($VPAC_1$) (h), V_{1a} (h), Ca^{2+} channel (L, verapamil site) (phenylalkylamines) (r), K_v^+ channel (r), SK_{Ca}^+ channel (r), Na^+ channel (site 2) (r), Cl^- channel (r), DA transporter (DAT) (h), NE transporter (NET).

MDS Pharma Services: α_{1A} (r), α_{1B} (r), α_{1D} (h), α_{2A} (h), α_{2C} (h), β_3 (h), AM_1 (h), AM_2 (h), Aldosterone (r), Anaphylatoxin C5a (h), AR (r), APJ (h), ANF (gp), BB1 (h), BB2 (h), BB3 (h), B_1 (h), Calcitonin (h), Ca^{2+} channel L-Type benzothiazepine (r), Ca^{2+} channel L-Type dihydropyridine (r), Ca^{2+} channel N-Type (r), CB_2 (h), CCR2B (h), CCR4 (h), CCR5 (h), CX3CR1 (h), Colchicine (r), CRF1 (h), $D_{4.2}$ (h), EGF (h), EPOR (h), ERα (h), ERβ (h), GPR103 (h), GPR8 (h), $GABA_A$, muscimol central (r), $GABA_{B1A}$ (h), $GABA_{B1B}$ (h), Gabapentin (r), Glucocorticoid (h), Glutamate (Kainate) (r), Glutamate (AMPA) (r) Glutamate (NMDA agonism) (r), Glutamate (NMDA, glycine) (r), Glutamate (NMDA, polyamine) (r), Glycine strychnine-sensitive (r), GHS, Ghrelin (h), H_3 (h), H_4 (h), IP_3 (r), Insulin (r), IL-1 (r), IL-2 (r), IL-6 (h), Leptin (m), LTB_4 (h), Leukotriene ($CysLT_1$) (h), Leukotriene ($CysLT_2$) (h), MC_1 (h), MC_3 (h), MC_5 (h), MT_2 (h), Motilin (h), FPR1 (h), FPRL1 (h), NMU_1 (h), NMU_2 (h), Ach. Nic (h), Ach. Nic α_1 bungarotoxin (h), Ach. Nic α_7 bungarotoxin (r), Phorbol ester (m), PAF (h), K^+ channel (K_{ATP}) (ha), K^+ channel HERG (h), PR-B (h), CRTH2 (h) DP (h), EP_2 (h), EP_4 (h), Rolipram (r), RyR3 (r), σ1 (h), σ2 (r), sst1 (h), sst2 (h), sst3 (h), sst4 (h), sst5 (h), Thyroid hormone (r), TRH (r), TGF-β (m), Adenosine transp (gp), Choline transp (r), GABA transporter (r), Monoamine transporter (rb), Urotensin II (h), Vanilloid (r), VEGF (h), V_{1B} (h), V_2 (h), Vitamin D_3 (h).

[##] *In vitro pharmacology screening package including the following assays: MDS Pharma Services:* A_1 (h), A_{2A} (h), A_3 (h), α_{1A} (r), α_{1B} (r), α_{1D} (h), α_{2A} (h), β_1 (h), β_2 (h), B_1 (h), B_2 (h), Ca^{2+} channel (L-Type benzothiazepine) (r), Ca^{2+} channel (L-Type dihydropyridine) (r), Ca^{2+} channel (N-Type) (r), D_1 (h), D_3 (h), $D_{4.2}$ (h), ET_A (h), ET_B (h), EGF (h), ERα (h), $GABA_A$ (agonist site) (r), $GABA_A$ (benzodiazepine, central) (r), $GABA_B$ (non-selective) (r), Glucocorticoid (r), Glutamate (Kainate) (r), Glutamate (NMDA, agonism) (r), Glutamate (NMDA, glycine) (r), Glutamate (NMDA, phencyclidine) (r), H_1 (h), H_2 (h), H_3 (h), IL-1 (m), Leucotriene ($CysLT_1$) (h), M_1 (h), M_2 (h), M_3 (h), NPY_1 (h), NPY_2 (h), Ach. Nic. (h), δ opiate (OP1, DOP) (h), κ opiate (OP2, KOP) (h), μ opiate (OP3, MOP) (h), Phorbol ester (m), PAF (h), K^+ channel (K_{ATP}) (ha), K^+ channel HERG (h), P2X (rb), P2Y (r), σ2 (r), Na^+ channel (site 2) (r), NK_1 (h), Testosterone (r), DA transporter (DAT) (r), GABA transporter (r), NE transporter (NET) (h).

tion-dependently increased cAMP formation in HEK-293F/h5-HT$_7$ cells and behaved as a full agonist, with efficacy and potency (E_{max} = 99 ± 1% and EC_{50} = 16 ± 1 nM) similar to those of 5-HT (E_{max} = 100 and EC_{50} = 11 nM) (Fig. 1).

AS-19 is a commercially available 5-HT$_7$ receptor agonist. Unfortunately, as indicated by Perez-García and Meneses [38], there is a lack of information in the literature regarding its affinity for receptors other than 5-HT$_7$. Hence, the affinity of AS-19 for a total of 70 receptors, transporters and ion channels was assayed in commercial *in vitro* screening packages (CEREP and MDS Pharma Services), and the resulting binding profile is presented in Table 1. AS-19 had high affinity for 5-HT$_7$ receptors (K_i = 0.6 nM) but also had affinity for the 5-HT$_{1A}$ (K_i = 89.7 nM), 5-HT$_{1B}$ (K_i = 490 nM), 5-HT$_{1D}$ (K_i = 6.6 nM) and 5-HT$_{5A}$ (K_i = 98.5 nM) receptors. No significant affinity of AS-19 was detected for any other 5-HT receptor subtypes and additional receptors, transporters and ion channels included in the screening packages. This compound was also tested in a functional assay (cAMP stimulation in HEK-293F/h5-HT$_7$ cells) and was found to behave as a potent (EC_{50} = 9 ± 1 nM) but partial 5-HT$_7$ receptor agonist, with a maximal effect reaching 77% of that of 5-HT (Fig. 1).

MSD-5a is also a 5-HT$_7$ receptor agonist showing high affinity for 5-HT$_7$ receptors (K_i = 0.6 nM), but its selectivity against 5-HT$_{1A}$ (K_i = 16 nM) and other receptors is not as high as that of the other agonists [50] (Table 1). It was described as a potent partial agonist at 5-HT$_7$ receptors, giving at maximum 80% of the response evoked by a full agonist such as 5-carboxamidotryptamine [50]. In our hands, it was also a potent (EC_{50} = 7 ± 1.5 nM) 5-HT$_7$ partial agonist with a maximal cAMP response corresponding to 61% of that evoked by 5-HT (Fig. 1).

3.2. 5-HT$_7$ receptor agonists dose-dependently inhibit capsaicin-induced mechanical hypersensitivity

Mice injected with capsaicin (1 μg) into the mid-plantar surface of the right hindpaw developed mechanical hypersensitivity 15 min after the injection. The pressure exerted by the filament (1 g) was below/close to the threshold triggering paw withdrawal in control mice, since withdrawal latencies for mice injected with 1% DMSO (capsaicin vehicle) were close to the 60 s cut-off. However, 15 min after subplantar capsaicin injection, the same pressure (1 g) triggered paw withdrawal with a markedly reduced latency (Fig. 2). This abnormal mechanical hypersensitivity results from capsaicin-induced sensitization, as previously described by others [17,24].

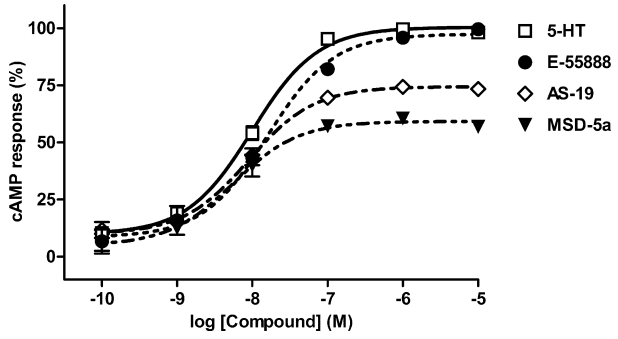

Fig. 1. Agonist-induced 5-HT$_7$ receptor-mediated cAMP formation. 5-HT$_7$ receptor-mediated cAMP formation was analyzed in HEK-293 cells stably transfected with the human 5-HT$_7$ receptor. Cells were treated with increasing concentrations of the indicated ligands, and cAMP formation was determined as described in Section 2. E-55888 reached similar levels of cAMP formation as 5-HT, while AS-19 and MSD-5a behaved as partial agonists. Mean dose–response curves ± SEM are shown from at least three independent experiments performed in duplicate.

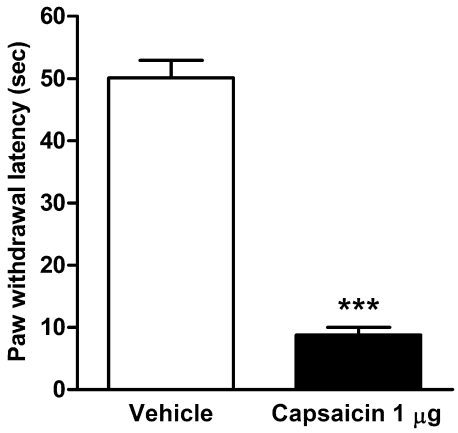

Fig. 2. Effect of subplantar capsaicin injection on the paw withdrawal latency to mechanical stimulation: mechanical hypersensitivity. Mice receiving subplantar injection of 1 μg capsaicin developed mechanical hypersensitivity, as evidenced by their significantly reduced paw withdrawal latencies to stimulation with a 1 g-pressure filament when compared to mice subplantarly injected with vehicle (1% DMSO). Each bar is the mean + SEM of four independent experiments using three mice in each. $^{***}p < 0.001$ *vs.* vehicle.

Systemic subcutaneous administration of three different 5-HT$_7$ receptor agonists, AS-19, MSD-5a and E-55888, dose-dependently inhibited capsaicin-induced mechanical hypersensitivity (Fig. 3). Significantly increased paw withdrawal latencies in mice treated with AS-19, MSD-5a and E-55888 were found at the doses of 3 (except for MSD-5a), 5 and 10 mg/kg compared to vehicle-treated mice. The order of efficacy was E-55888 > AS-19 > MSD-5a.

3.3. Blockade of the antinociceptive effect of 5-HT$_7$ receptor agonists by 5-HT$_7$ receptor antagonists

In order to confirm that activation of 5-HT$_7$ receptors was unambiguously responsible for the inhibition of capsaicin-induced mechanical hypersensitivity exerted by 5-HT$_7$ receptor agonists, the next series of experiments aimed at assessing whether co-administered 5-HT$_7$ receptor antagonists (SB-258719 and SB-269970) could prevent the effect of agonists.

First, we performed a dose–response study of the effect of 5-HT$_7$ receptor antagonists by themselves on capsaicin-induced mechanical hypersensitivity. When tested up to the dose of 10 mg/kg, neither SB-258719 nor SB-269970 exerted any effect (data not shown). Next, SB-258719 was administered in combination with AS-19, MSD-5a or E-55888. As shown in Fig. 4, SB-258719 at 5 mg/kg had no effect on its own but prevented the increase in paw withdrawal latencies induced by the agonists AS-19, MSD-5a and E-55888 at 10 mg/kg. The effect of AS-19 (5 mg/kg) was dose-dependently inhibited by SB-258719 co-administered at 2.5 and 5 mg/kg (Fig. 5). Blockade of AS-19-evoked antinociception was also successfully achieved with the other 5-HT$_7$ receptor antagonist, SB-269970, at a dose (10 mg/kg) that, on its own, did not modify paw withdrawal latencies after subplantar capsaicin injection (Fig. 5). Because (i) AS-19 has also some affinity for 5-HT$_{1A}$ receptors (see Table 1) and (ii) 5-HT$_{1A}$ receptor agonists are known to exert analgesic effects in a number of animal models [7], we also evaluated the possibility that 5-HT$_{1A}$ receptor stimulation by AS-19 could contribute to the antinociceptive effects exerted by this drug. For this purpose, AS-19 was co-administered with the selective 5-HT$_{1A}$ receptor antagonist WAY-100635 [14,18] at 0.3 mg/kg, a dose that had no effect on capsaicin-induced mechanical hypersensitivity but blocked the effects of the selective 5-HT$_{1A}$ receptor agonist F-13640 [6] (Figs. 5 and 6) and those of the prototypical 5-HT$_{1A}$ receptor agonist, 8-hydroxy-dipropylaminotetralin (8-OH-DPAT) [14]. Interestingly, the

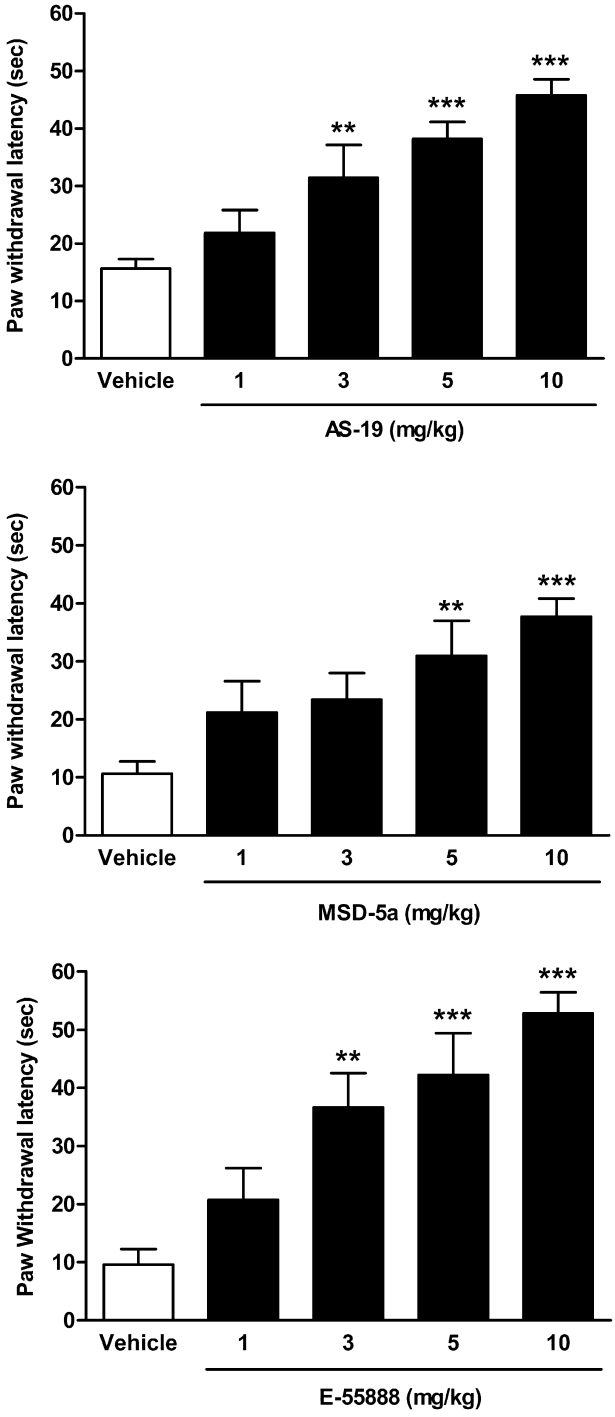

Fig. 3. Dose–response effects of 5-HT$_7$ receptor agonists on mechanical hypersensitivity induced by capsaicin. Mechanical hypersensitivity in mice receiving subplantar injection of 1 μg capsaicin was dose-dependently inhibited after subcutaneous treatment with three different 5-HT$_7$ receptor agonists: AS-19, MSD-5a, and E-55888. E_{max} = 52.8 ± 3.6, 45.8 ± 2.7 and 37.7 ± 3.1 s; and ED_{50} = 2.1 ± 1.2, 2.1 ± 1.4 and 2.5 ± 1.2 mg/kg for E-55888, AS-19 and MSD-5a, respectively. Each bar is the mean + SEM of three independent experiments using 3–4 mice in each. $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* vehicle.

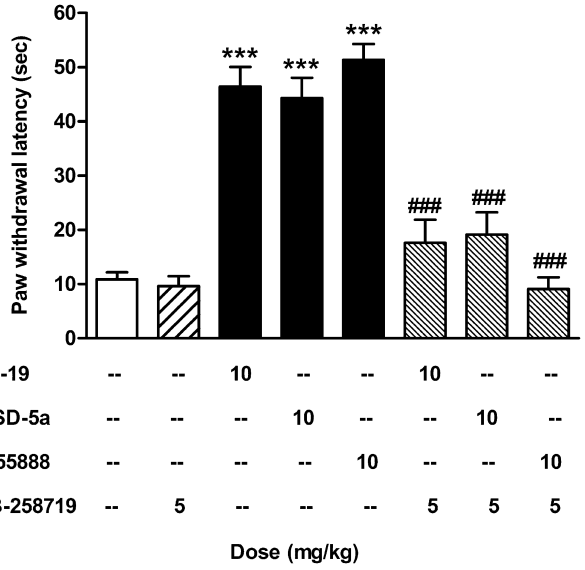

Fig. 4. Blockade of the antinociceptive effect of AS-19, MSD-5a and E-55888 by SB-258719. Mechanical hypersensitivity in mice receiving subplantar injection of 1 μg capsaicin was unaffected by the 5-HT$_7$ receptor antagonist SB-258719 (5 mg/kg), but when co-administered with the 5-HT$_7$ receptor agonists AS-19, MSD-5a or E-55888 (10 mg/kg), the antagonist blocked the effects of the agonists. Each bar is the mean + SEM of three independent experiments using 3–4 mice in each. $^{***}p < 0.001$ *vs.* vehicle; $^{###}p < 0.001$ *vs.* AS-19 (10 mg/kg), MSD-5a (10 mg/kg) or E-55888 (10 mg/kg).

reduction of mechanical hypersensitivity exerted by AS-19 at 5 mg/kg was unaffected by co-administration of WAY-100635 (0.3 mg/kg) (Fig. 5). This was in contrast to the inhibition found when SB-258719 or SB-269970 was co-administered (Figs. 4 and 5), indicating that activation of 5-HT$_7$ but not 5-HT$_{1A}$ receptors underlay the effect of AS-19. Doses of WAY-100635 higher than 0.3 mg/kg were not used because they produced, on their own, significant increases in paw withdrawal latencies (Fig. 6), in line with both the hypo- and hyperalgesic effects elicited through 5-HT$_{1A}$ receptors [1].

Altogether, these data obtained with three different 5-HT$_7$ receptor agonists and two different 5-HT$_7$ receptor antagonists

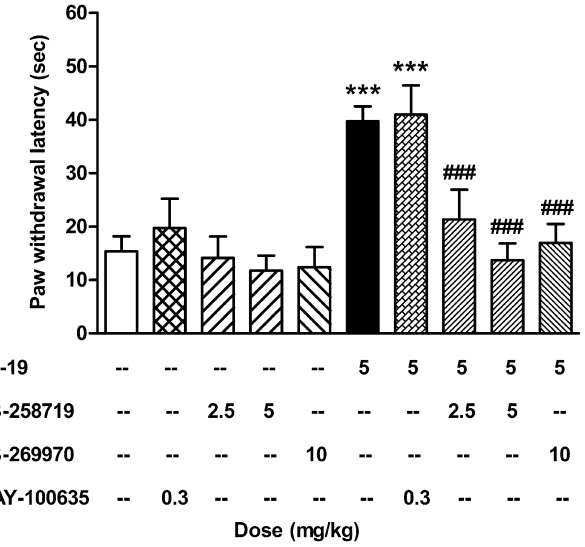

Fig. 5. Effects of SB-258719, SB-269970 and WAY-100635 on the antinociceptive action of AS-19. Mechanical hypersensitivity in mice receiving subplantar injection of 1 μg capsaicin was unaffected after subcutaneous treatment with SB-258719 (2.5 and 5 mg/kg), SB-269970 (10 mg/kg) or WAY-100635 (0.3 mg/kg). However, SB-258719 and SB-269970 but not WAY-100635 significantly inhibited the antinociceptive effect of AS-19 (5 mg/kg). Each bar is the mean + SEM of three independent experiments using 3–4 mice in each. $^{***}p < 0.001$ *vs.* vehicle; $^{###}p < 0.001$ *vs.* AS-19 (5 mg/kg).

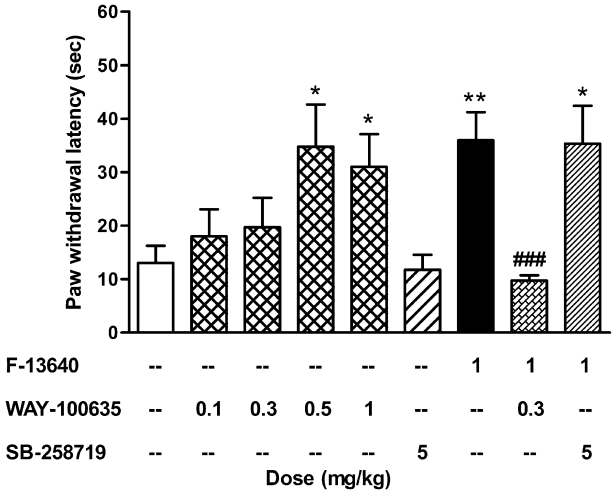

Fig. 6. Dose–response effect of WAY-100635 on capsaicin-induced mechanical hypersensitivity and blockade by this 5-HT_{1A} receptor antagonist of the antinociceptive effect of F-13640. Low doses (0.1 and 0.3 mg/kg) of the 5-HT_{1A} receptor antagonist WAY-100635 did not modify mechanical hypersensitivity in mice receiving subplantar injection of 1 μg capsaicin, but a significant antinociceptive effect was observed at higher doses (0.5 and 1 mg/kg). The reduction of mechanical hypersensitivity exerted by the 5-HT_{1A} receptor agonist F-13640 (1 mg/kg) was blocked by co-administration of WAY-100635 (0.3 mg/kg) but not SB-258719 (5 mg/kg). Each bar is the mean + SEM of three independent experiments using 3–4 mice in each. $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* vehicle; $^{###}p < 0.001$ *vs.* F-13640 (1 mg/kg).

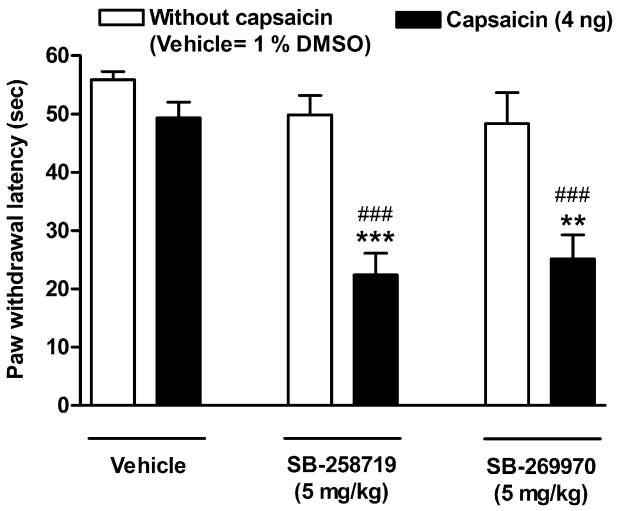

Fig. 7. Promotion of capsaicin-induced mechanical hypersensitivity by 5-HT_7 receptor antagonists. No hypersensitivity response to a low upward pressure (0.25 g) was detected in mice injected subplantarly with 4 ng of capsaicin or its vehicle. However, hypersensitivity was evidenced after subcutaneous treatment with the 5-HT_7 receptor antagonists SB-258719 and SB-269970 (5 mg/kg) in mice subplantarly injected with this low dose of capsaicin and mechanically stimulated with this low pressure. Each bar is the mean + SEM of four independent experiments using 3–4 mice in each. $^{**}p < 0.01$ *vs.* "SB-269970 without capsaicin"; $^{***}p < 0.001$ *vs.* "SB-258719 without capsaicin"; $^{###}p < 0.001$ *vs.* "vehicle group with capsaicin".

strongly suggest that activation of 5-HT_7 receptors inhibits mechanical hypersensitivity in mice sensitized by capsaicin.

3.4. 5-HT_7 receptor antagonists promote mechanical hypersensitivity

Because activation of 5-HT_7 receptors by selective agonists reduced mechanical hypersensitivity in mice sensitized by capsaicin, we tested whether, in contrast, 5-HT_7 receptor antagonists could promote hypersensitivity. To investigate this possibility, we reduced both the dose of capsaicin, from 1 μg to 4 ng, and the upward pressure exerted by the filament, from 1 to 0.25 g. Under these conditions, mice did not display mechanical hypersensitivity (Fig. 7). However, when treated with SB-258719 or SB-269970 (5 mg/kg), mice receiving this minor amount of capsaicin (4 ng) and exposed to low-pressure stimulation (0.25 g) showed a marked hypersensitivity (Fig. 7). Interestingly, the effect of 5-HT_7 receptor antagonists was not observed in mice subplantarly injected with capsaicin vehicle (1% DMSO), suggesting that a minimal sensitizing challenge with capsaicin is needed for the antagonists to exert their pronociceptive effect.

The pronociceptive effect of SB-258719 was dose-dependent and could be dose-dependently reversed by the co-administration of AS-19 (2.5–10 mg/kg) (Fig. 8). Promotion of hypersensitivity by the other antagonist, SB-269970, was also inhibited by AS-19 (Fig. 8), pointing to the involvement of 5-HT_7 receptors in such an effect.

4. Discussion

Intradermal injection of capsaicin (i.e., capsaicin sensitization) is a useful model for correlative behavioral, physiological and pharmacological studies in pain conditions involving sensitization and behavioral hyperalgesia and allodynia [16,17,23]. In fact, capsaicin-induced mechanical hypersensitivity is regarded as a predictive model of the antinociceptive action of analgesics in neuropathic pain [24]. In this study, sensitization by subplantar capsaicin injection in mice was used to pharmacologically assess the role of 5-HT_7 receptors in mechanical hypersensitivity. As there is a remarkable need of both a better knowledge of available 5-HT_7 receptor ligands and new selective 5-HT_7 receptor ligands, particularly agonists, we first investigated the affinity, selectivity and functionality of the 5-HT_7 receptor agonists used in the experiments. The *in vitro* pharmacological profile of E-55888, a new selective, potent and efficient 5-HT_7 receptor agonist, is also described herein for the first time.

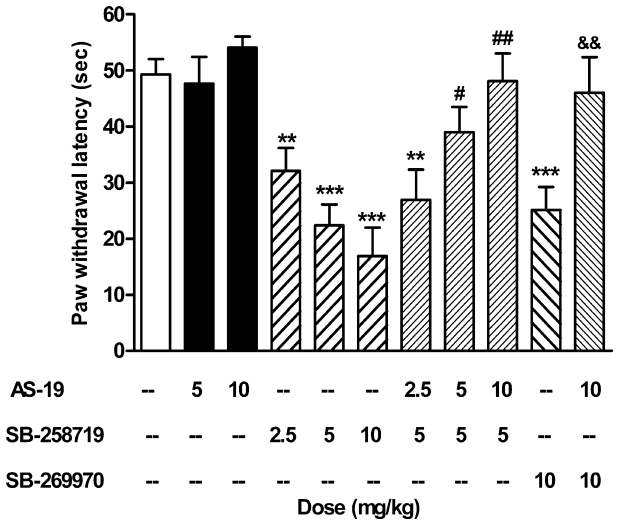

Fig. 8. Dose-dependent promotion of capsaicin-induced mechanical hypersensitivity by SB-258719 and reversion of the pronociceptive effects of SB-258719 and SB-269970 by AS-19. Subcutaneous treatment with AS-19 had no effect but subcutaneous treatment with SB-258719 dose-dependently promoted hypersensitivity in mice subplantarly injected with a minor dose of capsaicin (4 ng) and mechanically stimulated with a low upward pressure (0.25 g) (non-sensitizing conditions). AS-19 dose-dependently reversed the pronociceptive effects of SB-258719 and SB-269970 under these conditions. Each bar is the mean + SEM of three independent experiments using 3–4 mice in each. $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* vehicle; $^{#}p < 0.05$, $^{##}p < 0.01$ *vs.* SB-258719 (5 mg/kg); $^{&&}p < 0.01$ *vs.* SB-269970 (10 mg/kg).

Three 5-HT_7 receptor agonists were investigated at human 5-HT_7 receptors in HEK-293F cells using the cAMP signaling pathway as a functional read-out. MSD-5a behaved as a potent partial agonist, as previously described [50]. AS-19 also behaved as a potent partial agonist, with higher intrinsic efficacy than MSD-5a, whereas E-55888 was a potent full agonist. Remarkably, binding data showed that AS-19 and, particularly, E-55888, are selective for 5-HT_7 receptors, which makes both agonists suitable as pharmacological tools to investigate the physiology of 5-HT_7 receptors. In the case of E-55888, significant affinity was only found for 5-HT_7 and 5-HT_{1A} receptors among more than 170 receptors, transporters and ion channels tested. Furthermore, the affinity of this ligand was 280-fold higher for 5-HT_7 than for 5-HT_{1A} receptors.

By using these compounds, the present work provides the first demonstration of behavioral antinociception generated by systemic administration of selective 5-HT_7 receptor agonists. Subcutaneous administration of all three 5-HT_7 receptor agonists, AS-19, MSD-5a and E-55888, exerted a clear-cut dose-dependent inhibition of capsaicin-induced mechanical hypersensitivity. All three 5-HT_7 receptor agonists reduced the nociceptive response of capsaicin-sensitized mice close to baseline values found in control mice. The order of efficacy was E-55888 > AS-19 > MSD-5a, which matches their *in vitro* efficacy as 5-HT_7 receptor agonists. The effect of agonists was blocked by the systemic co-administration of two different 5-HT_7 receptor antagonists, SB-258719 and SB-269970. In contrast, the selective 5-HT_{1A} receptor antagonist, WAY-100635, was unable to reverse the effect of AS-19 at a dose that fully reversed the effect of the 5-HT_{1A} receptor agonist F-13640. These results are remarkable because: (i) the ligand binding profile of the 5-HT_7 receptor resembles that of the 5-HT_{1A} receptor [32,39,52]; (ii) 5-HT_7 receptor ligands used in this study showed submicromolar (10^{-7}–0^{-8} M) affinity for 5-HT_{1A} receptors; and (iii) antinociceptive actions have been attributed to 5-HT_{1A} receptor agonists [7]. In addition, SB-258719 was unable to block the effect of the 5-HT_{1A} receptor agonist F-13640, indicating that reduction of mechanical hypersensitivity exerted through 5-HT_7 and 5-HT_{1A} receptors can actually be pharmacologically differentiated, as they probably involve different target neurons and/or rely on different pathways.

In contrast to the antinociceptive effect exerted by agonists, a dose-dependent pronociceptive effect was observed when the 5-HT_7 receptor antagonist SB-258719 was given to mice injected with a low subactive dose of capsaicin and stimulated with a low subthreshold pressure. Interestingly, promotion of hypersensitivity by SB-258719 and SB-269970 was not observed in mice subplantarly injected with vehicle, indicating that a minimal capsaicin sensitizing challenge is needed for the antagonist to exert its pronociceptive effect. This suggests that endogenous activation of the 5-HT_7 receptor takes place after intraplantar capsaicin challenge (but not after vehicle), thereby allowing selective antagonists to exert a counteracting effect. Altogether, reduction of hypersensitivity by agonists and promotion of hypersensitivity by antagonists, which could be reversed by antagonists and agonists, respectively, highlight the role of 5-HT_7 receptors in the control of mechanical hypersensitivity secondary to a sensitizing stimulus.

Regarding the distribution of 5-HT_7 receptors, several lines of evidence in the literature already suggested that this receptor could be involved in pain control at the peripheral, spinal and/or supraspinal levels. Thus, Pierce et al. [40,41] detected the presence of 5-HT_7 receptor messenger RNA in rat and human dorsal root ganglia (DRG). In addition, Doly et al. [10] described by immunocytochemistry the localization of 5-HT_7 receptors at the lumbar level in small and medium-sized DRG cells as well as in the superficial laminae of the dorsal horn, which is consistent with a predominant role in nociception. Electron microscopic examination of the dorsal horn revealed both a presynaptic (in axon terminals from primary afferents but also from intrinsic cells) and a postsynaptic localization, mainly in cell bodies and synaptic differentiations of dendrites belonging to local interneurons. At the supraspinal level, the distribution of 5-HT_7 receptors in different cortical areas and several nuclei in the thalamus, midbrain, pons and medulla [33,35,48] is also compatible with a role in pain control.

To date, experimental data supporting the idea that 5-HT_7 receptors are involved in pain control are scarce. Recently, Dogrul and Seyrek [9] highlighted the contribution of spinal 5-HT_7 receptors to morphine analgesia. They found that 5-HT_7 receptor blockade by intrathecal administration of SB-269970 inhibited the antinociceptive effect of systemic morphine in the tail-flick test. This effect was not reproduced when 5-HT_{1A} and 5-HT_2 receptor antagonists were intrathecally injected, supporting the notion that the specific activation of spinal 5-HT_7 receptors plays a key role in the inhibitory pathway activated by morphine. It is unlikely that activation of 5-HT_7 receptors could directly inhibit primary afferents or nociceptive dorsal horn neurons because these receptors are positively coupled to adenylate cyclase and their stimulation is excitatory. However, activation of 5-HT_7 receptors localized on spinal inhibitory enkephalinergic or GABAergic interneurons, to evoke the release of enkephalins or GABA, would produce an inhibition of nociceptive transmission. Indeed, it has been recently reported that the GABAergic system is responsible for 5-HT_7 receptor-mediated antinociception in the spinal cord: intrathecal administration of the $GABA_A$ receptor antagonist bicuculline, but not of the opioid receptor antagonist naloxone, prevented the 5-HT_7 receptor agonist-mediated anti-hyperalgesic effects in rats after chronic constriction injury of the sciatic nerve [5].

Supraspinal areas, particularly the thalamus, have also been suggested to be involved in modulation of nociception by serotonin, and a role for 5-HT_7 receptors has been outlined. In particular, Díaz-Reval et al. [8] suggested that supraspinal 5-HT_7 receptors mediate the antinociceptive effect of *S*-(+)-ketoprofen because intracerebroventricular administration of methiothepin, a non-selective high affinity 5-HT_7 receptor antagonist, significantly inhibited the effect of this non-steroidal anti-inflammatory agent. Furthermore, administration of the mixed 5-HT_{1A}/5-HT_7 receptor agonist, 8-OH-DPAT, into the medial thalamus was shown to raise tailshock intensity thresholds to trigger vocalization, and this antinociceptive effect could be blocked by intrathalamic microinjection of the 5-HT_7 receptor antagonist, SB-269970 [19].

Although the above data support the idea that 5-HT_7 receptors participate in antinociceptive mechanisms, a pronociceptive role of peripheral and spinal 5-HT_7 receptors has also been proposed on the basis of apparently discrepant results. Using the formalin test, Rocha-González et al. [42] reported that 5-HT_7 receptor blockade by SB-269970 antagonized the increased formalin-evoked nociceptive responses caused by local (directly into the formalin injected paw) or intrathecal administration of 5-HT or 5-carboxamidotryptamine, a non-selective 5-HT_7 receptor agonist. It is thus possible that activation of 5-HT_7 receptors could elicit a spectrum of pro- and antinociceptive actions depending on the localization of the receptor and the stimulus quality and modality. Based on our results in the capsaicin model and those recently reported in the sciatic nerve chronic constriction injury model [5], if a balance exists between pro- and antinociceptive actions, the overall effect of systemically (s.c.) administered 5-HT_7 receptor agonists in sensitizing conditions is clearly analgesic (antiallodynic/anti-hyperalgesic).

In conclusion, the present data suggest the involvement of the 5-HT_7 receptor subtype in the control of pain in conditions involving central sensitization and raises the notion that systemically administered selective 5-HT_7 receptor agonists may represent a new potential therapeutic approach for pain alleviation. Further studies in other relevant animal models and/or focusing on the mechanism of action underlying 5-HT_7-mediated analgesia are needed to strengthen this interesting possibility.

Conflicts of interests

The authors state that there were no conflicts of interests in respect to the work reported in the paper.

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6.3. 5-HT$_7$ receptors in neuropathic pain

Pharmacological activation of 5-HT$_7$ receptors reduces nerve injury-induced mechanical and thermal hypersensitivity. *Pain 2010;149:483-494*

Alex Brenchat, Xavier Nadal, Luz Romero, Sergio Ovalle, Asunción Muro, Ricard Sánchez-Arroyos, Enrique Portillo-Salido, Marta Pujol, Ana Montero, Xavier Codony, Javier Burgueño, Daniel Zamanillo, Michel Hamon, Rafael Maldonado, José Miguel Vela

L'activació farmacològica dels receptors 5-HT$_7$ redueix la hipersensibilitat mecànica i tèrmica induïda per la lesió del nervi

Amb els resultats del present estudi vàrem demostrar la participació del receptor 5-HT$_7$ en la hipersensibilitat mecànica i tèrmica induïda per la lesió del nervi ciàtic dels ratolins. L'activació dels receptors 5-HT$_7$ a través de l'administració sistèmica de l'agonista del receptor 5-HT$_7$, AS-19 (1 i 10 mg/kg) va produir una reducció de la hipersensibilitat mecànica i tèrmica la qual vàrem revertir amb la coadministració de l'antagonista selectiu del receptor 5-HT$_7$, SB-258719. També interessant és que el bloqueig dels receptors 5-HT$_7$ amb SB-258719 (2.5 i 10 mg/kg) va promoure la hipersensibilitat mecànica (però no tèrmica) en ratolins amb lesió del nervi ciàtic i en ratolins *sham* (operats sense lesionar el nervi). El tractament crònic de dos vegades al dia durant 11 dies de l'agonista selectiu del receptor 5-HT$_7$, E-57431 (10 mg/kg) va demostrar mantenir l'eficàcia del tractament després de repetides administracions sistèmiques, no desenvolupant tolerància de l'efecte antial·lodínic i antihiperalgèsic. El receptor 5-HT$_7$ va resultar col·localitzar amb cèl·lules GABAèrgiques en l'asta dorsal de la medul·la espinal, suggerint que l'activació d'interneurones GABAèrgiques inhibidores a nivell espinal podrien contribuir a l'efecte analgèsic dels agonistes del receptor 5-HT$_7$. A més, vam detectar per immunohistoquímica un increment significatiu dels receptors 5-HT$_7$ en l'asta dorsal ipsilateral de la medul·la espinal després de la lesió del nervi, suggerint una regulació de l'expressió del receptor 5-HT$_7$ induïda pel dolor. Els resultats obtinguts en el present treball ens van permetre evidenciar el potencial interès terapèutic dels agonistes selectius del receptor 5-HT$_7$ en el tractament del dolor neuropàtic, i suggerir que l'activació del sistema GABAèrgic sembla ser el responsable dels efectes antinociceptius resultant de l'activació del receptor 5-HT$_7$.

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Pharmacological activation of 5-HT$_7$ receptors reduces nerve injury-induced mechanical and thermal hypersensitivity

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ABSTRACT

The involvement of the 5-HT$_7$ receptor in nociception and pain, particularly chronic pain (i.e., neuropathic pain), has been poorly investigated. In the present study, we examined whether the 5-HT$_7$ receptor participates in some modulatory control of nerve injury-evoked mechanical hypersensitivity and thermal (heat) hyperalgesia in mice. Activation of 5-HT$_7$ receptors by systemic administration of the selective 5-HT$_7$ receptor agonist AS-19 (1 and 10 mg/kg) exerted a clear-cut reduction of mechanical and thermal hypersensitivities that were reversed by co-administering the selective 5-HT$_7$ receptor antagonist SB-258719. Interestingly, blocking of 5-HT$_7$ receptors with SB-258719 (2.5 and 10 mg/kg) enhanced mechanical (but not thermal) hypersensitivity in nerve-injured mice and induced mechanical hypersensitivity in sham-operated mice. Effectiveness of the treatment with a 5-HT$_7$ receptor agonist was maintained after repeated systemic administration: no tolerance to the antiallodynic and antihyperalgesic effects was developed following treatment with the selective 5-HT$_7$ receptor agonist E-57431 (10 mg/kg) twice daily for 11 days. The 5-HT$_7$ receptor co-localized with GABAergic cells in the dorsal horn of the spinal cord, suggesting that the activation of spinal inhibitory GABAergic interneurons could contribute to the analgesic effects of 5-HT$_7$ receptor agonists. In addition, a significant increase of 5-HT$_7$ receptors was found by immunohistochemistry in the ipsilateral dorsal horn of the spinal cord after nerve injury, suggesting a "pain"-triggered regulation of receptor expression. These results support the idea that the 5-HT$_7$ receptor subtype is involved in the control of pain and point to a new potential use of 5-HT$_7$ receptor agonists for the treatment of neuropathic pain.

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1. Introduction

The serotonin (5-hydroxytryptamine [5-HT]) system has been recognized as one of the main neurotransmitter systems participating in pain transmission, processing and controlling. Both pronociceptive and antinociceptive effects have been attributed to 5-HT depending on the site and the receptor subtype it acts on [12,19,30,38,47]. Among the seven 5-HT receptor families identified so far, much of the pain research has focused on 5-HT$_{1A}$, 5-HT$_{1B/1D}$, 5-HT$_{2A/2C}$ and 5-HT$_3$ receptors [1,7,12,18,19,32,33], but the role played by other 5-HT receptors in nociception has been poorly or not thoroughly investigated. This is the case of the last identified 5-HT receptor, the 5-HT$_7$ receptor [25,44].

At the periphery, 5-HT$_7$ receptors have been found in dorsal root ganglia (DRG) [11,29,36,37]. Interestingly, increased levels of 5-HT$_7$ receptor messenger RNA have been reported in rat DRG after bee venom- [23] and complete Freund's adjuvant-induced inflammation [49]. Regarding its role, the only two studies addressing this issue suggest a pronociceptive role of peripheral 5-HT$_7$ receptors: (1) intraplantar injection of the 5-HT$_7$ receptor antagonist SB-269970 reduced formalin-induced nociception whereas intraplantar administration of non-selective 5-HT$_7$ receptor agonists such as 5-HT itself and 5-carboxamidotryptamine (5-CT) increased formalin-induced nociceptive behavior [39]; (2) intra-articular injection of the mixed 5-HT$_{1A}$/5-HT$_7$ receptor agonist, 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT), induced c-Fos expression in the dorsal horn of the rat spinal cord and this effect was prevented by intra-articular administration of the non-selective 5-HT$_7$ receptor antagonist methiothepin [29].

In the CNS, the presence of 5-HT$_7$ receptors in the spinal cord and supraspinal areas has also been reported [11,28,29,31,46]. In the spinal cord, the 5-HT$_7$ receptor was mainly found in the superficial laminae of the dorsal horn, postsynaptically in local interneurons, presynaptically in peptidergic fibers (including presumably primary afferents) and in astrocytes [11,29]. Four studies support

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an antinociceptive role of spinal and supraspinal 5-HT_7 receptors: (1) intrathecal administration of the 5-HT_7 receptor antagonist SB-269970 inhibited the antinociceptive effect of systemic morphine in the tail-flick test [10]; (2) spinal administration of SB-269970 also blocked the antinociceptive effects of morphine when the opioid was microinjected into the rostroventromedial medulla (RVM) [9]; (3) intracerebroventricular administration of methiothepin blocked the antinociceptive effect of the non-steroidal anti-inflammatory *S*-(+)-ketoprofen [8]; (4) microinjection of 8-OH-DPAT into the medial thalamus exerted antinociceptive effects in the tailshock test that were reversed by intrathalamic administration of SB-269970 [16].

In summary, data supporting a role for 5-HT_7 receptors in nociception are scarce and come mostly from studies using non-selective ligands. Particularly remarkable is the absence of data in chronic pain (i.e., neuropathic pain). We showed previously that subcutaneous administration of 5-HT_7 receptor agonists crossing the blood–brain barrier (BBB) dose-dependently inhibited capsaicin-induced mechanical hypersensitivity in mice [3]. It was thus argued that, in sensitizing conditions, the overall effect of activating 5-HT_7 receptors is antinociceptive. In the present study, the effect of systemically administered selective, BBB-penetrant 5-HT_7 receptor ligands were investigated in mice subjected to sensitizing neuropathic pain conditions. The cellular localization of spinal 5-HT_7 receptors and possible changes in 5-HT_7 receptor expression in the spinal dorsal horn following nerve injury were also investigated.

2. Methods

2.1. Animals

Male, 6- to 8-week-old, CD1 mice (Harlan Iberica, Spain) were used in these studies. Animals were housed in groups of five, provided with food and water *ad libitum* and kept in controlled laboratory conditions with the temperature maintained at 21 ± 1 °C and light in 12 h cycles (on at 07:00 h and off at 19:00 h). Experimental behavioral testing was carried out in a soundproof and air-regulated experimental room and was done in blind respect to treatment and surgical procedure. All experimental procedures and animal husbandry were conducted according to ethical principles for the evaluation of pain in conscious animals [51] and to ethical guidelines of the European Community on the Care and Use of Laboratory Animals (European Communities Council Directive of 24 November 1986, 86/609/ECC) and received approval by the local Ethical Committee.

2.2. Drugs

Drugs used for treatments were AS-19 (dimethyl-[5-(1,3,5-trimethyl-1*H*-pyrazol-4-yl)-1,2,3,4-tetrahydro-naphthalen-2(*S*)-yl]-amine) [17,41], SB-258719 (*N*,3-dimethyl-*N*-[1(*R*)-methyl-3-(4-methyl-1-piperidinyl)propyl]benzenesulfonamide hydrochloride) [12,22,35], and E-57431 (2-(2-(dimethylamino)ethyl)-4-(1,3,5-trimethyl-1*H*-pyrazol-4-yl)phenol). Table 1 summarizes the affinity and selectivity of these drugs. AS-19 is a potent selective 5-HT_7 receptor agonist [3], SB-258719 is a potent selective 5-HT_7 receptor antagonist [13,26,40], and E-57431 is a new potent selective 5-HT_7 receptor agonist developed by Laboratorios Esteve and described herein for the first time (Table 1). AS-19 was purchased from Tocris Bioscience (UK) and SB-258719 and E-57431 were synthesized for the purpose of this study at Esteve. AS-19 was dissolved in 1% DMSO and E-57431 and SB-258719 in aqueous solutions (0.5% hydroxypropyl methyl cellulose, HPMC; and physiological saline, respectively). Compounds and vehicles were administered through the subcutaneous (s.c.) or intraperitoneal (i.p.) routes, in a volume of 5 or 10 ml/kg, respectively. When two compounds were co-administered, they were sequentially injected s.c. in opposite flanks of the mice, immediately one after the other.

Table 1
Binding profile of the 5-HT_7 receptor ligands AS-19, SB-258719 and E-57431.

Receptor	Affinity [K_i (nM)]		
	AS-19	SB-258719	E-57431
h5-HT_{1A}	89.7 (149.5×)	n.s.	n.s.
r5-HT_{1B}	490 (816.6×)	n.s.	n.s.
h5-HT_{1D}	6.6 (11×)	n.s.	53 (112.7×)
h5-HT_{2A}	n.s.	n.s.	560 (1191.5×)
h5-HT_{2B}	n.s.	n.s.	n.s.
h5-HT_{2C}	n.s.	n.s.	n.s.
h5-HT_3	n.s.	–	n.s.
h5-HT_{4e}	–	–	n.s.
gp5-HT_4	n.s.	n.s.	n.s.
h5-HT_{5A}	98.5 (164.2×)	–	n.s.
h5-HT_6	n.s.	n.s.	n.s.
h5-HT_7	0.60	31.6	0.47
h5-HT transporter (SERT)	n.s.	–	n.s
Other receptors	n.s.[a]	n.s.[b]	n.s.[c]

n.s., not significant (K_i > 1 μM or % inhibition at 1 μM < 50%); –, data not available. gp, Guinea pig; h, human; ha, hamster; m, mouse; r, rat; rb, rabbit.
Data obtained from CEREP and MDS Pharma Services (E-57431); Brenchat et al. [3] (AS-19); and Forbes et al. [13] (SB-258719).
Data in brackets following K_i values represent the affinity ratio vs. 5-HT7 receptors calculated as K_i for the tested receptor/K_i for 5-HT_7 receptor. It is expressed as number-fold higher (×) for 5-HT_7 than for the tested receptor.
[a] See in Brenchat et al. [3] the panel of other receptors assayed.
[b] See in Forbes et al. [13] the panel of other receptors assayed.
[c] The following panel of other receptors was assayed (MDS Pharma Services): A_1 (h), A_{2A} (h), A_3 (h), α_{1A} (r), α_{1B} (r), α_{1D} (h), α_{2A} (h), α_{2C} (h), β_1 (h), β_2 (h), β_3 (h), AM_1 (h), AM_2 (h), Aldosterone (r), Anaphylatoxin C5a (h), Androgen (Testosterone) AR (r), AT_1 (h), AT_2 (h), APJ (h), ANF (gp), BB_1 (h), BB_2 (h), BB_3 (h), B_1 (h), B_2 (h), Calcitonin (h), $CGRP_1$ (h), Ca^{2+} channel (L-Type benzothiazepine) (r), Ca^{2+} channel (L-Type dihydropyridine) (r), Ca^{2+} channel (L-Type phenylalkylamine) (r), Ca^{2+} channel (N-Type) (r), CB_1 (h), CCR2B (h), CCR4 (h), CCR5 (h), CX3CR1 (h), CXCR2 (IL-$8R_B$) (h), CCK_1 (h), CCK_2 (h), Colchicine (r), CRF_1 (h), D_1 (h), D_{2S} (h), D_3 (h), $D_{4.2}$ (h), D_5 (h), ET_A (h), ET_B (h), EGF (h), EPOR (h), ERα (h), ERβ (h), GPR103 (h), GPR8 (h), $GABA_A$ (chloride channel, TBOB) (r), $GABA_A$ (flunitrazepam, central) (r), $GABA_A$ (muscimol, central) (r), $GABA_{B1A}$ (h), $GABA_{B1B}$ (h), Gabapentin (r), GAL1 (h), GAL2 (h), Glucocorticoid (h), Glutamate (AMPA) (r), Glutamate (Kainate) (r), Glutamate (NMDA, agonism) (r), Glutamate (NMDA, glycine) (r), Glutamate (NMDA, phencyclidine) (r), Glutamate (NMDA, polyamine) (r), Glycine, strychnine-sensitive (r), GHS, Ghrelin (h), H_1 (h), H_2 (h), H_3 (h), H_4 (h), I_2 (r), IP_3 (r), Insulin (r), IL-2 (m), IL-6 (h), Leptin (m), Leucotriene (LTB_4) (h), Leucotriene ($CysLT_1$) (h), Leucotriene ($CysLT_2$) (h), MC_1 (h), MC_3 (h), MC_4 (h), MC_5 (h), MT_1 (h), MT_2 (h), Motilin (h), M_1 (h), M_2 (h), M_3 (h), M_4 (h), M_5 (h), NMU_1 (h), NMU_2 (h), NPY_1 (h), NPY_2 (h), NT_1 (h), FPR1 (h), FPRL1 (h), Ach. Nic. (h), Ach. Nic. α_1 (h), Ach. Nic. α7(r), δ opiate (OP1, DOP) (h), κ opiate (OP2, KOP) (h), μ opiate (OP3, MOP) (h), ORL_1 (h), Phorbol ester (m), PAF (h) PDGF (m), K^+ channel (K_A) (r), K^+ channel (K_{ATP}) (ha), K^+ channel (SK_{CA}) (r), K^+ channel HERG (h), PR-B (h), CRTH2 (h), DP (h), EP_2 (h), EP_4 (h), Prostanoid, Thromboxane A_2 (TP) (h), P_{2X} (rb), P_{2Y} (r), RXRα (h), Rolipram (r), RyR3 (r), σ1 (h), σ2 (r), Na^+ channel (site 2) (r), SST1 (h), SST2 (h), SST3 (h), SST4 (h), SST5 (h), NK_1 (h), NK_2 (h), NK_3 (h), Thyroid Hormone (r), TRH (r), TGF-β (m), Adenosine transporter (gp), Choline transporter (r), Dopamine (DAT) transporter (h), GABA transporter (r), Monoamine transporter (rb), Norepinephrine transporter (NET) (h), TNF (non-selective) (h), Urotensin II (h), Vanilloid (r), VIP_1 (h), V_{1A} (h), V_{1B} (h), V_2 (h), Vitamin D_3 (h).

2.3. Binding profile and cAMP measurements

Binding affinities of E-57431 were determined by commercial radioligand binding assays by MDS Pharma and CEREP (see Table 1), according to their standard assay protocols (for details of the experimental conditions see http://discovery.mdsps.com/Catalog/OnlineCatalog/Profiling/Assays/AssayList.aspx?id=5 and http://www.cerep.fr/Cerep/Users/pages/catalog/binding/catalog.asp).

For the determination of efficacy and potency of the 5-HT_7 receptor agonist, cAMP measurements were performed using a system based on homogeneous time-resolved fluorescence (HTRF)

applied to human embryonic kidney (HEK)-293F cells that stably express the human 5-$HT_{7(a)}$ receptor, as previously described [3]. The HTRF cAMP kit from CisBio (CisBio Int., France) was used according to the instructions of the manufacturer. Briefly, after overnight incubation in serum-free medium, cells were added to 96-well plates (20,000 cells/well) in Ham's F12 (Gibco, Invitrogen Co., Spain) incubation buffer (40 μl/well) containing 1 mM 3-isobutyl-1-methyl-xanthine (IBMX; Sigma–Aldrich Co., Spain) and 20 μM pargyline (Sigma–Aldrich Co.). Then, 10 μl of different concentrations of E-57431 was added, and the plates were incubated for 30 min at 37 °C. The reaction was stopped by using a mixture of 25 μl of cryptate and 25 μl of XL-665 prepared in the lysis buffer supplied by the manufacturer. Plates were then incubated for an additional hour at room temperature, and cAMP contents were calculated from the 665 nm/620 nm ratio using a RubyStar Plate reader (BMG LabTech, Germany).

2.4. Neuropathic pain model: partial sciatic nerve ligation

The partial sciatic nerve ligation model was used to induce neuropathic pain, according to the method previously described [27,43]. This model consists of partial injury to the sciatic nerve at mid-thigh level. Surgery was performed under isoflurane (Iso-Flo®, Abbott-Laboratorios Esteve, Barcelona, Spain) anesthesia (induction: 3%; surgery: 2%). Briefly, mice were anaesthetized and the common sciatic nerve was exposed at the level of the mid-thigh of the right hindpaw. Partial nerve injury was produced at about 1 cm proximal to the nerve trifurcation by tying a tight ligature around approximately 33–50% of the diameter of the sciatic nerve using 9-0 non-absorbable virgin silk suture (Alcon surgical, USA). Ligature enclosed the outer 1/3–1/2 sciatic nerve whereas the rest of the nerve (inner 2/3–1/2) was leaved "uninjured". The muscle was then stitched with 6-0 silk suture and the skin incision was closed with wound clips. Control, sham-operated, mice underwent the same surgical procedure and the sciatic nerve was exposed, but not ligated.

2.5. Nociceptive behavioral tests

Mechanical hypersensitivity and thermal hyperalgesia were used as outcome measures of neuropathic pain and as indicators of the possible antinociceptive effect of treatments.

2.5.1. Evaluation of mechanical hypersensitivity

Acute administration. Mechanical hypersensitivity was quantified by determining the pressure threshold eliciting withdrawal of the ipsilateral hindpaw in response to stimulation with a von Frey filament applied onto the plantar surface (dynamic plantar aesthesiometer; Ugo Basile, Comerio, Italy) [20]. The electronic von Frey device applied a single non-flexible filament (0.5 mm in diameter) with increasing force (0.1 g/s; from 0 to 5 g) against the plantar surface over a 50-s period. The nocifensive paw withdrawal response automatically turned off the stimulus, and the pressure eliciting the response was recorded. For measurements, mice were placed individually into compartment enclosures in a test chamber with a framed metal mesh floor and allowed to acclimate for 1 h before testing. Paw withdrawal thresholds were measured in triplicate for each animal, allowing at least 30 s intervals between successive measurements.

Repeated administration. Mechanical hypersensitivity (allodynia) was quantified by measuring the hindpaw withdrawal response to manual von Frey filament stimulation [6]. Briefly, animals were placed into compartment enclosures in a test chamber with a framed metal mesh floor through which the von Frey monofilaments (bending force range from 0.008 to 2 g) (North Coast Medical, Inc., San Jose, CA, USA) were applied and thresholds were measured using the up–down paradigm. The filament of 0.4 g was used at first. Then, the strength of the next filament was decreased when the animal responded or was increased when the animal did not respond. This up–down procedure was stopped four measures after the first change in animal responding. The threshold of response was calculated by using the up–down Excel spreadsheet generously provided by Basbaum's laboratory (UCSF, San Francisco, USA). Clear paw withdrawal, shaking or licking was considered as a nociceptive-like response.

2.5.2. Evaluation of thermal hyperalgesia

Thermal hyperalgesia was assessed, in both acute and repeated administration experiments, using the plantar test by determination of the hindpaw withdrawal latency in response to a thermal stimulus (radiant heat) [15]. On the day of the test, mice were placed into plastic compartment enclosures on the glass surface of the plantar test device (Ugo Basile) and allowed to acclimate to their environment for 1 h before testing. The heat source, a mobile infrared photobeam, was then applied onto the plantar surface of the right hindpaw and latency time for withdrawal from the thermal stimulus was automatically determined. Response latency was defined as the time from the onset of exposure to the cessation of the photobeam when the photodiode motion sensor detected the withdrawal of the paw. The intensity of the infrared photobeam was adjusted based on previous studies to produce baseline response latencies ranging between 8 and 12 s in control untreated mice. A cut-off time of 20 s was imposed to prevent tissue damage in the absence of response. Paw withdrawal latencies were measured in triplicate for each animal, with at least one minute interval between successive measurements.

2.5.3. Experimental approach

Acute administration. The effectiveness of acute treatments with 5-HT_7 receptor ligands (AS-19 and SB-258719) on neuropathic pain-related behaviors as well as pharmacological reversion of the effects was investigated in independent groups of nerve-injured (n = 12) and sham-operated (n = 12) mice using either automatic von Frey or plantar test, depending on the experiment. Mice were habituated to the environment of each experimental test during 2 days. After the habituation period, responses in the test were established during 2 consecutive days to obtain pre-surgery baseline values. One day after baseline measurements, surgery to generate nerve injury or sham operation was carried out. Post-surgery responses of mice treated with vehicle were obtained on day 10 after surgery. On days 11–13 post-surgery, mice were treated s.c. with either three different doses (0.1, 1 and 10 mg/kg) of a 5-HT_7 receptor ligand (the agonist AS-19 or the antagonist SB-258719) following a *Latin square* design or with single compounds (day 11 the antagonist, and day 12 the agonist) followed by the combination of the two compounds (day 13) in reversion experiments. Finally, on day 14 after surgery, mice were administered with vehicle and responses (post-treatment values) were evaluated as an internal control to ensure that mechanical hypersensitivity and thermal hyperalgesia were not influenced by previous treatments. Behavioral evaluation was always performed 30 min after treatment with either vehicle or 5-HT_7 receptor ligands.

Repeated administration. The effectiveness of repeated administration of the 5-HT_7 receptor agonist E-57431 on the development of neuropathic pain-related behaviors was investigated in nerve-injured (n = 24; 12 receiving drug treatment and 12 vehicle) and sham-operated (n = 24; 12 receiving drug treatment and 12 vehicle) mice using manual von Frey and plantar tests. After the habituation period, baseline pre-surgery responses were established during two consecutive days for each test in the following sequence: von Frey test and plantar test (15 min interval

between each test). One day after baseline measurements, sciatic nerve injury or sham operation was induced. Treatment with vehicle or E-57431 (10 mg/kg) started the day of surgery (day 0) and was maintained for a period of 11 days. Treatments were administered by i.p. route twice daily (9:00 and 19:00 h). Animals were tested 30 min after the morning administration on days 3, 6 and 10 after the surgical procedure. On days 12, 15 and 20 after surgery all mice received vehicle and were tested using the same sequence (30 min von Frey test and 45 min plantar test) to know if post-treatment values of mechanical allodynia and thermal hyperalgesia were influenced by previous repeated treatments.

2.6. Rotarod motor coordination test

To investigate the possibility that treatments could affect motor coordination and thus the responsiveness of mice in the nociceptive behavioral tests, the motor performance of mice treated with 5-HT_7 receptor agonists (AS-19 or E-57431) or vehicle ($n = 10$ per group) was assessed by means of an automated rotarod (Panlab SL, Barcelona, Spain). Briefly, mice were required to walk against the motion of an elevated rotating drum at 10 rpm and the latency to fall down was recorded automatically. Before drug treatments, mice were trained and animals that were unable to stay moving on the rod for 240 s were discarded for the study. With the selected animals, rotarod latencies were measured 30, 60, 120 and 180 min after the i.p. administration of compounds or vehicle.

2.7. Immunohistochemistry

2.7.1. Antibodies

The antibody used to identify 5-HT_7 receptors is commercially available (formerly DiaSorin, Antony, France; now commercialized by ImmunoStar Inc., Hudson, USA, Cat. No. 24430). This antibody is an affinity-purified rabbit polyclonal antiserum specific for amino acids 8–23 of rat 5-HT_7 receptor, a sequence producing no significant alignments with other 5-HT receptors or non-related proteins (Protein Blast NCBI). The specificity of the antibody was tested by several methods, including Western blot and immunocytochemistry [4,31]. The antibody labeled cells transfected with the 5-HT_7 receptor gene but not untransfected ones. Western blot analysis has shown that a single band of 70 kDa, compatible with the size of the receptor, is labeled [4]. The immunolabeling was absent in transfected cells after preabsorption with the immunogen [31], and in tissue sections from rat spinal cord [11] and brain [31] pre-incubated with the immunogen. Finally, intraventricular injection of 8-OH-DPAT (mixed 5-HT_{1A}/5-HT_7 agonist) together with a specific 5-HT_{1A} antagonist induced c-fos expression only in cell bodies immunoreactive for this anti-5-HT_7 receptor antibody [31].

A commercially available mouse monoclonal antibody to GABA (clone GB-69; Sigma–Aldrich Co., Cat. No. A0310) was used for double 5-HT_7 receptor/GABA immunohistochemistry. The characterization and specificity of the monoclonal GABA antibody have been described by the provider (see product Datasheet). Cross-reactivity with glutamate was discarded based on the finding that immunolabeling of neurons was abolished by preabsorption of the antibody with a GABA–BSA conjugate, while pre-incubation with an L-glutamate-conjugate did not interfere with normal labeling [22]. In addition, comparable labeling was found when compared immunohistochemical localization of GABA with this antibody and the GABA-synthesizing enzyme glutamic acid decarboxylase [24].

2.7.2. Immunohistochemical procedure

Single immunoperoxidase labeling (quantification of 5-HT_7 receptor immunostaining). On day 11 after surgery, independent groups of nerve-injured ($n = 6$) and sham-operated ($n = 3$) mice not exposed to pharmacological treatments were deeply anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and perfused intracardially with cold saline followed by cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Spinal cords were removed and the L4–L5 segments were dissected out and postfixed for 4 h in the same fixative. Then, spinal cord segments were washed in PB and serial coronal sections (40 μm thick) were obtained using a vibratome (vibrocut FTB, Germany) and collected in phosphate-buffered saline (PBS) to be processed immunohistochemically as free-floating sections. Sections were pre-incubated with 0.3% H_2O_2 in PBS for 30 min at room temperature (RT) to block endogenous peroxidase activity and then, after washing three times with PBS, with normal goat serum diluted 1:100 in PBS for 1 h at RT to prevent unspecific staining. Sections were then incubated for 24 h at 4 °C with the 5-HT_7 receptor rabbit antiserum (ImmunoStar, Cat. No. 24430) diluted 1:125 in PBS with 1% bovine serum albumin (PBS–BSA) [11,31]. The sections were washed three times for 10 min each in PBS–BSA and incubated with anti-rabbit biotinylated antiserum diluted 1:200 in PBS–BSA (Vectastain Vector, USA) for 1 h at RT. After washing the sections three times in PBS–BSA, an avidin–biotin–peroxidase complex was applied (diluted 1:100 in PBS, Vectastain Vector) for 1 h at RT. The sections were washed again in PBS, then immersed in a chromogen solution containing 0.05% 3,3′-diaminobenzidine-tetrahydrochloride and 0.01% H_2O_2 in PBS for 5 min at RT and reaction was stopped by several washes in PBS. The immunostained sections were placed on gelatin-coated slides, air dried and dehydrated before being mounted on DPX (Fluka, Spain) for microscopic observation and photography.

Sections from nerve-injured and sham-operated mice were simultaneously processed for immunohistochemistry in order to avoid methodological variations that would affect the intensity of staining. Three L4–L5 spinal cord sections per mouse were randomly selected and fields containing the ipsilateral dorsal horn were digitized using a video camera (Olympus DP70) connected to a microscope (Olympus BX61) and interfaced to a computer. The boundary of the dorsal horn laminae was traced and the mean density of immunostaining was quantified based on the inverse computer grayscale (from 0 = white to 255 = black) using the National Institutes of Health (NIH) Image J software. Individual immunodensity values were corrected by subtracting the background (labeling in the white matter) for each section.

Double immunofluorescence labeling of 5-HT_7 receptor and GABA. On day 15 after surgery, independent groups of nerve-injured ($n = 3$) and sham-operated ($n = 3$) mice were anesthetized with ketamine/xylacin (50/10 mg/kg) and then intracardially perfused with heparinized phosphate buffer, followed by 4% paraformaldehyde. The lumbar region of the spinal cord was removed, cryopreserved in 30% sucrose solution at 4 °C, embedded in O.C.T., sliced in 25 μm sections on a cryostat and mounted on silane-coated slides.

The slides were incubated in the blocking solution (3% of normal goat serum, 0,05% Triton 100 in PB 0.1 M) for 1 h and then in a mixture containing polyclonal anti-5-HT_7 receptor antibody (1:250, ImmunoStar Inc.) and monoclonal anti-GABA antibody (1:750, Sigma–Aldrich Co.) in blocking solution at 4 °C overnight. The sections were washed three times for 10 min each in PB followed by incubation for 1 h with CY3-conjugated anti-rabbit secondary antibody (1:500, Jackson Immunoresearch, Baltimore, PA, USA) and CY2-conjugated anti-mouse secondary antibody (1:500, Jackson Immunoresearch) in blocking solution.

Confocal images were obtained using a Leica SP2 confocal microscope, adapted to an inverted Leica DM IRBE microscope. Tissue sections of the lumbar dorsal horn were examined with a 40× 1.25 NA oil immersion in Leica Plan Apochromatic objective at 2× zoom. CY2 and CY3 were excited with the 488 nm line of an Argon laser and the 543 nm line of a Green Neon laser, respectively, and

double immunofluorescence images of three stained sections were taken for each animal in a sequential mode. From each section, images were always recorded from the ipsilateral and contralateral dorsal horns.

2.8. Data analysis

For neuropathic pain-related behaviors, statistical analysis to test the effect of treatment in nerve-injured and sham-operated mice was made using an initial ANOVA followed by Newman–Keuls (acute administration) or Bonferroni (repeated administration) multiple comparison tests. For the rotarod test, statistic analysis to test the effect of treatments on latency was made using ANOVA followed by Newman–Keuls's post hoc comparison.

For the histological study, the expression of 5-HT$_7$ receptor was estimated as the density of immunostaining using anti-5-HT$_7$ receptor antibodies. Immunodensity values in nerve-injured mice in laminae I–II and laminae III–V of the ipsilateral dorsal horn were compared with values obtained in sham-operated mice using one-way ANOVA followed by Newman–Keuls's post hoc comparison.

Values presented in graphs are the mean ± SEM. The level of significance was set at $p < 0.05$.

3. Results

3.1. Selectivity, efficacy and potency of E-57431

E-57431 is a new highly selective, potent 5-HT$_7$ receptor agonist. It showed high affinity for 5-HT$_7$ receptors (K_i = 0.47 nM), some affinity for 5-HT$_{1D}$ (K_i = 53 nM) and 5-HT$_{2A}$ (K_i = 560 nM) and no significant affinity (K_i > 1 μM or % inhibition at 1 μM < 50%) for other 5-HT receptor subtypes and 160 additional targets including receptors, transporters and ion channels included in the commercial binding screening package (Table 1). When tested in a functional assay, E-57431 concentration-dependently increased cAMP formation in HEK-293F/h5-HT$_7$ cells (data not shown) and behaved as a full agonist, with high efficacy (E_{max} = 94.5 ± 1%; E_{max} for 5-HT considered 100%) and potency (EC_{50} = 21.5 ± 1 nM) at 5-HT$_7$ receptors.

Information on the selectivity (binding profile) of the rest of pharmacological tools used in this study has also been compiled in Table 1.

3.2. AS-19, a selective 5-HT$_7$ receptor agonist, inhibits mechanical hypersensitivity and thermal hyperalgesia secondary to nerve injury

Partial sciatic nerve ligation induced mechanical hypersensitivity and thermal hyperalgesia. Mechanical hypersensitivity was evidenced by a reduced pressure threshold evoking withdrawal of the ipsilateral hindpaw on day 10 post-surgery compared to baseline pre-surgery values (Fig. 1A). In turn, thermal hyperalgesia was evidenced by a decreased withdrawal latency of the ipsilateral hindpaw in response to a thermal stimulus on day 10 post-surgery compared to baseline pre-surgery values (Fig. 2A). Sham operation did not induce mechanical hypersensitivity (Fig. 1B) or thermal hyperalgesia (Fig. 2B) as no significant changes of the response were found in sham-operated mice 10 days after surgery compared to baseline pre-surgery values.

Systemic administration of the 5-HT$_7$ receptor agonist AS-19 on days 11–13 at doses of 1 and 10 mg/kg significantly inhibited mechanical hypersensitivity and thermal hyperalgesia (Figs. 1A and 2A). At these doses, AS-19 restored the withdrawal threshold in response to mechanical stimulation and the withdrawal latency in response to thermal stimulation of the nerve-injured hindpaw to baseline pre-surgery values.

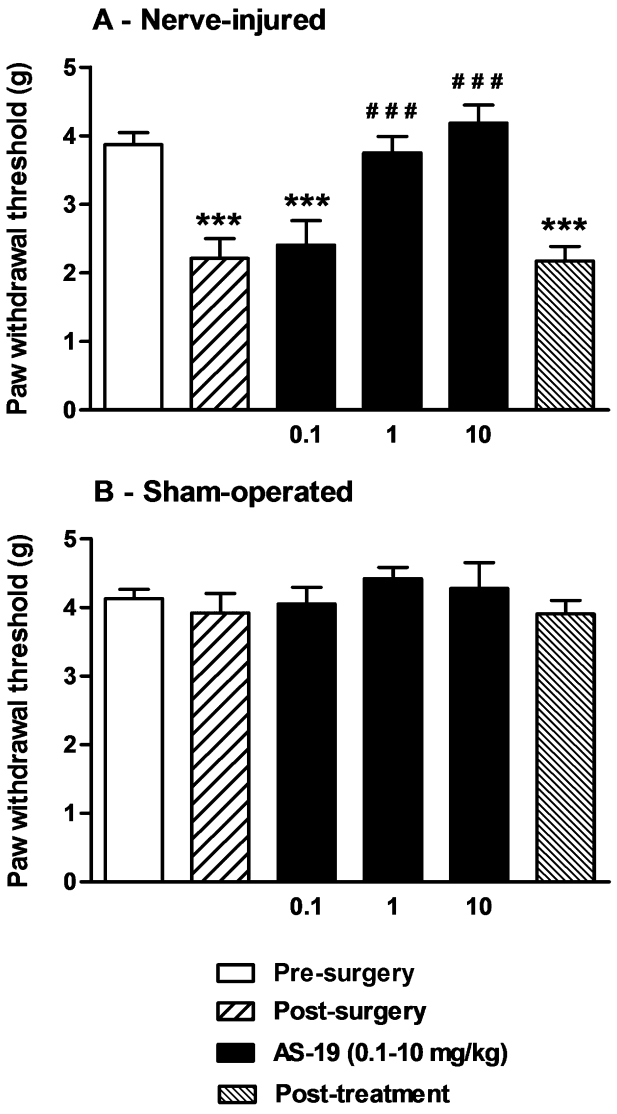

Fig. 1. Dose–response effect of AS-19 on mechanical hypersensitivity. Pressure threshold evoking withdrawal of the ipsilateral hindpaw in response to mechanical stimulation (electronic von Frey) in nerve-injured (A) and sham-operated (B) mice before surgery (pre-surgery), on day 10 after surgery following treatment with vehicle (post-surgery), on days 11–13 post-surgery after treatment with three different doses of AS-19 in a Latin square design, and finally on day 14 post-surgery after treatment with vehicle (post-treatment). Note that mechanical hypersensitivity developed after partial sciatic nerve ligation (but not after sham operation) and that AS-19 at doses of 1 and 10 mg/kg inhibited nerve injury-induced mechanical hypersensitivity. $^{***}p < 0.001$ vs. pre-surgery; $^{###}p < 0.001$ vs. post-surgery (ANOVA followed by Newman–Keuls multiple comparison test).

The reduction of mechanical hypersensitivity and thermal antihyperalgesic effects disappeared after the withdrawal of the AS-19 treatment (post-treatment values on day 14 were not significantly different from pre-treatment post-surgery values on day 10). Treatment with a lower dose (0.1 mg/kg) of AS-19 was ineffective as no modification of these behavioral manifestations of neuropathic pain was found compared to vehicle treatment (Figs. 1A and 2A). AS-19 did not produce significant effects at 0.1, 1 and 10 mg/kg in sham-operated mice (Figs. 1B and 2B).

3.3. SB-258719, a selective 5-HT$_7$ receptor antagonist, promotes mechanical hypersensitivity but not thermal hyperalgesia

Activation of 5-HT$_7$ receptors by the selective agonist AS-19 reduced mechanical and thermal hypersensitivities in nerve-injured mice, but does a selective 5-HT$_7$ receptor antagonist exert pronoci-

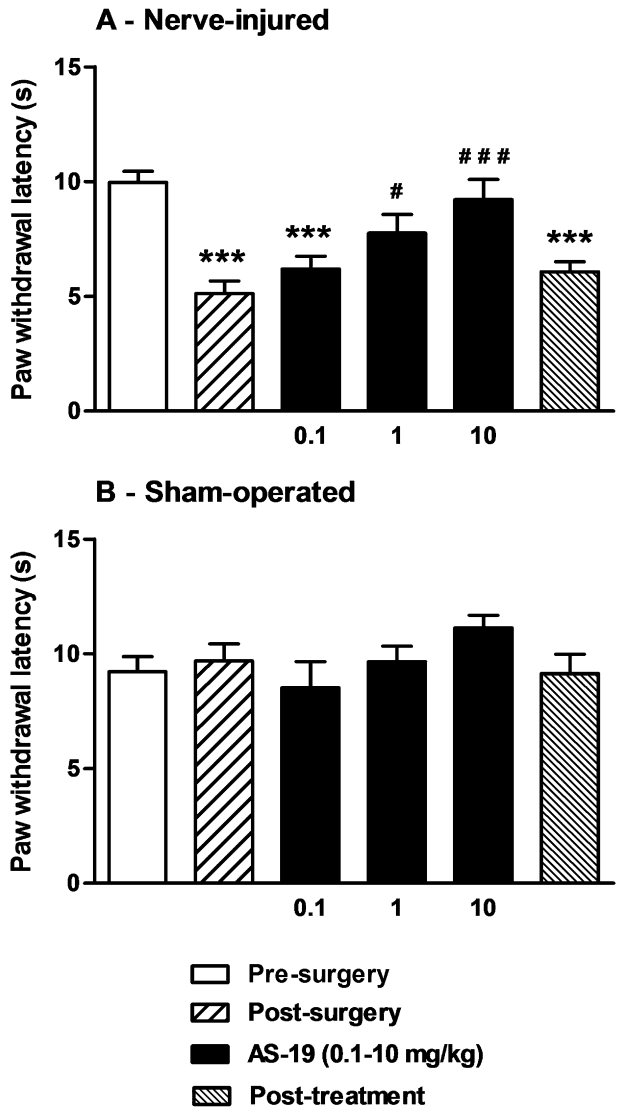

Fig. 2. Dose–response effect of AS-19 on thermal hyperalgesia. Latency of withdrawal of the ipsilateral hindpaw in response to thermal stimulus (plantar test) in nerve-injured (A) and sham-operated (B) mice before surgery (pre-surgery), on day 10 after surgery following treatment with vehicle (post-surgery), on days 11–13 post-surgery after treatment with three different doses of AS-19 in a Latin square design, and finally on day 14 post-surgery after treatment with vehicle (post-treatment). Note that thermal hyperalgesia developed after partial sciatic nerve ligation (but not after sham operation) and that AS-19 at doses of 1 and 10 mg/kg exerted antihyperalgesic effects. $^{***}p < 0.001$ vs. pre-surgery; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs. post-surgery (ANOVA followed by Newman–Keuls multiple comparison test).

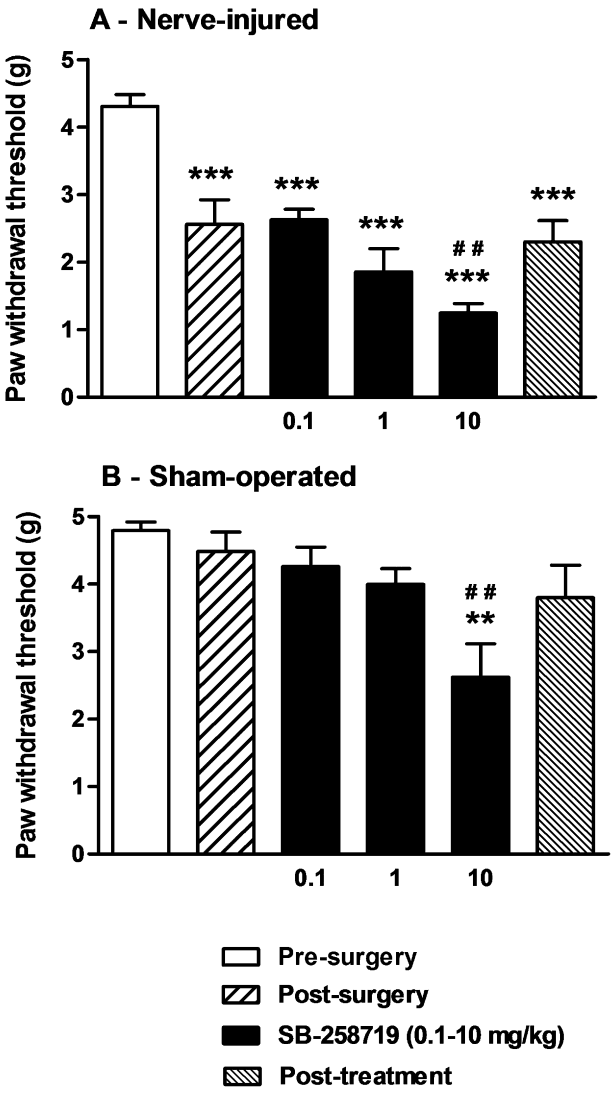

Fig. 3. Dose–response effect of SB-258719 on mechanical hypersensitivity. Pressure threshold evoking withdrawal of the ipsilateral hindpaw in response to mechanical stimulation (electronic von Frey) in nerve-injured (A) and sham-operated (B) mice before surgery (pre-surgery), on day 10 after surgery following treatment with vehicle (post-surgery), on days 11–13 post-surgery after treatment with three different doses of SB-258719 in a Latin square design, and finally on day 14 post-surgery after treatment with vehicle (post-treatment). Note that mechanical hypersensitivity developed after partial sciatic nerve ligation (but not after sham operation) and that SB-258719 at the dose of 10 mg/kg significantly promoted mechanical hypersensitivity in both nerve-injured and sham-operated mice. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. pre-surgery; $^{\#\#}p < 0.01$ vs. post-surgery (ANOVA followed by Newman–Keuls multiple comparison test).

ceptive effects? To investigate this possibility, we administered the 5-HT_7 receptor antagonist SB-258719 to nerve-injured and sham-operated mice.

We observed that subcutaneous treatment with SB-258719 at doses of 2.5 and 10 mg/kg significantly decreased the mechanical threshold evoking withdrawal of the ipsilateral, nerve-injured hindpaw below post-surgery values (Figs. 3A and 5A). In addition, SB-258719 was able to induce mechanical hypersensitivity in sham-operated mice when subcutaneously administered at doses of 2.5 and 10 mg/kg (Figs. 3B and 5B). Promotion of mechanical hypersensitivity did not occur at lower doses (0.1 and 1 mg/kg) (Fig. 3A).

Contrary to mechanical hypersensitivity, thermal hyperalgesia was not promoted by the 5-HT_7 receptor antagonist SB-258719 at any tested dose in mice exposed either to sciatic nerve injury or to sham operation (Fig. 4).

3.4. Reversion of the inhibitory effects of AS-19 on mechanical and thermal hypersensitivity by SB-258719

In order to confirm that the activation of 5-HT_7 receptors was unambiguously responsible for the inhibition of nerve injury-induced mechanical and thermal hypersensitivities, we used the 5-HT_7 receptor antagonist SB-258719 to pharmacologically reverse the inhibitory effects exerted by the 5-HT_7 receptor agonist AS-19. As shown in Figs. 5A and 6A, the effects on mechanical and thermal hypersensitivity elicited by AS-19 (1 mg/kg) in nerve-injured mice were significantly reduced when the agonist was co-administered with SB-258719 (2.5 mg/kg). Similarly, the promotion of mechanical hypersensitivity by SB-258719 (2.5 mg/kg) in sham-operated mice was blocked by co-administration of AS-19 (1 mg/kg) (Fig. 5B).

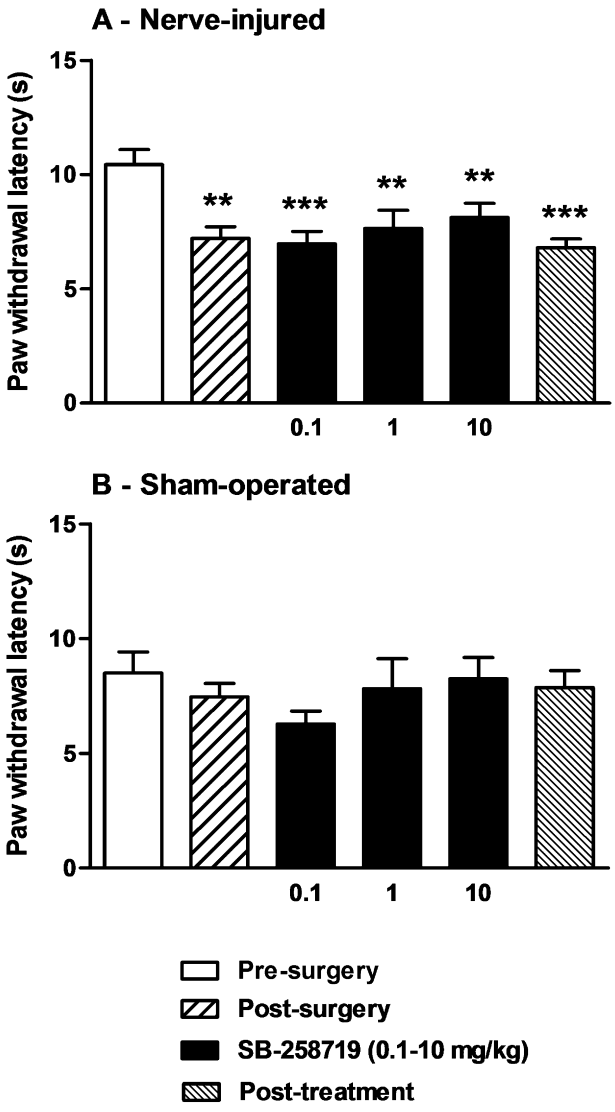

Fig. 4. Dose–response effect of SB-258719 on thermal hyperalgesia. Latency of withdrawal of the ipsilateral hindpaw in response to thermal stimulus (plantar test) in nerve-injured (A) and sham-operated (B) mice before surgery (pre-surgery), on day 10 after surgery following treatment with vehicle (post-surgery), on days 11–13 post-surgery after treatment with three different doses of SB-258719 in a Latin square design, and finally on day 14 post-surgery after treatment with vehicle (post-treatment). Note that thermal hyperalgesia developed after partial sciatic nerve ligation (but not after sham operation) and that SB-258719 did not exert any significant effect. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. pre-surgery (ANOVA followed by Newman–Keuls multiple comparison test).

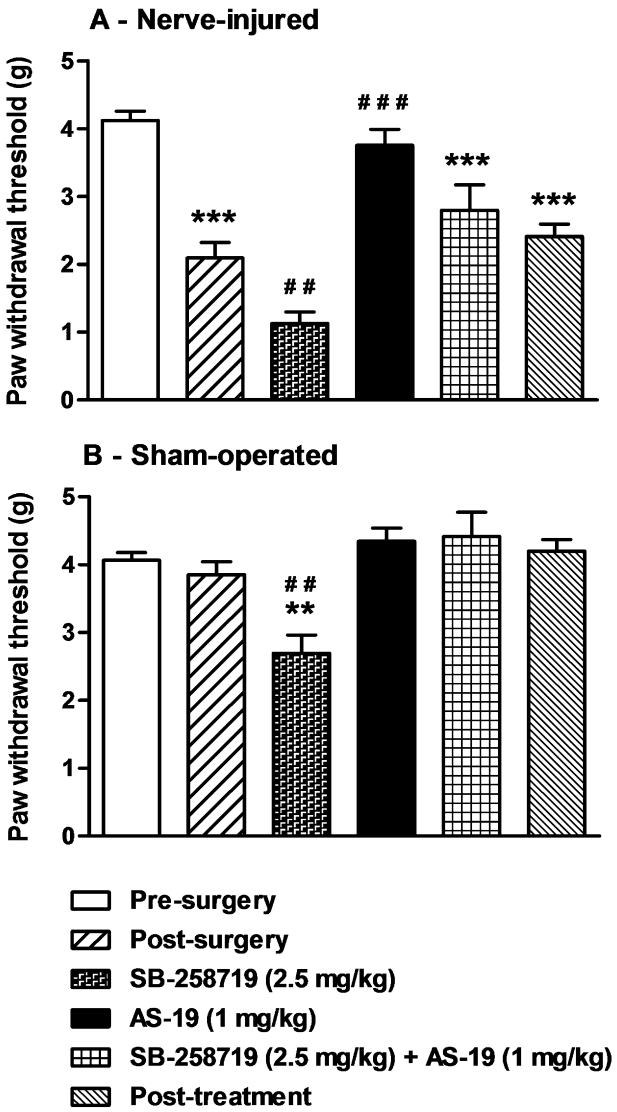

Fig. 5. Reversion of the effects of AS-19 and SB-258719 on mechanical hypersensitivity. Pressure threshold evoking withdrawal of the ipsilateral hindpaw in response to mechanical stimulation (electronic von Frey) in nerve-injured (A) and sham-operated (B) mice before surgery (pre-surgery), on day 10 after surgery following treatment with vehicle (post-surgery), on day 11–13 post-surgery after treatment with SB-258719, AS-19 or their combination, and finally on day 14 post-surgery after treatment with vehicle (post-treatment). Note that SB-258719 (2.5 mg/kg) promoted mechanical hypersensitivity in both nerve-injured and sham-operated mice. In contrast, AS-19 (1 mg/kg) reduced hypersensitivity only in nerve-injured mice. Combination of SB-258719 and AS-19 resulted in the blockade of their respective, opposite effects. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. pre-surgery; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. post-surgery (ANOVA followed by Newman–Keuls multiple comparison test).

3.5. Effectiveness of the treatment with the 5-HT$_7$ receptor agonist E-57431 was maintained after repeated administration

The effectiveness of repeated administration of the 5-HT$_7$ receptor agonist E-57431 on the development of neuropathic pain-related behaviors was investigated in nerve-injured and sham-operated mice. Mice were administered i.p. twice daily with vehicle or E-57431 (10 mg/kg) for a period of 11 days after the surgery. On days 3, 6 and 10 of treatment mice were sequentially assessed for mechanical hypersensitivity (allodynia) (evaluated by manual von Frey stimulation 30 min after the morning administration) and thermal (heat) hyperalgesia (evaluated by the plantar test 45 min after the morning administration).

As expected, mechanical allodynia and thermal hyperalgesia developed in vehicle-treated mice exposed to sciatic nerve injury from day 3 after surgery when compared to sham-operated mice (Fig. 7). In contrast, mechanical allodynia and thermal hyperalgesia were significantly attenuated in nerve-injured mice treated with E-57431 throughout the treatment period. Mechanical allodynia was significantly reduced on day 3 in nerve-injured mice receiving subchronically E-57431 (respect to values in vehicle-treated nerve-injured mice) and the antiallodynic efficacy of the treatment increased progressively on days 6 and 10 (Fig. 7A). Regarding thermal hyperalgesia, it was completely blocked on day 3 in nerve-injured mice receiving subchronically E-57431 (values were undistinguishable from values obtained in sham-operated mice) and this level of efficacy was maintained on days 6 and 10 of treatment (Fig. 7B). Neuropathic pain-related

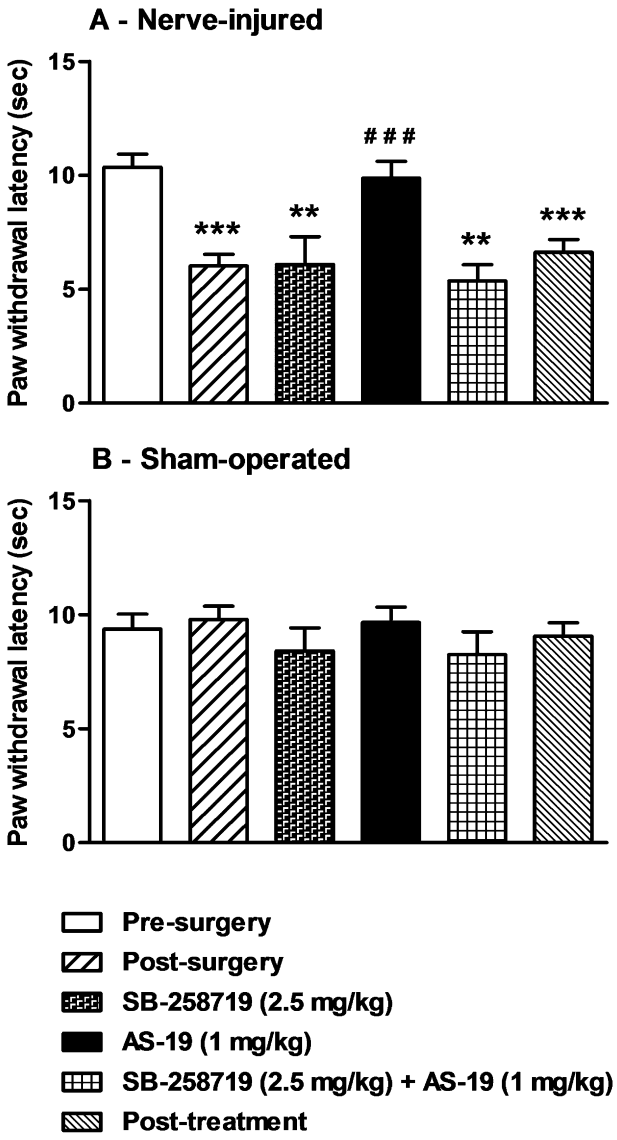

Fig. 6. Reversion of the effects of AS-19 on thermal hyperalgesia. Latency of withdrawal of the ipsilateral hindpaw in response to thermal stimulus (plantar test) in nerve-injured (A) and sham-operated (B) mice before surgery (pre-surgery), on day 10 after surgery following treatment with vehicle (post-surgery), on day 11–13 post-surgery after treatment with SB-258719, AS-19 or their combination, and finally on day 14 post-surgery after treatment with vehicle (post-treatment). Note that AS-19 (1 mg/kg) exerted antihyperalgesic effect in nerve-injured mice whereas SB-258719 (2.5 mg/kg) was devoid of effect. However, when combined, SB-258719 blocked the antihyperalgesic effect of AS-19. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. pre-surgery; $^{###}p < 0.001$ vs. post-surgery (ANOVA followed by Newman–Keuls multiple comparison test).

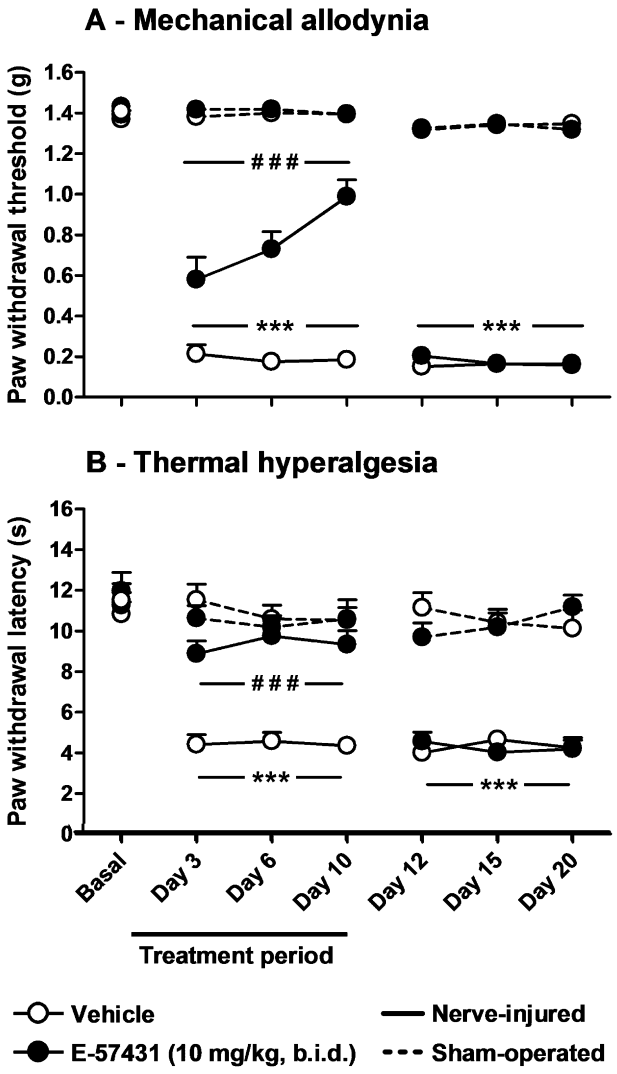

Fig. 7. Effect of repeated administration of E-57431 on the development of neuropathic pain-related behaviors. Mechanical allodynia using manual von Frey filaments (A) and thermal hyperalgesia using the plantar test (B) were assessed in the ipsilateral hindpaw of nerve-injured and sham-operated mice after daily administration of the 5-HT_7 agonist E-57431 (10 mg/kg) or vehicle, twice a day for 11 days. Treatment with E-57431 or vehicle started the day of surgery (day 0) and was maintained up to day 10. Behavioral testing was done before surgery (basal, pre-surgery values), after surgery on days 3, 6 and 10 of treatment (30–45 min after the morning administration), and on days 12, 15 and 20 post-surgery when the treatment was withdrawn (30–45 min after vehicle administration). Note that both mechanical and thermal hypersensitivity were significantly inhibited in nerve-injured mice subchronically treated with E-57431 and that the efficacy of the treatment was maintained (thermal hyperalgesia) or increased (mechanical allodynia) throughout the treatment period. Note also that neuropathic pain-related behaviors reverted back to baseline nerve injury values when the treatment with E-57431 was withdrawn (days 12, 15 and 20). Treatments were devoid of effects in sham-operated mice. $^{***}p < 0.001$ vehicle nerve-injured vs. vehicle sham-operated; $^{###}p < 0.001$ E-57431 nerve-injured vs. vehicle nerve-injured. (ANOVA followed by Bonferroni multiple comparison test).

behaviors reverted back to baseline nerve injury values when the treatment with E-57431 was withdrawn (post-treatment mechanical allodynia and thermal hyperalgesia on days 12, 15 and 20 were undistinguishable from values of nerve-injured mice that received vehicle treatment).

3.6. Effect of AS-19 and E-57431 on motor performance (rotarod test)

Animals treated with different increasing doses of AS-19 and E-57431 were tested in the rotarod test 30, 60, 120 and 180 min post-treatment to rule out possible treatment-related locomotor disturbing effects on the results of the pain experiments.

The latency to fall down from the rotarod was recorded and significant differences between groups were found after the administration of compounds at the highest doses. Both AS-19 and E-57431 induced the maximum effects 30 min after their i.p. administration (data not shown). At 30 min, the dose–response curve revealed significant effects on motor coordination at 40 and 80 mg/kg, but not at 10 and 20 mg/kg for both compounds (Fig. 8). Thus, at the maximum dose used in nociceptive behavioral tests (10 mg/kg), compounds were devoid of motor disturbing effects.

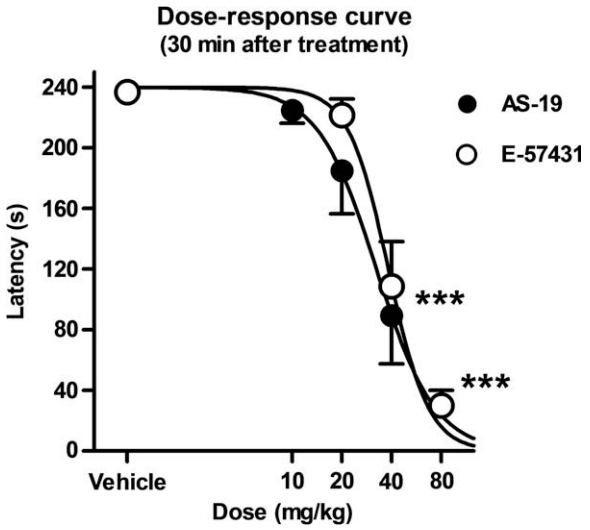

Fig. 8. Effect of AS-19 and E-57431 on the rotarod test. The latency to fall-down from the rotarod was recorded in mice 30 after single administration of AS-19 and E-57431 at different doses. The dose–response curve revealed significant motor disturbing effects at doses higher than 20 mg/kg for both compounds. ED_{50} = 32.45 ± 1.13 mg/kg for AS-19; and 38.35 ± 1.08 mg/kg for E-57431. ***$p < 0.001$ vs. vehicle (ANOVA followed by Newman–Keuls multiple comparison test).

3.7. Nerve injury increases 5-HT$_7$ receptor immunoreactivity in the dorsal horn of the spinal cord

We next investigated whether changes in 5-HT$_7$ receptor expression were induced after nerve injury in the spinal cord by immunohistochemistry. Immunolabeling of the 5-HT$_7$ receptor was observed mainly in the two superficial laminae of the dorsal horn. At the light microscope level, immunoreaction was mostly found in the perikarya of some cells (probably neurons based on their distribution) and the neuropile (including probably dendritic processes). Interestingly, when expression levels were quantified on day 11 after surgery, the density of 5-HT$_7$ receptor immunoreactivity was found to be significantly increased in both laminae I–II and III–V of the ipsilateral dorsal horn of L4–L5 segments in nerve-injured compared to sham-operated mice (Fig. 9A–C).

3.8. The 5-HT$_7$ receptor co-localized with GABAergic cells in the dorsal horn of the spinal cord

Double immunofluorescence labeling of 5-HT$_7$ receptor and GABA on spinal cord sections from nerve-injured and sham-operated mice on day 15 after surgery revealed that the 5-HT$_7$ receptor co-localized with GABAergic cells in the dorsal horn of the spinal cord. This co-localization was mainly revealed in cell bodies of GABAergic interneurons in laminae III–V (Fig. 9D–F). In the superficial laminae I and II, immunostaining for 5-HT$_7$ receptor did not clearly co-localized with the GABA neurotransmitter. The number of GABAergic cell bodies that expressed 5-HT$_7$ receptor was similar in the ipsilateral and contralateral horns of both sham-operated and nerve-injured mice.

4. Discussion

In the present work, using the partial sciatic nerve ligation model of neuropathic pain in mice [27], we investigated the effects of 5-HT$_7$ receptor ligands on nerve injury-induced mechanical and thermal (heat) hypersensitivities. A new 5-HT$_7$ receptor agonist, E-57431, with more than 100-fold selectivity over a range of other receptors, was described. The effect of acute and repeated administration of 5-HT$_7$ receptor agonists, the cellular localization of spinal 5-HT$_7$ receptors and changes in 5-HT$_7$ receptor expression in the spinal cord secondary to nerve injury were investigated.

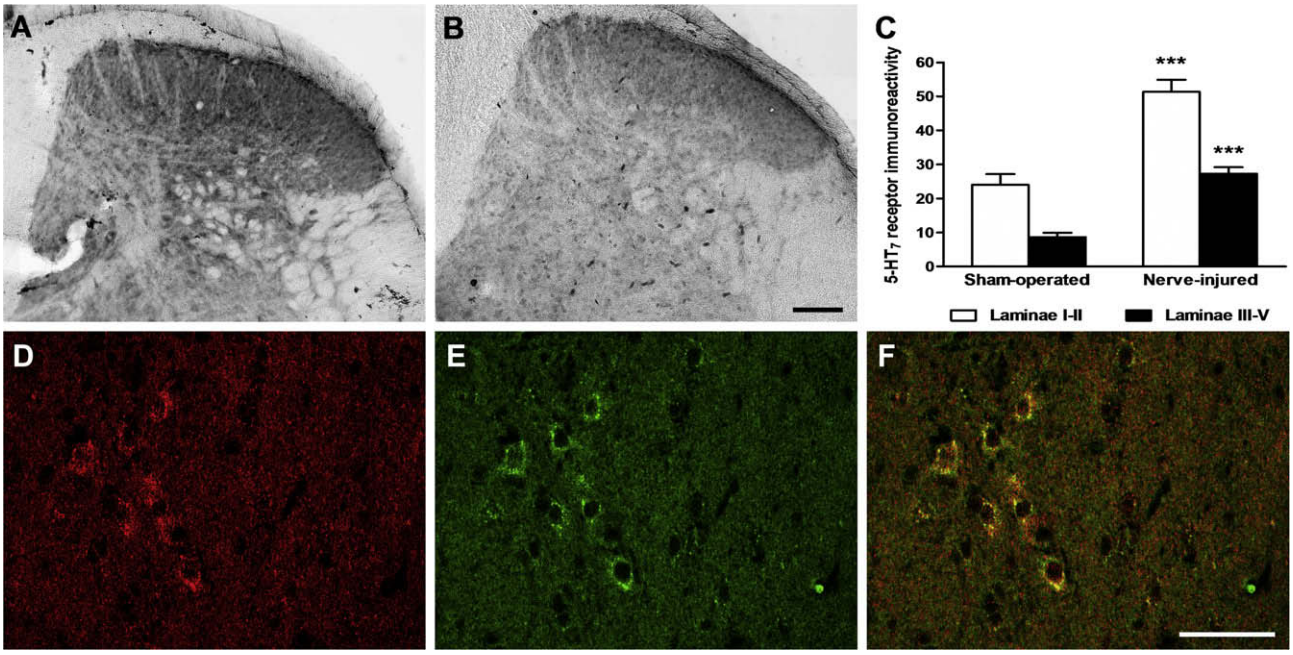

Fig. 9. 5-HT$_7$ receptor immunoreactivity in the dorsal horn of the lumbar spinal cord and co-localization with GABAergic neurones. Immunohistochemical labeling of 5-HT$_7$ receptors in the ipsilateral dorsal horn of the spinal cord 11 days after sciatic nerve injury (A) or sham operation (B). Note that, when quantified, immunoreactivity for 5-HT$_7$ receptors was significantly increased in both laminae I–II and III–V of the ipsilateral dorsal horn at lumbar L4–L5 levels in nerve-injured compared to sham-operated mice (C). Confocal immunofluorescence microscopy showing 5-HT$_7$ receptor (D), GABA (E) and double 5-HT$_7$ receptor/GABA (F) immunostaining in the ipsilateral laminae III–V of the lumbar spinal cord of sciatic nerve-injured mice. ***$p < 0.001$ vs. corresponding laminae of sham-operated mice (ANOVA followed by Newman–Keuls multiple comparison test). Scale bar in A and B = 200 μm; in D–F = 50 μm.

Activation of 5-HT$_7$ receptors by acute systemic administration of the 5-HT$_7$ receptor agonist AS-19 exerted a clear-cut dose-dependent inhibition of nerve injury-induced mechanical hypersensitivity and thermal hyperalgesia. Co-administration of the selective 5-HT$_7$ receptor antagonist SB-258719 blocked the effects of the agonist. Similarly, in a previous study describing the effect of systemically administered BBB-penetrant 5-HT$_7$ receptor ligands following neurogenic sensitization with capsaicin, 5-HT$_7$ receptor agonists inhibited mechanical hypersensitivity in mice and co-administration of 5-HT$_7$ receptor antagonists prevented this effect [3]. Here, using the same 5-HT$_7$ receptor ligands, we show that the overall effect of activating 5-HT$_7$ receptors is antinociceptive (antiallodynic/antihyperalgesic) in sensitizing conditions involving nerve injury. Interestingly, no tolerance to the effect was evidenced following repeated systemic administration of the selective 5-HT$_7$ receptor agonist E-57431 twice daily for 11 days. The effectiveness was maintained or even slightly increased throughout the treatment period but neuropathic pain-related behaviors reverted back to baseline nerve injury values when the treatment was withdrawn. This suggests an improvement of "disease symptoms" related to the presence and influence of the drug at the time of the test. Finally, it is important to note that effectiveness of the treatment with 5-HT$_7$ receptor agonists was not masked by non-specific motor effects, as no motor incoordination was found in the rotarod test at the doses used in both acute and repeated administration experiments.

Desensitization and down-regulation following agonist exposure are common among G protein-coupled receptors, but there is some discrepancy at this regard for 5-HT$_7$ receptors. Down-regulation has been described in some studies (e.g., in the hypothalamus after fluoxetine treatment for 21 days) [44] but data indicating that 5-HT$_7$ receptors are not readily down-regulated by long-term exposure to agonists is also available [21]. Actually, significantly increased 5-HT$_7$ receptor mRNA expression has been reported in raphe nuclei, hippocampus and prefrontal cortex after treatment with the 5-HT$_7$ receptor agonist AS-19 [35]. In the present study, activity of the 5-HT$_7$ receptor agonist E-57431 in nerve-injured mice was maintained throughout subchronic (11 days) treatment. Therefore, if desensitization and/or down-regulation phenomena occur, they do not have noticeable consequences in the particular conditions of our study (e.g., they could require longer exposure to the agonist to become apparent) or are compensated by nerve injury-induced receptor up-regulation (see discussion later).

Available data in the literature suggest a pronociceptive role of 5-HT$_7$ receptors when activation occurs at the periphery [29,39]. This inference is based on the use of non-selective agonists (5-HT, 5-CT, 8-OH-DPAT) locally administered (intraplantarly or intra-articularly) in the context of tissue injury and inflammation. Peripheral tissue injury causes the release of 5-HT from platelets and mast cells, and the endogenous indolamine acts in combination with other inflammatory mediators to excite and sensitize afferent nerve fibers [45]. Activation of 5-HT$_{2A}$ and 5-HT$_3$ receptor subtypes present on C-fibers was already shown to underlie such a peripheral pronociceptive effect of 5-HT [32,45]. However, whether or not peripheral 5-HT$_7$ receptors contribute to 5-HT-evoked pain in inflammatory and neuropathic pain conditions needs to be confirmed.

In contrast, an antinociceptive role for central 5-HT$_7$ receptors has been suggested at both spinal [9,10] and supraspinal [8,16] levels based on the effects of local (intrathecal, intracerebroventricular or intrathalamic) administration of non-selective ligands in nociceptive (tail-flick, paw-flick or tailshock) or inflammatory (intrarticular, gout-like) pain models. Regarding such an antinociceptive role, it is important to note that agonists acting at 5-HT$_7$ receptors cannot directly inhibit primary afferents, second-order nociceptive dorsal horn neurons or third order supraspinal neurons because stimulation of the 5-HT$_7$ receptor has excitatory effects [5]. Therefore, an indirect action through the activation of 5-HT$_7$ receptors localized on inhibitory enkephalinergic or GABAergic interneurons, to evoke the release of enkephalins or GABA, is presumably required to inhibit nociceptive transmission. In this way, immunohistochemical studies revealed that the 5-HT$_7$ receptor is located postsynaptically on local interneurons within the superficial laminae of the dorsal horn [11,29]. Our observations at the confocal microscope level showing the co-localization of 5-HT$_7$ receptors and GABA in neurons of the dorsal horn of the spinal cord provide further support to this hypothesis. In addition, it has been recently reported that spinal GABAergic interneurons are involved in 5-HT$_7$ receptor-mediated antinociception [2,48]. This is based on the finding that intrathecal pre-treatment with the GABA$_A$ receptor antagonist bicuculline, but not with the GABA$_B$ receptor antagonist phaclofen or the opioid receptor antagonist naloxone, prevented the antihyperalgesic effects exerted by 5-HT$_7$ receptor agonists in rats with constriction injury to the sciatic nerve [2,48]. Accordingly, the activation of spinal inhibitory GABAergic interneurons could underlie or at least contribute to the analgesic effects of 5-HT$_7$ receptor agonists.

A dose-dependent promotion of mechanical hypersensitivity, but not heat hyperalgesia, was observed after treatment with the 5-HT$_7$ receptor antagonist SB-258719. Differential endogenous 5-HT tone and/or modulation by 5-HT$_7$ receptors of sensory/nociceptive pathways depending on the nature (mechanical vs. thermal) and intensity (allodynic/subthreshold vs. hyperalgesic/suprathreshold) of the stimulus could explain the different effects on the response to mechanical and thermal stimuli exerted by the 5-HT$_7$ receptor antagonist. Interestingly, treatment with SB-258719 not only promoted mechanical hypersensitivity in nerve-injured mice but it also induced mechanical hypersensitivity in sham-operated mice. We found a comparable result in the capsaicin model: two different 5-HT$_7$ receptor antagonists, SB-258719 and SB-269970, promoted mechanical hypersensitivity when administered to mice subplantarly injected with a low subactive dose of capsaicin [3]. This suggests that endogenous activation of the 5-HT$_7$ receptor occurs, thereby allowing antagonists to exert a counteracting effect.

Descending 5-HTergic pathways projecting into the spinal cord can either suppress (descending inhibition) or potentiate (descending facilitation) nociceptive messages depending on the 5-HT receptor involved and its localization [9,30,33,47]. Regarding the descending inhibitory control, blockage of receptors involved in such a tonic brake control by 5-HT would suppress the inhibitory tone thus promoting hypersensitivity of nociceptive pathways. It is thus plausible on the basis on the present study showing that 5-HT$_7$ receptor agonists inhibit and a 5-HT$_7$ receptor antagonist promotes mechanical hypersensitivity, that 5-HT$_7$ receptors could participate, in concert with other 5-HT receptors [19,42], in the endogenous 5-HTergic inhibitory control of pain. In this way, the RVM is an important source of descending modulation of pain at the level of the spinal cord, and the antinociceptive effect of morphine microinjected into the RVM is known to involve the activation of spinal 5-HT$_7$ receptors [9,10].

Increased expression of 5-HT$_7$ receptors was found in the ipsilateral dorsal horn of the spinal cord of nerve-injured compared to sham-operated mice. In particular, we found a significant increase of 5-HT$_7$ immunoreactivity in laminae I–II and III–V of the dorsal horn in the ipsilateral side of the spinal cord eleven days after nerve injury. Increased 5-HT$_7$ receptor expression induced by nerve injury in the dorsal horn could represent a physiological,

compensatory, protective spinal mechanism relevant to the control of nociception in neuropathic pain conditions.

5-HT has been reported to exert algesic or analgesic effects depending on its site of action and the receptor subtype it acts on [12,19,30,33,47,50]. Based on the results reported here in the partial sciatic nerve ligation model and those recently reported in the capsaicin model in mice [3] as well as after constriction injury to the sciatic nerve in rats [2,48], it is clear that systemically administered 5-HT_7 receptor agonists crossing the BBB and acting in the CNS [3,14,34] exert an analgesic (antiallodynic/antihyperalgesic) effect in sensitizing neurogenic/neuropathic conditions. We hypothesize that, if a balance exists between pro- and antinociceptive actions depending on the localization of the 5-HT_7 receptor, the antinociceptive effect at some CNS sites may counteract the pronociceptive effect at the periphery or at other CNS sites. Activation of inhibitory GABAergic interneurons in the spinal cord, and possibly in other CNS locations, seems to be the most likely mechanism of action accounting for antinociception. The up-regulation of 5-HT_7 receptors in the dorsal horn of the spinal cord after sciatic nerve injury suggests a "pain"-triggered regulation of receptor expression that may be relevant for the effectiveness of 5-HT_7 receptor agonists.

Taken together, the results of the present study support the involvement of the 5-HT_7 receptor subtype in the control of pain and point to a new potential use of 5-HT_7 receptor agonists for the treatment of neuropathic pain. Nevertheless, this study is limited to a specific type of the experimental neuropathic pain. Further studies in different experimental pain conditions, using recently developed ligands with high affinity and selectivity for the 5-HT_7 receptor and focusing on the site and mechanism of action underlying 5-HT_7 receptor-mediated analgesia would be particularly useful.

Summary

The results of the present study support the involvement of the 5-HT_7 receptor subtype in the control of pain.

Conflicts of interests

The authors state that there were no conflicts of interests in respect to the work reported in the paper.

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6.4. Site of action of 5-HT$_7$ receptors in neurogenic and neuropathic pain

Role of peripheral versus spinal 5-HT$_7$ receptors in the modulation of pain under sensitizing conditions. *Eur J Pain 2012;16:72-81*

Alex Brenchat, Daniel Zamanillo, Michel Hamon, Luz Romero, José Miguel Vela

Paper dels receptor 5-HT$_7$ a nivell perifèric i espinal en la modulació del dolor en condicions de sensibilització.

L'objectiu del present treball va ser estudiar la contribució dels receptors 5-HT$_7$ presents a nivell perifèric i central responsables de l'efecte antinociceptiu observat amb l'administració sistèmica dels agonistes selectius del receptor 5-HT$_7$. Amb aquest propòsit, vàrem mesurar la hipersensibilitat mecànica secundària a la capsaicina després d'administrar l'agonista del receptor 5-HT$_7$ E-57431 per la via oral (nivell sistèmic), via intratecal (nivell espinal) i intraplantar (nivell perifèric) en les rates.

Vàrem observar una inhibició dosi-depenent de la hipersensibilitat mecànica induïda per capsaicina després d'administrar per la via oral l'agonista selectiu del receptor 5-HT$_7$ E-57431 (1,25 - 10 mg/kg). L'administració intratecal de 100 µg de E-57431 va resultar també inhibir la hipersensibilitat mecànica induïda per capsaicina. Contràriament a l'activació espinal, vàrem administrar per la via intraplantar l'agonista del receptor 5-HT$_7$ E-57431 (0.01, 0.1 i 1µg) resultant en la promoció de la hipersensibilitat mecànica induïda per capsaicina, reforçant el paper pronociceptiu dels receptors 5-HT$_7$ en la perifèria. L'agonista E-57431 el vàrem també administrar per via sistèmica e intratecal en un model de lesió del nervi ciàtic en rates per tal de confirmar els resultats obtinguts en el model de la capsaicina. L'administració intraperitoneal de 10 mg/kg e intratecal de 100 µg va resultar també inhibir la hipersensibilitat mecància induïda per la lesió del nervi ciàtic en rates. Els resultats del present estudi ens van permetre demostrar utilitzant un model de dolor neuropàtic (lligadura del nervi ciàtic) i un model de dolor neurogènic (sensibilització per capsaicina), que l'efecte antinociceptiu observat amb l'administració sistèmica d'un agonista selectiu del receptor 5-HT$_7$ es deu possiblement a l'activació dels receptors 5-HT$_7$ a nivell espinal, contrarrestant el paper pronociceptiu dels receptors 5-HT$_7$ en la perifèria.

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ORIGINAL ARTICLE

Role of peripheral versus spinal 5-HT$_7$ receptors in the modulation of pain undersensitizing conditions

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Abstract

Several studies have suggested that 5-HT$_7$ receptors are involved in nociceptive processing but the exact contribution of peripheral versus central 5-HT$_7$ receptors still needs to be elucidated. In the present study, the respective roles of peripheral and spinal 5-HT$_7$ receptors in the modulation of mechanical hypersensitivity were investigated under two different experimental pain conditions. In a first set of experiments, the selective 5-HT$_7$ receptor agonist, E-57431, was systemically, intrathecally or peripherally (intraplantarly) administered to rats sensitized by intraplantar injection of capsaicin. Oral administration of E-57431 (1.25–10 mg/kg) was found to exert a clear-cut dose-dependent reduction of capsaicin-induced mechanical hypersensitivity. Interestingly, intrathecal administration of E-57431 (100 μg) also inhibited mechanical hypersensitivity secondary to capsaicin injection. In contrast, a dose-dependent enhancement of capsaicin-induced mechanical hypersensitivity was observed after local intraplantar injection of E-57431 (0.01–1 μg). In a second set of experiments, E-57431 was systemically or intrathecally administered to rats submitted to neuropathic pain (spared nerve injury model). Significant inhibition of nerve injury-induced mechanical hypersensitivity was found after intraperitoneal (10 mg/kg) as well as intrathecal (100 μg) administration of E-57431 in this chronic pain model. These studies provide evidence that, under sensitizing neurogenic/neuropathic conditions, activation of 5-HT$_7$ receptors exerts antinociceptive effects at the level of the spinal cord and pronociceptive effects at the periphery. The antinociceptive effect mediated by central 5-HT$_7$ receptors seems to predominate over the pronociceptive effect at the periphery when a selective 5-HT$_7$ receptor agonist is systemically administered.

1. Introduction

Serotonin (5-hydroxytryptamine [5-HT]) is involved in pain transmission, processing and control (Eide and Hole, 1993; Millan, 2002; Kayser et al., 2010). Much of pain research has focused on 5-HT$_{1A}$, 5-HT$_{1B/1D}$, 5-HT$_{2A/2C}$ and 5-HT$_3$ receptors (Eide and Hole, 1993; Oyama et al., 1996; Obata et al., 2000; Kayser et al., 2002, 2010; Ahn and Basbaum, 2006; Colpaert, 2006; Pichon et al., 2010), but the role played by other receptors, including 5-HT$_7$ receptors, just begins to be elucidated. Interestingly, both pronociceptive and antinociceptive roles have been suggested for 5-HT$_7$ receptors.

A first series of investigations showed that systemic administration of selective 5-HT$_7$ receptor agonists inhibited capsaicin-induced and nerve injury-induced hypersensitivity in mice. As expected from 5-HT$_7$ receptor-mediated effects, antinociceptive-like responses elicited by agonists were reversed by 5-HT$_7$ receptor antagonists, and pronociceptive effects were observed when selective 5-HT$_7$ receptor antagonists

were systemically administered (Brenchat et al., 2009, 2010). Nevertheless, the effect of systemically administered 5-HT$_7$ receptor ligands could represent an overall balance of discrepant, opposite effects at different locations because 5-HT$_7$ receptors are expressed at different sites compatible with a role in pain control, including dorsal root ganglia (DRG) (Pierce et al., 1997; Meuser et al., 2002; Doly et al., 2005) and spinal (Meuser et al., 2002; Doly et al., 2005) and supraspinal (thalamus, midbrain, pons and medulla) (Stowe and Barnes, 1998; Neumaier et al., 2001; Martin-Cora and Pazos, 2004) regions in the CNS.

Peripheral tissue injury causes the release of 5-HT from platelets and mast cells, which acts in combination with other inflammatory mediators to excite afferent fibers (Sommer, 2004). Indeed, convergent data in the literature suggest a pronociceptive role of 5-HT$_7$ receptor at the periphery (Meuser et al., 2002; Rocha-González et al., 2005) based on investigations with nonselective 5-HT$_7$ receptor agonists, such as 5-HT itself, 5-carboxamidotryptamine (5-CT; binding affinity K_i values at human 5-HT receptors: 5-HT$_{1A}$ = 0.34 nM; 5-HT$_{1D}$ = 0.70 nM; 5-HT$_7$ = 0.93 nM) and 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT; binding affinity K_i values at human 5-HT receptors: 5-HT$_{1A}$ = 0.06 nM; 5-HT$_{1D}$ = 47 nM; 5-HT$_7$ = 467 nM) (Boess and Martin, 1994; Doménech et al., 1997), administered locally (intraplantarly or intra-articularly) in the context of tissue injury and inflammation.

On the other hand, contradictory results have been reported with regard to the spinal contribution of 5-HT$_7$ receptors in nociception. A pronociceptive role of spinal 5-HT$_7$ receptors was initially proposed by Rocha-González et al. (2005), who found that spinal administration of the 5-HT$_7$ receptor antagonist SB-269970 inhibited the increased formalin-evoked nociceptive responses elicited by intrathecal administration of 5-CT. However, other studies showed that intrathecal administration of 5-HT$_7$ receptor antagonists inhibited the antinociceptive effects of morphine, tramadol and its metabolite O-Desmethyltramadol, and WIN 55,212-2 and ACEA, which led to the suggestion that activation of spinal 5-HT$_7$ receptors, via descending serotonergic pathways, is required for opioid and CB$_1$ receptor-mediated antinociception (Dogrul and Seyrek, 2006; Dogrul et al., 2009; Seyrek et al., 2010; Yanarates et al., 2010). Differences in animal models, experimental approaches and ligands used as pharmacological tools may account for the apparent discrepancies regarding the role played by spinal 5-HT$_7$ receptors in nociception.

In an attempt to solve these discrepancies and to determine the respective contributions of spinal versus peripheral activation of 5-HT$_7$ receptors, we compared the effects of systemic, intrathecal or peripheral administration of a selective 5-HT$_7$ receptor agonist, E-57431 (binding affinity K_i values at human 5-HT receptors: 5-HT$_7$ = 0.47 nM; 5-HT$_{1D}$ = 53 nM; 5-HT$_{2A}$ = 560 nM; and >1000 nM for the rest) (Brenchat et al., 2010), on mechanical hypersensitivity secondary to either intraplantar injection of capsaicin or spared injury to the sciatic nerve in the rat.

2. Methods

2.1 Animals

Male Wistar rats weighing 230–300 g (Charles River, France) were used in these studies. Rats were housed in groups of three, provided with food and water *ad libitum* and kept in controlled laboratory conditions with temperature maintained at 21 ± 1 °C and 12-h light/dark cycles with light on at 07:00 h. Experiments were carried out in a sound-attenuated, air-regulated, experimental room. All experimental procedures and animal husbandry were conducted according to ethical principles for the evaluation of pain in conscious animals (Zimmermann, 1983) and to ethical guidelines of the European Communities Council Directive of 24 November 1986 (86/609/ECC). The experimental work received approval by the Local Ethical Committee.

2.2 Drugs

Capsaicin (8-methyl-N-vanillyl 6-nonamide) was purchased from Sigma-Aldrich Co. (Spain) and dissolved in 1% dimethylsulfoxide (DMSO). E-57431 (2-(2-(dimethylamino)ethyl)-4-(1,3,5-trimethyl-1H-pyrazol-4-yl)phenol hydrochloride) (MW 309.83 g/mol) was originated and synthesized by Laboratories Esteve (Barcelona, Spain). E-57431 is a potent 5-HT$_7$ receptor agonist with more than 100× selectivity over other 5-HT receptor subtypes and non-5-HT receptors, transporters and ion channels (Brenchat et al., 2010). E-57431 was dissolved in 0.5% (hydroxypropyl)methyl cellulose (Sigma–Aldrich) for systemic administration (2 ml/kg), in physiological saline for intraplantar administration (10 μl), and in artificial cerebrospinal fluid (NaCl 147 mmol/l, KCl 2.7 mmol/l, $CaCl_2$ 1.2 mmol/l and $MgCl_2$ 0.85 mmol/l; CMA Microdialysis, N. Chelmsford, MA, USA) for intrathecal administration (10 μl).

2.3 Intrathecal catheterization

Chronic catheterization of the spinal subarachnoid space was performed as described previously

(Storkson et al., 1996; Pogatzki et al., 2000). After basal measurements, surgery to generate intrathecal catheterization was carried out. Rats were anesthetized with pentobarbital (60 mg/kg, i.p.). The lumbar region was shaved, prepared with povidone-oidine, made kyphotic and incised 2–3 cm longitudinally in the midline at the level of the iliac crests. The space between the lumbar vertebrae L5 and L6 was punctured with a 22G hypodermic needle. Tail-flick or hind paw retraction indicated an intrathecal location. 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 through the needle cranially. The needle and the stylet were removed and the catheter withdrawn so that 5 cm extended outside of the lumbar musculature. Superglue-3 gel (Loctite®) was used to fix the catheter to the fascia. The distal end of the 28G PU catheter was connected 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 tunneled under the skin to the cervical region, flushed with CSF and sealed with a cautery pen. The dead volume of the catheter was 20 µl. The skin was then closed and animals allowed to recover. Pharmacological evaluation was performed 3 days (capsaicin model) and 14 days (spared nerve injury) after catheterization. Catheterized rats had no detectable motor deficits.

2.4 Capsaicin model

Sensitization by intraplantar capsaicin injection results in hypersensitivity to both thermal and mechanical stimuli (Gilchrist et al., 1996). In this study, sensitization by intraplantar capsaicin injection was used to assess the effect of treatments on the response to mechanical stimulation (von Frey test) in rats.

Capsaicin was administered (50 µl) by intraplantar (i.pl.) injection into the mid dorsal surface of the right hind paw. An equal volume of solvent (1% DMSO) was used in the control group. The intraplantar injection was performed by means of a 100 µl 1710 TLL Hamilton® microsyringe (Teknokroma, Spain) with a $30^{1/2}$- gauge needle. In a pilot study, different doses of capsaicin (0.1, 1, 10, 20 and 30 µg) were tested for their ability to induce mechanical hypersensitivity (i.e., allodynia) using von Frey filaments (data not shown). The capsaicin dose of 30 µg was able to induce robust and persistent paw withdrawal responses to subthreshold (i.e., allodynic) mechanical stimuli and was then used to evaluate the antinociceptive (i.e., antiallodynic) effects of the selective 5-HT$_7$ receptor agonist E-57431. The dose of 10 µg of capsaicin was unable to induce mechanical hypersensitivity and was then used to explore possible pronociceptive (i.e., proallodynic) effects of E-57431. Basal measurements were always done before capsaicin or vehicle injection and drug treatments.

2.4.1 Systemic treatment

The antinociceptive effects of oral administration of the selective 5-HT$_7$ receptor agonist E-57431 were investigated after intraplantar injection of capsaicin (30 µg/50 µl). Prior to pharmacological treatments, the von Frey test was done 30 min after intraplantar injection of capsaicin to discard animals that did not develop capsaicin-induced mechanical hypersensitivity. E-57431 (1.25, 2.5, 5 and 10 mg/kg) or vehicle was orally administered 30 min after intraplantar injection of capsaicin. Mechanical hypersensitivity was evaluated before capsaicin injection (basal), before administration (pre-treatment) and 30 min after oral administration of E-57431 or vehicle, corresponding to 60 min after capsaicin injection.

2.4.2 Intrathecal treatment

Intrathecal treatments were performed 3 days after intrathecal catheterization. Prior to pharmacological treatments, the von Frey test was done 30 min after intraplantar injection of capsaicin (30 µg/50 µl) to discard animals that did not develop capsaicin-induced mechanical hypersensitivity. E-57431 (100 µg/10 µl) or vehicle was intrathecally administered 30 min after intraplantar injection of capsaicin. Mechanical hypersensitivity was evaluated before capsaicin injection (basal), before intrathecal administration (time 0) and 30, 60 and 90 min after intrathecal administration of E-57431 or vehicle, corresponding to 60, 90 and 120 min after capsaicin injection.

2.4.3 Local intraplantar treatment

To study peripheral, local effects, E-57431 (0.01, 0.1 and 1 µg) or vehicle was intraplantarly administered into the right hind paw 10 min before capsaicin (10 µg/50 µl) or vehicle (DMSO 1%) injection into the same paw, and mechanical sensitivity was evaluated after a further 20 min period. To assess whether the effect of intraplantar injection of E-57431 was local, the 5-HT$_7$ receptor agonist was also administered into the left (contralateral) hind paw while capsaicin was injected into the right hind paw, and the corresponding effect on mechanical hypersensitivity was assessed in the right hind paw.

2.5 Spared nerve injury

This partial denervation model of neuropathic pain was induced according to the method previously described by Decosterd and Woolf (2000). After basal measurements, surgery to generate nerve injury was carried out. Rats were anesthetized with pentobarbital (60 mg/kg i.p.) and the skin on the lateral surface of the thigh was incised. A section was made directly through the biceps femoris muscle to expose the sciatic nerve and its three terminal branches: the sural, common peroneal and tibial nerves. The procedure comprised ligation and axotomy of the tibial and common peroneal nerves leaving the sural nerve intact. The common peroneal and the tibial nerves were tight-ligated with 5.0 silk and sectioned distal to the ligation. Great care was taken to avoid any contact with or stretching of the intact sural nerve. Muscle and skin were then closed in two layers. Control, sham-operated rats underwent the same surgical procedure and the sciatic nerve was exposed, but neither ligated nor sectioned. Behavioral testing using von Frey filament stimulation was assessed on days 2, 7, and 14 after the surgical procedure in independent groups of nerveinjured and sham-operated rats to monitor the development of mechanical hypersensitivity.

2.5.1 Systemic and intrathecal treatments

On days 15–18 post-surgery, when neuropathic pain-related behaviors (i.e., mechanical allodynia) had clearly developed, two independent experiments using different routes of administration (intraperitoneal or intrathecal) were performed to investigate the effect of the selective 5-HT$_7$ receptor agonist E-57431: (1) E-57431 (10 mg/kg) or vehicle was administered i.p. on day 15 post-surgery and von Frey filament testing was performed before nerve-injury (basal), before intraperitoneal administration (time 0) and 15, 30, 60 and 90 min thereafter; (2) three different doses of E-57431 (10 μg, 30 μg and 100 μg) or vehicle were administered intrathecally on days 15–18 post-surgery following a *Latin square* design and allodynia-like responses were determined before nerve-injury (basal), before intrathecal administration (time 0) and 15, 30, 60, 90 and 120 min thereafter.

2.6 von Frey test

Behavioral testing was performed in blinded conditions for drugs and doses tested in independent groups of rats. Mechanical hypersensitivity was quantified as previously described (Chaplan et al., 1994) by determining the pressure thresholds eliciting withdrawal of the ipsilateral and contralateral hind paws in response to graded stimulation with von Frey filaments applied onto the plantar surface. The skin area stimulated with von Frey filaments was the mid plantar surface of the hind paw in the capsaicin model (area surrounding the site of capsaicin injection in the ipsilateral paw) and the lateral plantar surface of the hind paws in the case of nerve-injured rats. Stimulation of the toes and heel was avoided. Rats were placed under plastic boxes above a wire mesh floor, which allowed full access to the paws. Behavioral acclimation was allowed for at least 30 min. Mechanical paw withdrawal thresholds were measured using the up-down testing paradigm (Dixon, 1980; Chaplan et al., 1994) by applying von Frey filaments in log increments of force (0.4, 0.6, 1, 2, 4, 6, 10, and 15 g) to the test area for about 3 s with an interstimuli interval of approximately 1 min. The 2-g stimulus was applied first. Whenever a withdrawal response to a given probe occurred, the next smaller von Frey filament was applied. Whenever a negative response occurred, the next higher force was applied. The test continued until: (1) the responses to four identical stimuli after the first change in response had been obtained or (2) a negative response to the highest von Frey filament or a positive response to the lowest von Frey filament had occurred. Abrupt paw withdrawal, licking, and shaking were regarded as positive responses. The 50% paw withdrawal threshold values were derived according to a previously described method (Chaplan et al., 1994).

At least a 50% reduction of the baseline threshold was established as acceptance criterion to select animals developing tactile allodynia both 30 min after capsaicin (30 μg) injection and 14 days after nerve injury. That is, the withdrawal response must be evoked when the paw is stimulated using a von Frey hair with bending force ≤7.5 g to select animals developing tactile allodynia for pharmacological studies.

2.7 Data analyses

Data are presented as mean paw withdrawal threshold in grams ± SEM. Statistical analysis to test significant differences among groups was made using ANOVA followed by Bonferroni's post hoc comparison. The level of significance was set at $p < 0.05$. Data analysis and graphing were done using GraphPad Prism software (version 4.0; GraphPad Software, Inc., USA).

3. Results

3.1 Dose-response antinociceptive effect of orally administered E-57431 in the capsaicin model

Rats intraplantarly injected with capsaicin (30 μg) into the midplantar surface of the right hind paw (ipsilateral paw) developed mechanical hypersensitivity, evidenced by a reduction of the mechanical threshold triggering withdrawal of the ipsilateral paw in the von Frey test 30 min after injection (Fig. 1). In particular, 83% of rats injected with capsaicin developed mechanical hypersensitivity (≥50% reduction of the baseline threshold) and were thus selected to investigate the role of 5-HT$_7$ receptors. No significant changes in the response to mechanical stimuli were observed in the contralateral paw (data not shown).

Oral administration of the selective 5-HT$_7$ receptor agonist E-57431 30 min after capsaicin injection dose-dependently reversed capsaicin-induced mechanical hypersensitivity in the ipsilateral paw (Fig. 1). Significantly increased paw withdrawal thresholds in rats treated with E-57431 were found 30 min after oral administration of 5 and 10 mg/kg compared to vehicle-treated rats. Treatments with lower doses (1.25 and 2.5 mg/kg) of E-57431 did not significantly modify this evoked, pain-related behavior compared to vehicle treatment (Fig. 1). No significant changes in the response to mechanical stimuli were observed in the contralateral paw (data not shown).

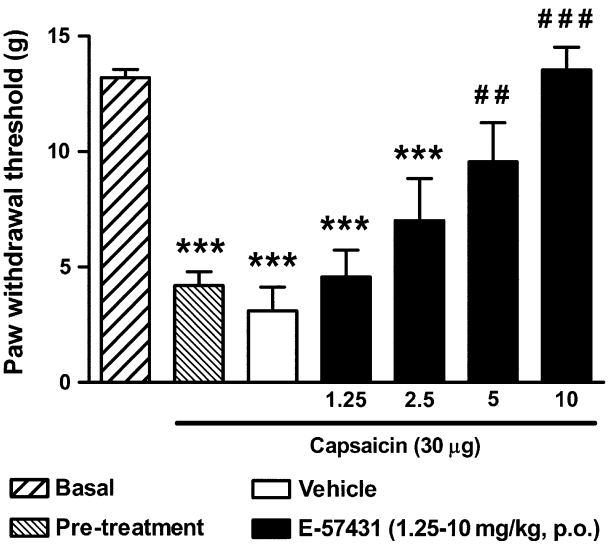

Figure 1 Dose-response antinociceptive effect of the 5-HT$_7$ receptor agonist E-57431 administered orally on capsaicin-induced mechanical hypersensitivity. Mechanical hypersensitivity secondary to intraplantar injection of 30 μg of capsaicin was evaluated by using the von Frey test. After basal measurements, capsaicin (30 μg) was injected into the right hind paw (ipsilateral paw) and mechanical hypersensitivity was evidenced at 30 min (pre-treatment) in the ipsilateral paw. The effect of treatments (E-57431 or vehicle) was evaluated 30 min after oral administration, corresponding to 60 min after capsaicin injection. Mechanical hypersensitivity in the ipsilateral paw was dose-dependently inhibited by oral treatment with the selective 5-HT$_7$ receptor agonist E-57431. Note that E-57431 exerted significant antinociceptive (antiallodynic) effects at doses of 5 and 10 mg/kg. No effects were detected in the contralateral paw (not shown). Each bar represents the mean ± SEM (n = 7–11 per group). ***$p < 0.001$ versus basal; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus vehicle (ANOVA followed by Bonferroni multiple comparison test).

3.2 Antinociceptive effect of intrathecally administered E-57431 in the capsaicin model

As activation of 5-HT$_7$ receptors after systemic administration of E-57431 inhibited capsaicin-induced mechanical hypersensitivity in rats, we investigated whether this antinociceptive effect could have involved spinal 5-HT$_7$ receptors. For this purpose, E-57431 was administered intrathecally after intraplantar injection of capsaicin.

Three days after intrathecal catheterization, 74% of rats injected with capsaicin (30 μg) into the midplantar surface of the right hind paw developed mechanical hypersensitivity (≥50% reduction of the baseline threshold) 30 min after capsaicin sensitization and were thus selected for the study (Fig. 2). No significant changes in the response to mechanical stimuli were observed in the contralateral paw (data not shown).

Intrathecal administration of E-57431 at the dose of 100 μg inhibited capsaicin-induced mechanical hypersensitivity in the ipsilateral paw (Fig. 2). Significantly increased paw withdrawal thresholds in rats intrathecally administered with E-57431 were found from 30 to 90 min post-treatment compared to vehicletreated rats. No significant changes were observed in the contralateral paw (Fig. 2).

3.3 Dose-response pronociceptive effect of E-57431 intraplantarly injected in the capsaicin model

Here we investigated whether peripheral activation of 5-HT$_7$ receptors could exert pronociceptive effects (i.e., promote mechanical hypersensitivity). To assess this possibility, E-57431 or vehicle was administered locally, through intraplantar injection, and the dose of capsaicin was reduced from 30 to 10 μg, i.e. a dose unable to induce significant withdrawal responses when the paw was mechanically stimulated at subthreshold pressures, but providing a minimal

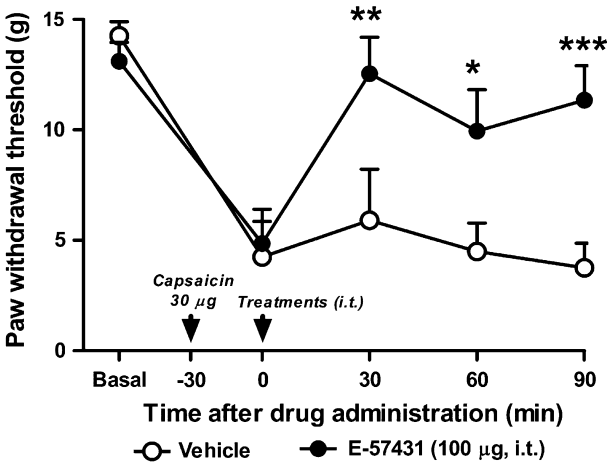

Figure 2 Antinociceptive effect of the 5-HT$_7$ receptor agonist E-57431 administered intrathecally on capsaicin-induced mechanical hypersensitivity. Mechanical hypersensitivity secondary to intraplantar injection of 30 µg of capsaicin was evaluated by using the von Frey test in intrathecally catheterized rats. After basal measurements, capsaicin (30 µg) was injected into the right hind paw (ipsilateral paw) 3 days after intrathecal catheterization. Mechanical hypersensitivity was evidenced in the ipsilateral from 30 min after the injection of capsaicin. The effect of treatments was evaluated 30, 60 and 90 min after intrathecal administration of E-57431 or vehicle, corresponding to 60, 90 and 120 min after capsaicin injection. Mechanical hypersensitivity secondary to intraplantar injection of 30 µg of capsaicin in the ipsilateral paw was significantly reversed by intrathecal administration of E-57431 (100 µg) at all three time points evaluated. No effects were observed in the contralateral paw (not shown). Each symbol represents the mean ± SEM (n = 7–10 per group). *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle (ANOVA followed by Bonferroni multiple comparison test).

sensitizing challenge to study pronociceptive effects of drugs, as previously reported (Brenchat et al., 2009). In these conditions, intraplantar injection of E-57431 dose-dependently enhanced mechanical hypersensitivity prompted by the local injection of capsaicin (10 µg) 30 min before. Significant pronociceptive effects were observed after injection of 1 µg of E-57431 (Fig 3). Lower doses of E-57431 (0.01 µg and 0.1 µg) did not significantly modify the paw withdrawal threshold compared to vehicle treatment. Interestingly, the pronociceptive effect of intraplantar E-57431 was not observed in rats injected with vehicle (1% DMSO) only (no capsaicin), suggesting that a minimal sensitizing challenge with capsaicin was needed for the 5-HT$_7$ receptor agonist to exert its pronociceptive effect (Fig. 3). In contrast, intraplantar injection of E-57431 (1 µg) into the left (contralateral) hind paw was ineffective as it did not induce mechanical hypersensitivity in the right, ipsilateral paw receiving the 10 µg dose of capsaicin (Fig. 3). Accordingly, the pronociceptive effect of intraplantar administration of 1 µg of E-57431 into the ipsilateral paw was very probably mediated locally and did not involve any systemic action. No significant changes were observed in the contralateral paw (not shown).

3.4 Antinociceptive effect of intraperitoneally administered E-57431 in the spared nerve injury model of neuropathic pain

In order to assess the effect of the selective 5-HT$_7$ receptor agonist E-57431 in another model of pain involving sensitization, we investigated whether mechanical allodynia could be affected by this ligand in rats rendered neuropathic by spared nerve injury. Mechanical hypersensitivity in nerve-injured animals was evidenced in 82% of rats by a reduction (≥50%) of the pressure threshold evoking withdrawal of the ipsilateral hind paw 14 days postsurgery compared to basal pre-surgery values (Fig. 4). Mechanical sensitivity to von Frey filament stimulation was unchanged in the contralateral paw of nerve-injured rats (not

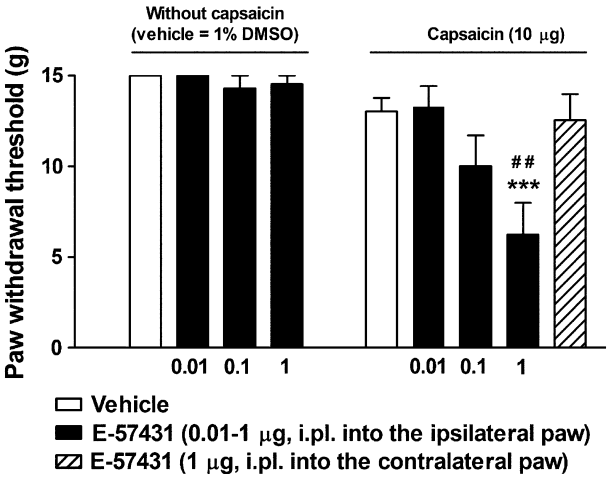

Figure 3 Dose-dependent enhancement of capsaicin-induced mechanical hypersensitivity by intraplantar administration of the 5-HT$_7$ receptor agonist E-57431. Mechanical thresholds were evaluated by using the von Frey test. No mechanical hypersensitivity was observed in rats intraplantarly injected with a low dose of capsaicin (10 µg) or its vehicle (1% DMSO) in the right, ipsilateral hind paw. However, mechanical hypersensitivity was evidenced after intraplantar administration of E-57431 (1 µg) in the ipsilateral hind paw intraplantarly injected with this low dose of capsaicin (but not its vehicle). Note that no mechanical hypersensitivity in the capsaicin (10 µg)-injected ipsilateral paw was observed after intraplantar administration of E-57431 (1 µg) into the contralateral paw. No significant changes were observed in the contralateral paw (not shown). Each bar represents the mean ± SEM (n = 6–14 per group). ***p < 0.001 versus "vehicle group with capsaicin"; ##p < 0.01 versus "E-57431 (1 µg) group without capsaicin" (ANOVA followed by Bonferroni multiple comparison test).

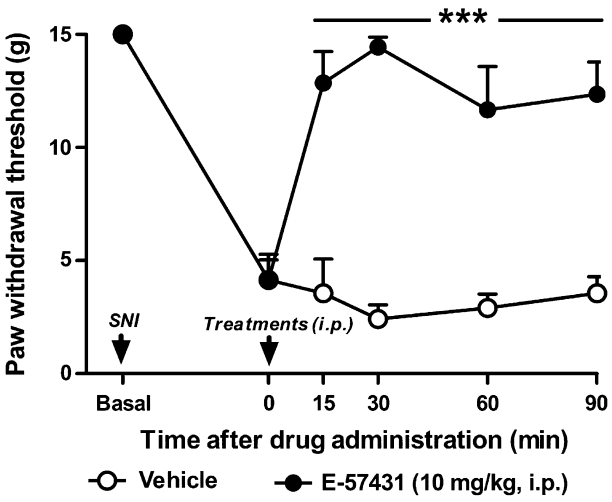

Figure 4 Antinociceptive effect of the 5-HT$_7$ receptor agonist E-57431 administered intraperitoneally on mechanical hypersensitivity induced by spared nerve injury. Mechanical hypersensitivity secondary to spared nerve injury was evaluated using the von Frey test. Measurements were done before surgery (basal), 14 days after surgery (time 0) and on day 15 post-surgery, 15, 30, 60 and 90 min after the intraperitoneal administration of E-57431 or vehicle. Mechanical hypersensitivity was evidenced in the ipsilateral hind paw of nerve-injured rats and was reversed by intraperitoneal administration of the selective 5-HT$_7$ receptor agonist E-57431 at 10 mg/kg. In contrast, no change in mechanical sensitivity was observed in the contralateral hind paw (not shown). Each symbol represents the mean ± SEM ($n = 6$–9 per group). ***$p < 0.001$ versus basal; ###$p < 0.001$ versus vehicle (ANOVA followed by Bonferroni multiple comparison test).

shown). In the same way, sham operation did not induce mechanical hypersensitivity as no significant changes in the response to mechanical stimulation were found in sham-operated rats 14 days after surgery compared to basal pre-surgery values (not shown).

Intraperitoneal administration of E-57431 at the dose of 10 mg/kg on day 15 post-surgery significantly inhibited mechanical hypersensitivity from 15 to 90 min after treatment, restoring the pressure threshold value triggering withdrawal of the nerveinjured, ipsilateral hind paw to basal pre-surgery levels (Fig. 4). No significant changes were elicited by E-57431 treatment in the contralateral paw of nerve-injured rats (not shown).

3.5 Antinociceptive effect of intrathecally administered E-57431 in the spared nerve injury model of neuropathic pain

Based on above results, we also tested whether the inhibition of nerve injury-induced mechanical hypersensitivity after systemic administration of E-57431 could be reproduced by selectively activating spinal 5-HT$_7$ receptors. For this purpose, E-57431 was intrathecally administered on days 15–18 after spared nerve injury. Mechanical hypersensitivity in these animals was evidenced in 69% of rats by a reduction (≥50%) of the pressure threshold evoking withdrawal of the ipsilateral hind paw 14 days post-surgery compared to basal pre-surgery values (Fig. 5). In contrast, mechanical sensitivity to von Frey filament stimulation was unchanged in the contralateral paw of nerve-injured rats (data not shown).

Intrathecal administration of E-57431 (100 μg) inhibited nerve injury-induced mechanical hypersensitivity as shown by significant increases in pressure threshold values to trigger ipsilateral paw withdrawal 15, 60 and 90 min after treatment (Fig. 5). Lower doses of E-57431 (10 and 30 μg i.t.) were ineffective. E-57431 did not significantly modify pressure threshold values determined in the contralateral paw (not shown).

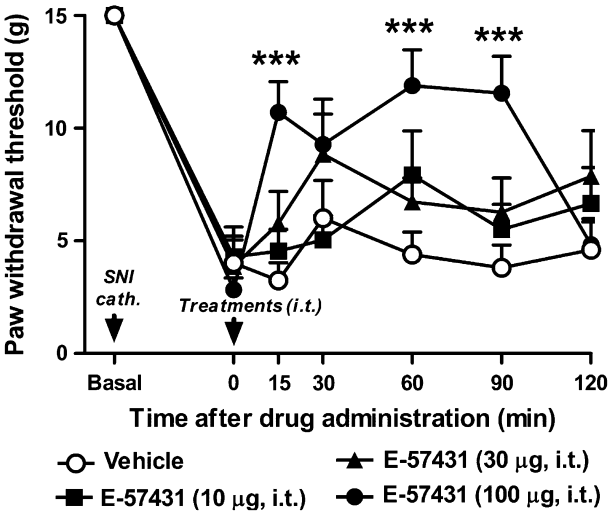

Figure 5 Antinociceptive effects of the 5-HT$_7$ receptor agonist E-57431 administered intrathecally on mechanical hypersensitivity induced by spared nerve injury. Mechanical hypersensitivity secondary to nerve injury was evaluated by using the von Frey test. After basal measurements, surgeries for spared nerve injury (SNI) and intrathecal catheterization (cath.) were performed and mechanical sensitivity was evaluated 15, 30, 60, 90 and 120 min after intrathecal administration of E-57431 (10, 30 and 100 μg) or vehicle following a *Latin square* design on days 15–18 after surgery. Mechanical hypersensitivity secondary to spared nerve injury in the ipsilateral paw was reversed by intrathecal administration of the selective 5-HT$_7$ receptor agonist E-57431 at 100 μg. Intrathecal administration of 10 and 30 μg of E-57431 did not exert any significant effect. No effects were observed in the contralateral paw (not shown). Each symbol represents the mean ± SEM ($n = 9$ per group). ***$p < 0.001$ versus vehicle (ANOVA followed by Bonferroni multiple comparison test).

4. Discussion

The present work provides demonstration in animal pain models involving central sensitization that activation of spinal 5-HT$_7$ receptors exerts antinociceptive effects whereas activation of peripheral 5-HT$_7$ receptors exerts pronociceptive effects. The antinociceptive effect at the central level seems to overcome the pronociceptive effect at the peripheral level as an overall antinociception is observed when 5-HT$_7$ receptor agonists are systemically administered (Brenchat et al., 2009, 2010). As a pharmacological tool in the experiments described herein we used the new 5-HT$_7$ receptor agonist E-57431, whose affinity, selectivity and functionality have been reported in detail (Brenchat et al., 2010).

Intradermal injection of capsaicin (i.e., capsaicin sensitization) is a useful model for correlative studies in pain conditions involving sensitization and behavioral hyperalgesia and allodynia (Gilchrist et al., 1996), and is regarded as a predictive model to assess antinociceptive action of analgesics in neuropathic pain (Joshi et al., 2006). In previous studies in mice, we showed that systemic administration of different selective 5-HT$_7$ receptor agonists inhibited capsaicin-induced mechanical hypersensitivity (Brenchat et al., 2009). Similarly, we found that systemic administration of selective 5-HT$_7$ receptor agonists, including E-57431, decreased partial nerve injury-induced mechanical and thermal hypersensitivity down to basal values found in control intact mice (Brenchat et al., 2010). Interestingly, no tolerance to the antinociceptive effects was evidenced following repeated (11 days) systemic administration of E-57431 (Brenchat et al., 2010).

In agreement with previous findings in mice, we found here that mechanical hypersensitivity could be reversed by acute systemic (oral or intraperitoneal) treatment with the selective 5-HT$_7$ receptor agonist E-57431 in both the capsaicin and spared nerve injury models of pain signaling sensitization in rats. Our results also showed that intrathecal administration of E-57431 was as effective as systemic administration of the drug to inhibit mechanical hypersensitivity in both models. Interestingly, agonists acting at spinal 5-HT$_7$ receptors cannot directly inhibit primary afferents or second-order nociceptive dorsal horn neurons because stimulation of the 5-HT$_7$ receptor has excitatory effects (Lovenberg et al., 1993; Chapin and Andrade, 2001). Therefore, an indirect action through activation of 5-HT$_7$ receptors localized on inhibitory interneurons is presumably required to inhibit nociceptive transmission. 5-HT$_7$ receptors in the spinal cord have been described on primary afferents and astrocytes but are also located postsynaptically on local interneurons within the superficial laminae of the dorsal horn (Meuser et al., 2002; Doly et al., 2005). Actually, a previous study at the light microscope level showed that 5-HT$_7$ receptors co-localize with GABA in neurons of the dorsal horn of the spinal cord (Brenchat et al., 2010) and it has been reported that spinal GABAergic interneurons, but not enkephalinergic interneurons, are involved in 5-HT$_7$ receptor-mediated antinociception (Bourgoin et al., 2008; Viguier et al., 2009). This is based on the finding that intrathecal pretreatment with the GABA$_A$ receptor antagonist bicuculline, but not the GABA$_B$ receptor antagonist phaclofen or the opioid receptor antagonist naloxone, prevented the antihyperalgesic effects exerted by 5-HT$_7$ receptor agonists in rats with constriction injury to the sciatic nerve (Bourgoin et al., 2008; Viguier et al., 2009). In line with these results, recent data also suggest that, at the spinal level, 5-HT$_7$ receptor stimulation modulates pain signaling through Cl^- conductance-dependent mechanisms (Viguier et al., 2010). Accordingly, activation of spinal inhibitory GABAergic interneurons could underlie, or at least contribute to, the analgesic effects of 5-HT$_7$ receptor agonists. Finally, we previously reported a significant increase of 5-HT$_7$ receptor immunoreactivity in the dorsal horn of the ipsilateral side of the spinal cord after sciatic nerve injury in mice (Brenchat et al., 2010). The upregulation of 5-HT$_7$ receptors in the spinal cord after nerve injury suggests a "pain"-triggered regulation of receptor expression that may be relevant for the effectiveness of 5-HT$_7$ receptor agonists. Indeed, 5-HT$_7$ receptor upregulation might represent a compensatory, protective, spinal mechanism to reduce excessive nociception in neuropathic pain conditions.

In contrast to the antinociceptive (antiallodynic) effect found after systemic or spinal administration, a clear-cut pronociceptive (proallodynic) effect was found when E-57431 was administered intraplantarly into the ipsilateral hind paw injected with a low subactive dose of capsaicin. No effects were found when E-57431 was intraplantarly administered into the contralateral paw, suggesting that peripheral, local mechanisms (i.e., activation of 5-HT$_7$ receptors on nerve endings) were responsible for its pronociceptive action. Interestingly, promotion of mechanical hypersensitivity did not occur when E-57431 was intraplantarly administered to rats receiving only vehicle (without capsaicin), indicating that a minimal sensitizing challenge with capsaicin was needed for the 5-HT$_7$ receptor agonist to exert its pronociceptive

action. This suggests that recruitment of additional mechanisms other than activation of 5-HT$_7$ receptors is required to induce mechanical hypersensitivity at the periphery. In fact, 5-HT released from platelets and mast cells at the periphery acts on different 5-HT receptor subtypes and in combination with other inflammatory mediators to excite afferent nerve fibers (Sommer, 2004). Activation of 5-HT$_{2A}$ and 5-HT$_3$ receptor subtypes present on C-fibers has already been reported to contribute to 5-HT-mediated peripheral pronociceptive effects (Obata et al., 2000; Sommer, 2004). Our data allow the conclusion that 5-HT$_7$ receptors, in particular those expressed by small and medium-sized DRG neurons (Meuser et al., 2002; Doly et al., 2005), also probably contribute to 5-HT-mediated pronociceptive effects at the periphery. Indeed, this conclusion is supported by several convergent data in the literature: (1) intraplantar injection of the 5-HT$_7$ receptor antagonist SB-269970 reduced formalin-induced nociception whereas intraplantar administration of non-selective 5-HT$_7$ receptor agonists such as 5-HT itself and 5-CT increased formalin-induced nociceptive behavior (Rocha-González et al., 2005); (2) intra-articular injection of the mixed 5-HT$_{1A}$/5-HT$_7$ receptor agonist 8-OH-DPAT induced c-Fos expression in the dorsal horn of the rat spinal cord and this effect was prevented by intra-articular administration of the non-selective 5-HT$_7$ receptor antagonist methiothepin (Meuser et al., 2002).

It is plausible that the antinociception resulting from activation of spinal 5-HT$_7$ receptors could overcome the pronociceptive effect mediated by peripheral 5-HT$_7$ receptors and thus be responsible for the overall antinociceptive effect found after systemic administration of selective 5-HT$_7$ receptor agonists in conditions involving central sensitization (this study; Brenchat et al., 2009, 2010). Indeed, it was recently demonstrated that the antinociceptive effects of morphine, tramadol and its metabolite O-Desmethyltramadol, and CB$_1$ receptor agonists were blocked by intrathecal administration of the selective 5-HT$_7$ receptor antagonist SB-269970, which suggests that spinal 5-HT$_7$ receptors are ultimately critical for opiate- and CB$_1$-mediated antinociception, and act as effector targets for descending inhibitory serotonergic pathways (Dogrul et al., 2009; Seyrek et al., 2010; Yanarates et al., 2010).

Taken together, the data reported herein support the idea that 5-HT$_7$ receptors play an important role in physiological mechanisms controlling nociception and pain, particularly in conditions involving sensitization of nociceptive pathways. Activation of 5-HT$_7$ receptors may result in pronociceptive or antinociceptive effects depending on their location. Mechanical hypersensitivity is promoted when activation occurs at peripheral receptors and inhibited when activation concerns 5-HT$_7$ receptors in the spinal cord, but an overall antinociception results from systemic administration of 5-HT$_7$ receptor agonists. Further studies in other experimental pain conditions, focusing on other CNS locations and/or exploring underlying mechanisms, would be particularly useful for a thorough assessment of the therapeutic potentialities of the 5-HT$_7$ receptor as a pharmacological target to develop new drugs to alleviate pain (i.e., clinical manifestations of neuropathic pain). Unfortunately, drugs selectively acting through central 5-HT$_7$ receptor activation have not yet been clinically assayed.

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6.5. *In vivo* specificity studies in 5-HT$_7$ receptor knockout mice

Assessment of 5-HT$_7$ receptor agonists selectivity using nociceptive and thermoregulation tests in knockout versus wild-type mice. *Adv Pharmacol Sci (submitted)*

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Estudi de la selectivitat dels agonistes pel receptor 5-HT$_7$ utilitzant tests de dolor i termoregulació en ratolins genoanul·lats i salvatges pel receptor 5-HT$_7$.

Basat en resultats preclínics, els agonistes pel receptor 5-HT$_7$ representen un potencial terapèutic pel tractament del dolor en condicions de sensibilització central. L'objectiu d'aquest estudi fou investigar el efectes diana específics dels agonistes pel receptor 5-HT$_7$ en el model de la formalina en ratolins genoanul·lats pel receptor 5-HT$_7$ i salvatges.

Aquest estudi va mostrar que els ratolins genoanul·lats pel receptor 5-HT$_7$ i salvatges mostraven la mateixa sensibilitat a estímuls tèrmics nocius i químics com la formalina. L'administració subcutània dels agonistes pel receptor 5-HT$_7$ AS-19 (10 mg/kg), E-55888 (20 mg/kg) i E-57431 (10 mg/kg) reduïren la resposta nociceptiva de la fase II de la formalina en el ratolins salvatges però no en els ratolins genoanul·lats pel receptor 5-HT$_7$.

Aquestes dades suggereixen que l'efecte antinociceptiu observat en el test de la formalina dels agonistes pel receptor 5-HT$_7$ AS-19, E-55888 i E-57431 es deu possiblement a l'activació específica dels receptor 5-HT$_7$ i confirma el potencial terapèutic dels receptors 5-HT$_7$ pel tractament de dolor.

Assessment of 5-HT_7 receptor agonists selectivity using nociceptive and thermoregulation tests in knockout versus wild-type mice

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Original report

Abstract

No study has ever examined the effect of 5-HT_7 receptor agonists on nociception by using 5-HT_7 receptor knockout mice. Basal sensitivity to noxious heat stimuli and formalin-induced nociception in both phase I and II of the formalin test did not differ in 5-HT_7 receptor knockout mice and paired wild-type controls. Similarly, there was no significant difference in basal body temperature between both genotypes. Subcutaneous administration of 5-HT_7 receptor agonists AS-19 (10 mg/kg), E-55888 (20 mg/kg) and E-57431 (10 mg/kg) significantly reduced formalin-induced licking/biting behavior during the phase II of the test in wild-type but not in 5-HT_7 receptor knockout mice. At these active analgesic doses, none of the three 5-HT_7 receptor agonists modified the basal body temperature neither in wild-type nor in 5-HT_7 receptor knockout mice. However, a significant decrease in body temperature was observed at a higher dose (20 mg/kg) of AS-19 and E-57431 in both genotypes. Our data strongly suggest that the 5-HT_7 receptor agonists AS-19, E-55888 and E-57431 produce antinociception in the formalin test by activating 5-HT_7 receptors. These results also strengthen the idea that the 5-HT_7 receptor plays a role in thermoregulation, but by acting in concert with other receptors.

Key words:

Serotonin 5-HT_7 receptor; Formalin; Body temperature; 5-HT_7 receptor knockout mice; Pain; Thermoregulation

1. Introduction

The 5-HT_7 receptor has been cloned from different genomes and its binding profile is consistent across species and between cloned and native receptors [1, 2]. In recent years, considerable efforts have focused on the development of selective 5-HT_7 receptor agonists and antagonists. To date, the search for 5-HT_7 receptor antagonists has led to the discovery of LY215840 [3], SB-258719 [4], DR4004 [5], SB-269970 [6] and SB-656104-A [7]. Regarding 5-HT_7 receptor agonists, AS-19 [8, 9], MSD-5a [10], LP-44 [11], LP-211 [12], E-55888 [13] and E-57431 [14] have been developed. However, most of these agonists display rather modest selectivity because their affinity for the 5-HT_7 type is only 11-fold higher than for 5-HT_{1D} in case of AS-19 [13], 28.6-fold higher than for 5-HT_{1A} in case of MSD-5a [10], and 33-fold higher than for dopamine D2 receptor [15] and 5-14-fold higher than for 5-HT_{1B}, 5-HT_{2B}, 5-HT_{2C} and 5-HT_{5A} in case of LP-211 [16]. Indeed, among 5-HT_7 receptor agonists, only E-55888 and E-57431 seem to have a satisfactory selectivity with affinity for the 5-HT_7 receptor 280-fold higher than for 5-HT_{1A} and 112.7-fold higher than for 5-HT_{1D}, respectively [13] (see Table 1). When tested in a functional assay, 5-HT_7 receptor agonists concentration-dependently increased cAMP formation in HEK-293F/h5-HT_7 cells. AS-19 has been found to behave as a potent (EC50 = 9 ± 1 nM) but partial 5-HT_7 receptor agonist, with a maximal effect reaching 77% of that of 5-HT [13]. However, E-55888 and E-57431 behaves as full agonists, with efficacy (Emax = 99 ± 1% and 94.5 ± 1%, respectively) and potency (EC50 = 16 ± 1 nM and 21.5 ± 1nM) similar to those of 5-HT, as previously described [13, 14].

From data obtained with these pharmacological tools, it has been claimed that 5-HT_7 receptors are involved in a number of physiological and pathophysiological phenomena such as nociception and thermoregulation. Data supporting a role for 5-HT_7 receptors in pain control mostly suggest an antinociceptive effect of 5-HT_7 receptor activation in the CNS and, in contrast, a pronociceptive effect of 5-HT_7 receptor activation in the periphery [17 - 23]. However, an overall antinociceptive effect has been observed following systemic administration of the selective 5-HT_7 receptor agonists AS-19, E-55888 and E-57431 to rodents suffering from neuropathic pain [13, 14].

On the other hand, 5-HT_7 receptors have been involved in the control of body temperature based on studies using some 5-HT_7 receptor agonists (5-CT, 8-OH-DPAT and LP-211), 5-HT_7 receptor antagonists (SB-258719 and SB-269970) and 5-HT_7 receptor knockout mice.

Activation of 5-HT_7 receptors has been reported to decrease body temperature in a complex manner, in concert with other serotonergic receptors such as the 5-HT_{1A} receptor and/or non-serotonergic receptors [16, 24 – 28].

In addition to pharmacological studies using 5-HT_7 receptor agonists and antagonists, the 5-HT_7 receptor knockout mice may provide a relevant tool to explore the functions of this receptor, and to assess the specificity of ligands supposed to interact selectively with it. Accordingly, the present study examines the effects of the so-called 5-HT_7 receptor agonists AS-19, E-55888 and E-57431 on formalin-induced pain behavior and thermoregulation in 5-HT_7 receptor knockout and paired wild-type mice in order to determine the *in vivo* functional selectivity of these ligands at this specific receptor type.

Table 1

Binding profiles of the 5-HT_7 receptor agonists AS-19, E-55888 and E-57431.

Receptor	**Affinity [K_i (nM)]**		
	AS-19	**E-55888**	**E-57431**
h**5-HT_{1A}**	89.7 (149.5 x)	700 (280 x)	n.s.
r**5-HT_{1B}**	490 (816.6 x)	n.s.	n.s.
h**5-HT_{1D}**	6.6 (11 x)	n.s.	53 (112.7 x)
h**5-HT_{2A}**	n.s	n.s.	560 (1191.5 x)
h**5-HT_{2B}**	n.s.	n.s.	n.s.
h**5-HT_{2C}**	n.s.	n.s.	n.s.
h**5-HT_3**	n.s.	n.s.	n.s.
h**5-HT_{4e}**	–	n.s.	n.s.
gp**5-HT_4**	n.s.	–	n.s.
h**5-HT_{5A}**	98.5 (164.2 x)	n.s.	n.s.
h**5-HT_6**	n.s.	n.s.	n.s.
h**5-HT_7**	0.6	2.5	0.47
h**5-HT** transporter (SERT)	n.s.	n.s.	n.s.
Other receptors	n.s.[a]	n.s.[a]	n.s.[b]

n.s., not significant (K_i > 1 µM or less than 50% inhibition of specific radioligand binding at 1 µM); –, data not available.
gp, guinea pig; h, human; r, rat.
Data obtained from Brenchat et al., [13, 14]
Data in parentheses after K_i values represent the affinity ratio vs. 5-HT_7 receptors calculated as K_i for the tested receptor/K_i for 5-HT_7 receptor. It is expressed as number-fold higher (x) for 5-HT_7 than for the tested receptor.
[a] See the panel of other receptors assayed [13].
[b] See the panel of other receptors assayed [14].

2. Materials and methods

2.1. Animals

Male, 5- to 8-week-old, 5-HT_7 receptor knockout (5-$HT_7R^{-/-}$) C57BL/6J mice and their wild-type 5-$HT_7R^{+/+}$ siblings used in this study we re provided by Deltagen (California, USA). Embryonic stem cells derived from the 129/OlaHsd mouse substrain were used to generate chimeric mice. F1 mice were generated by breeding with C57BL/6 females. F2 homozygous mutant mice were produced by intercrossing F1 heterozygous males and females. Successive mating of heterozygous progeny to the inbred C57BL/6J strain was performed for at least 8 generations before the knockout and wild-type homozygous offsprings were used in the present study. Animals were housed in groups of five, provided with food and water *ad libitum* and kept in controlled laboratory conditions with ambient temperature maintained at 21 ± 1 ºC and light in 12 h cycles (on at 07:00 h and off at 19:00 h). Experiments were carried out in a sound-attenuated, air-regulated, experimental room. All experimental procedures and animal husbandry were conducted according to ethical principles for the evaluation of pain in conscious animals [29], and to ethical guidelines of the European Communities Council Directive of 24 November 1986 (86/609/ECC). The experimental work received approval by the Local Ethical Committee.

2.2. Drugs

Formaldehyde (37 wt.% solution) was purchased from Panreac (Spain) and dissolved in physiological saline. Drugs used for treatments were AS-19 (dimethyl-[5-(1,3,5-tri-methyl-1H-pyrazol-4-yl)-1,2,3,4-tetrahydro-naphthalen-2(S)-yl]-amine) [8, 9], E-55888 (dimethyl-{2-[3-(1,3,5-trimethyl-1H-pyrazol-4-yl)-phenyl]-ethyl}-amine dihydrochloride) [13], and E-57431 (2-(2-(dimethylamino)ethyl)-4-(1,3,5-tri-methyl-1H-pyrazol-4-yl)phenol) [14]. AS-19 is a potent selective 5-HT_7 receptor agonist commercially available from Tocris Bioscience (UK) whereas E-55888 and E-57431 are 5-HT_7 receptor agonists developed by Esteve. All three 5-HT_7 receptor agonists were synthesized for the purpose of this study at Esteve, dissolved in aqueous solutions containing 0.5 % (hydroxypropyl)methyl cellulose (Sigma-Aldrich, Spain) and administered in a volume of 5 ml/kg through the subcutaneous (s.c.) route. Doses of drugs were referred to their salt forms. All treatments were performed under blind conditions in independent groups of mice and evaluated 30 min after administration.

2.3. Nociceptive behavioral tests

2.3.1. Tail flick test

Animals were placed in a loose Plexiglas restrainer with their tail extruding through a hole to perform the tail flick test as previously described [30]. A photobeam was placed on the tail about 4 cm from the tip. The latency to tail flick response was recorded automatically to the nearest 0.1 s. The intensity of the radiant heat source was adjusted to yield baseline latencies between 3 and 5 s in wild-type mice. A cut-off latency of 10 s was imposed to avoid damage of tail tissues.

2.3.2. Tail immersion test

Animals were placed in a loose Plexiglas restrainer with their tail extending through a hole in the water bath of the apparatus (Stuart Bibby Sterilin Ltd, Water Baths SWB1D, UK), as previously described [31]. The lower 2/3 of the tail was immersed in hot water maintained at a constant temperature of 52.0 ± 0.5 ºC. The latency between tail immersion and attempts to remove the tail from the hot water bath was recorded. A cut-off latency of 15 s was imposed to avoid damage of tail tissues.

2.3.3. Hot plate test

Animals were placed individually on the surface of the hot plate apparatus (PanLab, LE-7406, Spain) surrounded by a Plexiglas cylinder (20 cm in diameter, 25 cm high). The temperature of the surface was maintained at 55.0 ± 0.5 ºC, according to the method previously described [32]. The time between placement and the occurrence of forepaw licking (FPL), hindpaw licking (HPL) or jump was recorded as response latency. A cut-off latency of 240 s was established to avoid damage of paw tissues.

2.3.4. Formalin test

Formalin (20 µL of a 2.5 % formalin solution; 0.92 % of formaldehyde) was injected into the dorsal surface on the right hind paw, as previously described [33]. The formalin test is a valid and reliable model of nociception with two distinct periods of high licking activity that have different nociceptive mechanisms, an early phase lasting the first 5 min and a late phase lasting from 15 to 45 min after the injection of formalin. Mice were placed on a paper surface surrounded by a Plexiglas cylinder (20 x 25 cm) and the time spent licking and biting the injected paw was measured using a chronometer. A time-course of the licking/biting

behaviors was monitored during 45 minutes after formalin injection to evaluate possible differences between genotypes. Drugs were subcutaneously administered 15 min before formalin injection and their effects quantified in phase I (0-5 min) and phase II (15-30 min).

2.3.5. Rectal temperature

The body temperature was recorded using a precision thermometer (YSI 4600) equipped with a flexible probe (YSI 402). This probe was lubricated with vaseline and inserted 2 cm into the rectum. Temperature recordings were made 20 s following insertion of the probe, as previously described [27, 28].

2.4. Data analysis

Data are presented as mean values ± S.E.M. Statistical analysis to test significant differences among groups was made using ANOVA followed by Bonferroni's post hoc comparison. Unpaired Student's *t* test was used to test differences between two groups. The level of significance was set at $p < 0.05$. Data analysis and graphing were done using GraphPad Prism software (version 4.0; GraphPad Software, Inc., USA).

3. Results

3.1. Similar response to noxious thermal stimuli and formalin-induced nociception in 5-HT$_7$ receptor knockout and wild-type mice

Sensitivity to noxious heat measured as the latency of response to thermal stimulation in the tail flick, tail immersion and hot plate tests was similar in 5-HT$_7$ receptor knockout mice and paired wild-type mice (Fig. 1). No significant differences between the two genotypes were found in tail withdrawal latency in the tail flick (t_{49}=1.09, p=0.28) and tail immersion (t_{49}=1.82, p=0.08) tests (Fig. 1A). Both genotypes showed also the same latency for all measured behaviors in the hot plate test (Fig. 1B): forepaw licking (t_{49}=1.69, p=0.10), hindpaw licking (t_{49}=0.73, p=0.47) and jump (t_{48}=0.81, p=0.42), suggesting that 5-HT$_7$ receptor knockout mice perceive and respond normally to acute thermal nociceptive stimuli. In addition, formalin-induced licking and biting of the paw injected with formalin in 5-HT$_7$ receptor knockout mice did not differ from wild-type mice. Repeated measures ANOVA (time x genotype) showed a significant effect of time ($F_{8,162}$=24.30, p<0.001), but no effect of genotype ($F_{1,162}$=1.76, p=0.19) and no interaction between these two factors ($F_{8,162}$=0.90, p=0.52). A slightly greater licking/biting time was observed 25 min after formalin injection in the 5-HT$_7$ receptor knockout in comparison with wild-type mice, but differences were not significant (Fig. 1C).

3.2. 5-HT$_7$ receptor agonists inhibited selectively phase II of formalin-induced nociceptive behavior in wild-type but not in 5-HT$_7$ receptor knockout mice

To examine the *in vivo* functional specificity of 5-HT$_7$ receptor agonists, AS-19, E-55888 and E-57431 were subcutaneously administered to wild-type and 5-HT$_7$ receptor knockout mice and treated animals were then subjected to nociceptive tests. Doses of AS-19 (10 mg/kg), E-55888 (20 mg/kg) and E-57431 (10 mg/kg) that were effective in reversing allodynia/hyperalgesia following capsaicin sensitization and nerve injury [13, 14] were used in these experiments.

No significant effects were exerted by 5-HT$_7$ receptor agonists on the response to thermal stimuli in the tail flick, tail immersion and hot plate tests, and the formalin-induced phase I nociceptive behavior was not modified when 5-HT$_7$ receptor agonists were administered to wild-type or 5-HT$_7$ receptor knockout mice (data not shown). However, all three 5-HT$_7$

receptor agonists inhibited phase II of the formalin-induced nociceptive behavior in wild-type mice, as evidenced by a reduction in the duration of licking/biting of the hindpaw injected with formalin (Fig. 2). Two-way ANOVA (treatment x genotype) showed a significant effect of treatment after AS-19 administration ($F_{1,38}$=10.34, p=0.003), without genotype effect ($F_{1,38}$=0.04, p=0.84) and a significant interaction between these two factors ($F_{1,38}$=4.86, p=0.03). The comparison between treatments revealed a significant reduction of licking/biting time after AS-19 administration at 10 mg/kg in wild-type mice (p<0.001; Fig. 2). Two-way ANOVA calculated for E-57431 also showed a significant effect of treatment after E-57431 administration ($F_{1,39}$=15.04, p<0.001), without genotype effect ($F_{1,39}$=2.64, p=0.11) and a significant interaction between these two factors ($F_{1,39}$=14.91, p<0.001). The comparison between treatments revealed a significant reduction of licking/biting time after E-57431 administration at 10 mg/kg in wild-type mice (p<0.001; Fig. 2). In addition, a significant difference was found between genotypes when E-57431 was administered at 10 mg/kg (p<0.01; Fig. 2). In the same way, two-way ANOVA calculated for E-55888 also showed a significant effect of treatment after E-55888 administration ($F_{1,35}$=22.3, p<0.001), without genotype effect ($F_{1,35}$=2.42, p=0.13) and a significant interaction between these two factors ($F_{1,35}$=13.32, p<0.001). The comparison between treatments revealed a significant reduction of licking/biting time after E-55888 administration at 20 mg/kg in wild-type mice (p<0.001; Fig. 2). In addition, a significant difference was found between genotypes when E-55888 was administered at 20 mg/kg (p<0.01; Fig. 2). Interestingly, none of the three 5-HT_7 receptor agonists exerted significant effects on formalin phase II nociceptive behavior in 5-HT_7 receptor knockout mice (Fig. 2).

3.3. Selective doses of 5-HT_7 receptor agonists produced no effect on body temperature in 5-HT_7 receptor knockout and wild-type mice

The *in vivo* specificity of the 5-HT_7 receptor agonists (AS-19, E-55888 and E-57431) was further examined using 5-HT_7 receptor knockout and paired wild-type mice in the paradigm based on 5-HT_7 receptor-mediated hypothermia [27]. Basal body temperature did not significantly differ in 5-HT_7 receptor knockout and paired wild-type mice (36.1±0.1 °C and 35.8±0.1 ºC, respectively; Fig. 3).

Two-way ANOVA (treatment x genotype) showed a significant effect of treatment after AS-19 administration ($F_{2,49}$=62.17, p<0.001, with genotype effect ($F_{1,49}$=6.88, p=0.01) and a significant interaction between these two factors ($F_{2,49}$=4.77, p=0.01). The comparison between treatments revealed a significant reduction of body temperature after AS-19 administration at 20 mg/kg in wild-type and 5-HT_7 receptor knockout mice (p<0.001; Fig. 3). In addition, a significant difference was found between genotypes when AS-19 was administered at 20 mg/kg (p<0.001; Fig. 3). Two-way ANOVA calculated for E-57431 also showed a significant effect of treatment after E-57431 administration ($F_{2,49}$=35.85, p<0.001), without genotype effect ($F_{1,49}$=0.31, p=0.58) and a significant interaction between these two factors ($F_{2,49}$=5.82, p=0.005). The comparison between treatments revealed a significant reduction of body temperature after E-57431 administration at 20 mg/kg in wild-type and 5-HT_7 receptor knockout mice (p<0.001; Fig. 3). In addition, a significant difference was found between genotypes when E-57431 was administered at 20 mg/kg (p<0.05; Fig. 3). However, two-way ANOVA calculated for E-55888 did not show significant differences on treatment after E-55888 administration ($F_{1,35}$=0.02, p=0.89), neither genotype effect ($F_{1,35}$=0.91, p=0.35) nor interaction between these two factors ($F_{1,35}$=0.21, p=0.65). The comparison between treatments and genotypes did not reveal significant reduction of body temperature after E-55888 administration at 20 mg/kg (p>0.05; Fig. 3).

Subcutaneous administration of doses of AS-19 (10 mg/kg), E-55888 (20 mg/kg) and E-57431 (10 mg/kg), which exerted analgesic effects in phase II formalin-induced pain, did not significantly change body temperature neither in 5-HT_7 receptor wild-type nor in knockout mice (Fig. 3). However, administration of a higher dose (20 mg/kg s.c.) of the 5-HT_7 receptor agonists AS-19 and E-57431 significantly reduced body temperature in both genotypes (Fig. 3), suggesting that at such a high dose the selectivity window of AS-19 and E-57431 was overstepped. Nevertheless, because AS-19 at the 20 mg/kg dose produced a significantly higher body temperature reduction in wild-type than in 5-HT_7 receptor knockout mice (3.8 *vs*. 2.4 ºC respect to vehicle group),, it might be inferred that part of its hypothermic effect was actually mediated by 5-HT_7 receptor activation. In contrast, E-57431 at the same high dose (20 mg/kg) produced a significantly lower body temperature reduction in wild-type than in 5-HT_7 receptor knockout mice (1.1 *vs*. 2.3 ºC respect to vehicle group (Fig. 3).

4. Discussion

In this study, the *in vivo* target-specific effects of the 5-HT_7 receptor agonists AS-19, E-55888 and E-57431 on nociception (i.e., formalin-induced nociception) and thermoregulation were examined using 5-HT_7 receptor knockout mice. The three 5-HT_7 receptor agonists exerted antinociceptive effects in the phase II of the formalin test in wild-type but not 5-HT_7 receptor knockout mice, suggesting that their analgesic effect is actually 5-HT_7 receptor-mediated. Analgesic doses of 5-HT_7 receptor agonists did not change body temperature neither in 5-HT_7 receptor knockout nor in wild-type mice. However, a reduction in body temperature was observed in both genotypes when the dose of the agonists were increased up to levels exceeding their selectivity window.

The 5-HT_7 receptor knockout mice offer a complementary approach to classical pharmacology, and might provide insights into the functional implications of 5-HT_7 receptors. To date, data obtained with these mutants suggest the involvement of 5-HT_7 receptors in depression, schizophrenia, sleep, learning, locomotion and hypothermia [25, 28, 34 -37].

In this work, we demonstrated that sensitivity to noxious heat measured as the latency time of response to thermal stimulation in the tail flick, tail immersion and hot plate tests did not differ in 5-HT_7 receptor knockout compared to wild-type mice, as previously described [38]. In addition, the formalin-induced nociceptive behavior of 5-HT_7 receptor knockout mice was not different from wild-type mice as no significant differences in licking/biting time were found between both genotypes, for either phase I or phase II of the formalin test. These results suggest that basic mechanisms for transduction, transmission and perception of, as well as response to, nociceptive stimuli are intact in mice lacking 5-HT_7 receptors. Nevertheless, as previously reported, the loss-of-function of the missing 5-HT_7 receptor could induce possible adaptive changes which could compensate for some alterations, thereby resulting in wild-type-like responses [38 - 40].

Subcutaneous administration of 5-HT_7 receptor agonists was devoid of activity in acute nociceptive tests (i.e., thermal and formalin-induced phase I nociception) but exerted clear cut antinociceptive effects in phase II of the formalin test in wild-type mice. This observation is in line with previous reports describing antinociceptive effects of 5-HT_7 receptor agonists in neurogenic and neuropathic pain conditions involving central sensitization [13-14]. Indeed, thermal nociception and early phase response in the formalin test are caused predominantly

by direct activation of peripheral C-fibers, whereas the late response (phase II) in the formalin test involves functional changes in the dorsal horn of the spinal cord (i.e., central sensitization) [41 -43]. Activation of spinal 5-HT_7 receptors has been shown to exert antinociceptive effects and to play a role in the analgesic effects of opioids [17, 44 - 47]. The mechanism underlying this effect has not been fully elucidated. It is unlikely that activation of spinal 5-HT_7 receptors could directly inhibit primary afferents or nociceptive dorsal horn neurons because the stimulation of this Gs-protein coupled receptor is excitatory. However, 5-HT_7 receptors are also expressed by GABA interneurons in the dorsal horn of the spinal cord and evidence has been reported that the antinociceptive effects of 5-HT_7 receptor agonists could be mediated through activation of 5-HT_7 receptors localized on these inhibitory interneurons [14, 48, 49].

To further assess the *in vivo* specificity of the 5-HT_7 receptor agonists used in this study, we examined in 5-HT_7 receptor knockout mice the effects of the 5-HT_7 receptor agonists AS-19, E-55888 and E-57431 on both the formalin-induced nociception and the body temperature. Under our experimental conditions, 5-HT_7 receptor agonists, at doses effective to reduce phase II formalin-induced nociceptive behavior, affected body temperature neither in wild-type mice nor in 5-HT_7 knockout mutants. However, AS-19 and E-57431 at the high dose of 20 mg/kg significantly reduced body temperature not only in wild-type but also in 5-HT_7 receptor knockout mice, indicating a non-5-HT_7 receptor-mediated effect possibly due to interactions of these compounds with other 5-HT receptors when their selectivity window is surpassed. In line with this interpretation, we found that E-55888, the most selective 5-HT_7 receptor agonist based on *in vitro* radioligand binding assays (Table 1), even at the dose of 20 mg/kg, did not exert any effect on body temperature in both genotypes. Taken together, the finding that the less selective agonists (AS-19 and E-57431) at high doses reduced body temperature in both wild-type and knockout mice whereas the most selective one (E-55888) did not, suggests that activation of 5-HT_7 receptors alone is not enough to affect body temperature. This does not rule out the possibility that 5-HT_7 receptors might contribute to the regulation of body temperature by acting in concert with other serotonergic and/or non-serotonergic receptors. Indeed, we found a significantly higher hypothermic effect induced by AS-19 (20 mg/kg) in wild-type compared to 5-HT_7 receptor knockout mice, suggesting that 5-HT_7 receptors may promote the decrease in body temperature when other mechanisms are also recruited. Based on the known *in vitro* pharmacological profile of AS-19 (Table 1), it can

be proposed that 5-HT_{1D} and/or 5-HT_{1A} receptors might be involved in the observed hypothermic effects because activation of these receptor types has been reported to induce hypothermia [16, 28, 50]. In case of the other agonist E-57431, not only 5-HT_{1D} receptor activation (Table 1), but also actions at other, not yet identified, 5-HT_7 receptor-unrelated targets, might account for its hypothermic effect at high dose, especially because E-57431 exerted a higher hypothermic effect in 5-HT_7 receptor knockout than in wild-type mice. Overall, as previously reported, 5-HT_7 receptors appear to be involved in a complex manner in thermoregulation, probably through mechanisms implicating direct/indirect interactions between 5-HT_7 receptors and other, yet unknown, molecular targets [16].

5. Conclusions

Data obtained in this study strengthen the notion that 5-HT_7 receptors play a role in nociceptive control in pain conditions involving central sensitization and add further support to their fine-tuning effects in body temperature homeostasis through possible actions in concert with other molecular targets. In addition, this study provides evidence that formalin-induced nociceptive behaviors and body temperature in 5-HT_7 receptor knockout mice are useful models and relatively simple approaches to assess *in vivo* specificity of 5-HT_7 receptor agonists.

Conflicts of interests

There were no conflicts of interests with respect to the work reported in the paper.

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Figure Legends

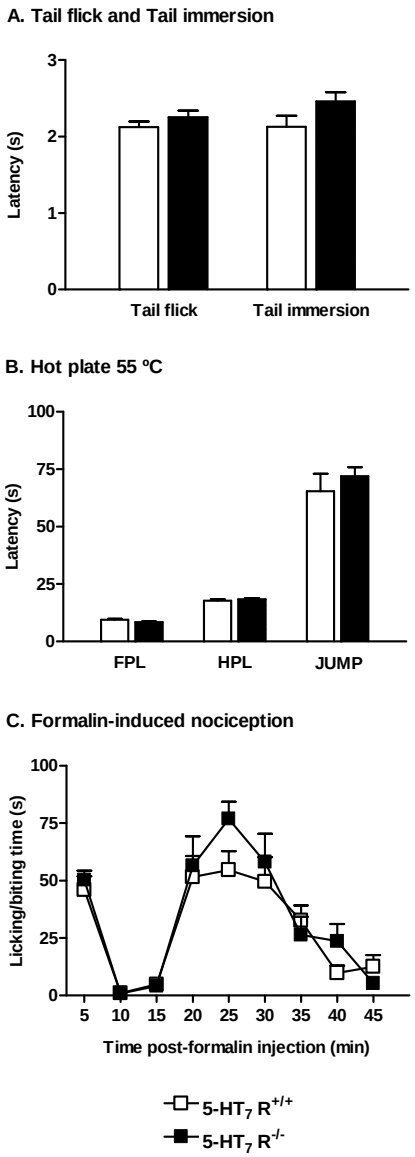

Fig. 1. Nociceptive behavior of wild-type and 5-HT_7 receptor knockout mice in the tail flick and tail immersion tests (A), hot plate 55 ºC test (B) and formalin test (C). Both genotypes showed similar latency for all measured behaviors in the tail flick, tail immersion and hot plate tests. Formalin-induced licking and biting of the hind paw injected with formalin in 5-HT_7 receptor knockout mice did not significantly differ from those in wild-type mice either in phase I (0 - 5 min) or in phase II (15 - 45 min). Only a slight but not significant increase of the licking/biting time was observed 25 min after formalin injection in 5-HT_7 receptor knockout mice compared to wild-type mice. Each bar or symbol represents the mean ± S.E.M. (n = 10 - 12 per group). Forepaw licking, FPL; hindpaw licking, HPL. No significant differences were observed in thermal nociception (unpaired Student's *t* test) or formalin-induced nociceptive behaviors (Two-Way ANOVA).

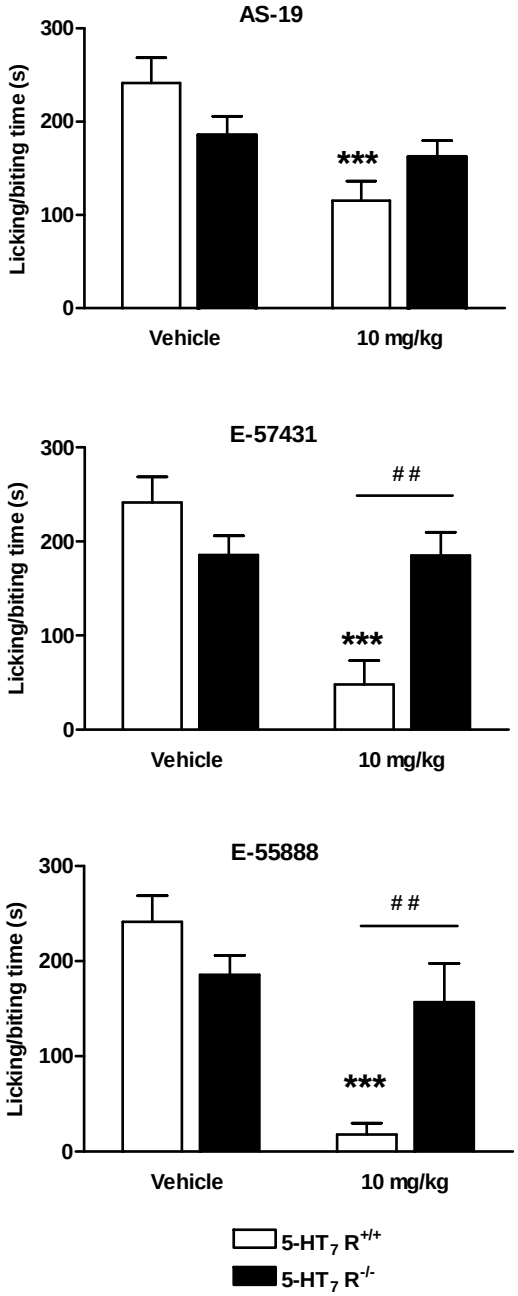

Fig. 2. Effects of 5-HT_7 receptor agonists AS-19, E-57431 and E-55888 on formalin-induced nociceptive behaviors during phase II in wild-type and 5-HT_7 receptor knockout mice. Subcutaneous administration of AS-19 (10 mg/kg), E-57431 (10 mg/kg) and E-55888 (20 mg/kg) significantly reduced the licking/biting time of the hind paw injected with formalin in wild-type but not in 5-HT_7 receptor knockout mice. Each bar represents the mean ± S.E.M. (n = 7 - 12). *** p<0.001 vs. vehicle corresponding group; $^{\#\#}$ p<0.01 vs. corresponding dose in 5-HT_7 receptor knockout mice (Bonferroni multiple comparison test post-ANOVA).

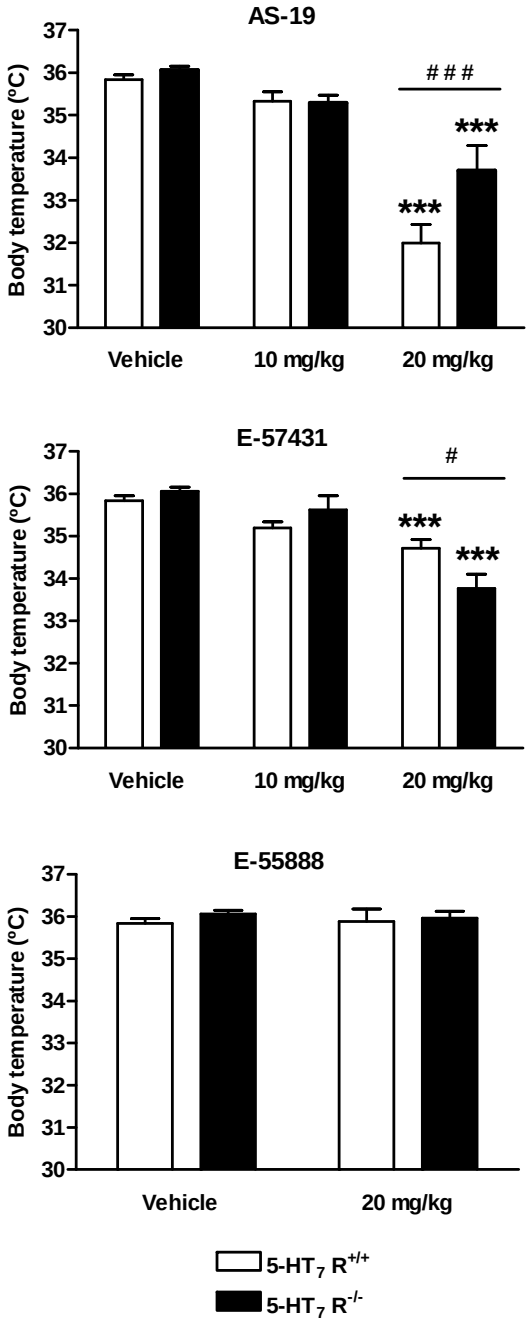

Fig. 3. Effects of 5-HT_7 receptor agonists AS-19, E-57431 and E-55888 on body temperature in wild-type and 5-HT_7 receptor knockout mice. Subcutaneous administration of AS-19 and E-57431 at a higher dose (20 mg/kg) significantly reduced body temperature in both wild-type and 5-HT_7 receptor knockout mice and showed significant differences between both genotypes. However, E-55888 at 20 mg/kg did not reduce the body temperature neither in wild-type nor in 5-HT_7 knockout mice. Each bar represents the mean ± S.E.M. (n = 8 - 12). *** $p<0.001$ vs. vehicle corresponding group; $^{\#}$ $p<0.05$; $^{\#\#\#}$ $p<0.001$ vs. corresponding dose in 5-HT_7 receptor knockout mice (Bonferroni multiple comparison test post-ANOVA).

VII. GENERAL DISCUSSION

Altogether, our results suggest an antinociceptive role of 5-HT_7 receptors under sensitizing neurogenic/neuropathic conditions that seems to be mediated by activation of 5-HT_7 receptors localized on GABAergic inhibitory interneurons of the spinal cord. In contrast, we observed a pronociceptive role of 5-HT_7 receptors localized at the periphery.

The antinociceptive role of central 5-HT_7 receptors seems to predominate over the pronociceptive role at the periphery when selective 5-HT_7 receptor agonists are systemically administered. It is plausible that the antinociception resulting from activation of spinal 5-HT_7 receptors could overcome the pronociceptive effect on peripheral 5-HT_7 receptors and thus be responsible for the overall antinociceptive effect found after systemic administration of selective 5-HT_7 receptor agonists in conditions involving central sensitization.

Taken together, data reported herein support the idea that 5-HT_7 receptors play a role in physiological mechanisms controlling pain. Physiological, compensatory and/or protective neuroplastic adaptations that take place in conditions involving central sensitization could explain why 5-HT_7 receptor agonists exert antinociceptive effects when sensitization occurs, but not in response to acute nociceptive pain stimuli.

- **Lack of response of 5-HT_7 receptor ligands to acute nociceptive pain and potentiation of morphine antinociception after systemic 5-HT_7 receptors activation**

We observed that sensitivity to noxious heat of 5-HT_7 receptor knockout mice, measured as the latency time of response to thermal stimulation in the tail-flick, tail immersion and hot plate tests, did not differ from wild-type mice (*section 6.5*). These results support a previous published study where no differences in sensitivity to noxious heat stimulus were found, respect to wild-type mice, in 5-HT_7 receptor knockout mice using the tail-flick test (Roberts et al., 2004).

In addition, no effect on noxious heat stimulus-evoked activity was found pharmacologically after subcutaneous administration of 5-HT_7 receptor ligands (the 5-HT_7 receptor agonist E-55888 or the 5-HT_7 receptor antagonist SB-258719) in the tail-flick test (*section 6.1*).

In contrast, when we explored the interaction of 5-HT_7 receptors with the opioidergic system, we observed that the analgesic effect of oral morphine was dramatically potentiated when morphine was co-administered with the selective 5-HT_7 receptor agonist E-55888 (10 mg/kg) by subcutaneous route. Activation of 5-HT_7 receptors improved significantly the analgesic

potency (effective dose, ED_{50}) of morphine (2.6 times), from 31.6 to 12.2 mg/kg. The analgesic effect of oral morphine was significantly potentiated from 15 to 120 min after co-administration with the 5-HT_7 receptor agonist E-55888, but not by the 5-HT_7 receptor antagonist SB-258719. Interestingly, the potentiating effect of morphine-induced analgesia by E-55888 was reversed by the selective 5-HT_7 receptor antagonist SB-258719 (*section 6.1*).

No significant differences in the concentration of morphine or its metabolite morphine-3-glucuronide M3G in the plasma and the brain were observed when morphine was co-administered with E-55888. These results suggest no pharmacokinetic interaction between morphine and the selective 5-HT_7 receptor agonist E-55888 and support a pharmacodynamic interaction responsible for the potentiation of morphine antinociception (*section 6.1*).

As already mentioned, systemic administration of a selective 5-HT_7 receptor agonist *per se* is not enough to reproduce the antinociception exerted by opioids in acute thermal nociceptive models (i.e., tail-flick test), in line with the knowledge involving multiple molecular mechanisms, cellular targets and pathways/locations to elicit opioid analgesia. These nociceptive behavioral tests (tail-flick and hot plate tests) involve the activation of unmyelinated C-polymodal nociceptors which are greatly attenuated by opioids, such as morphine. The lack of antinociceptive effects in acute thermal nociceptive models observed when 5-HT_7 receptor ligands are administered suggests no direct contribution of the 5-HT_7 receptor subtype in mediating acute thermal-evoked responses through small calibre unmyelinated nociceptive afferents. However, spinal activation of 5-HT_7 receptors seems to be ultimately critical for opiate-induced nociception, likely through the mediation of the descending serotonergic inhibitory pathway (see figure 16). This is supported by the observation that the antinociceptive effect of morphine and tramadol administered either systemically or into the rostroventromedial medulla (RVM) were blocked by intrathecal administration of the 5-HT_7 receptor antagonist SB-269970 (Dogrul et al., 2009; Yanarates et al., 2010).

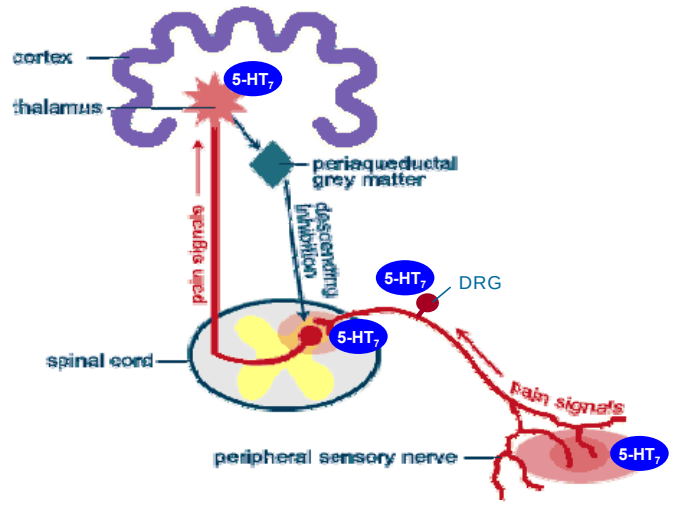

Fig 16. Main sites of action of 5-HT$_7$ receptors in pain control. 5-HT$_7$ receptors exert a role in pain transmission at different locations involved in nociception (thalamus, spinal cord, dorsal root ganglion, periphery).

- **Effect of 5-HT$_7$ receptor agonists and antagonists in pain conditions involving central sensitization**

Systemic administration of selective 5-HT$_7$ receptor agonists (AS-19, MSD-5a and E-55888) dose-dependently reversed capsaicin-induced mechanical hypersensitivity to levels close to basal values found in control mice. In contrast, a dose-dependent promotion of mechanical hypersensitivity after administration of 5-HT$_7$ receptor antagonists (SB-258719 and SB-269970) was observed in mice injected with a low subactive dose of capsaicin and stimulated with a low subthreshold pressure. In addition, reduction of mechanical hypersensitivity by agonists and promotion of hypersensitivity by antagonists were reversed by antagonists and agonists, respectively (*section 6.2*). Altogether, our initial data highlighted the role of 5-HT$_7$ receptors in the control of mechanical hypersensitivity secondary to a sensitizing stimulus.

Interestingly, we did not observe promotion of hypersensitivity by the selective 5-HT$_7$ receptor antagonists SB-258719 and SB-269970 in mice subplantarly injected with vehicle, indicating that a minimal capsaicin sensitizing challenge is needed for the antagonist to exert its pronociceptive effect (*section 6.2*). This suggests that endogenous activation of 5-HT$_7$ receptors takes place after intraplantar capsaicin challenge (but not after vehicle) and thus it may be counteracted by 5-HT$_7$ receptor antagonists.

Comparable results were found in the partial sciatic nerve ligation model in mice. We observed that systemic administration of selective 5-HT$_7$ receptor agonists (AS-19 and E-

57431) dose-dependently inhibited nerve injury-induced mechanical and thermal hypersensitivity to levels close to basal values found in control mice (*section 6.3*). In contrast, we found a dose-dependent promotion of mechanical hypersensitivity, but not heat hyperalgesia, in nerve injured and sham-operated mice after treatment with the selective 5-HT_7 receptor antagonist SB-258719 (*section 6.3*). Differential endogenous 5-HT tone and/or modulation by 5-HT_7 receptors of sensory/nociceptive pathways depending on the nature (mechanical vs. thermal) and intensity (allodynic/subthreshold vs. hyperalgesic/suprathreshold) of the stimulus could explain the different effects on the response to mechanical and thermal stimuli exerted by the 5-HT_7 receptor antagonist.

In line with these results, experiments performed in rats showed a dose-response antinociceptive effect of systemically administered E-57431 in the capsaicin model and spared nerve injury model of neuropathic pain (*section 6.4*). In addition, subcutaneous administration of 5-HT_7 receptor agonists (AS-19, E-55888 and E-57431) reduced the formalin-induced nociception in the phase II in mice (*section 6.5*). It is important to note that central sensitization occurs not only in relation to nerve injury in chronic neuropathic pain models but also following sensitization with capsaicin (Torebjork et al., 1992; Laird et al., 2001b) and formalin (particularly during phase II) (Coderre and Melzack, 1992; Vissers et al., 2003). It is also important to note that the effectiveness of the treatment with 5-HT_7 receptor agonists when evaluated pain behaviors was not masked by non-specific motor effects, as no motor incoordination was found in the rotarod test at the analgesic doses used in these studies.

No tolerance to the analgesic (antiallodynic/antihyperalgesic) effect was evidenced following repeated systemic administration of the selective 5-HT_7 receptor agonist E-57431 twice daily for 11 days to nerve-injured mice (*section 6.3*). The effectiveness was maintained or even slightly increased throughout the treatment period but neuropathic pain-related behaviors reverted back to baseline nerve injury values when the treatment was withdrawn. This suggests an improvement of ‘‘disease symptoms” related to the presence and influence of the drug at the time of the test. Down-regulation of receptor expression has been described in some studies (e.g., in the hypothalamus after fluoxetine treatment for 21 days) (Sleight et al., 1995) but data indicating that 5-HT_7 receptors are not readily down-regulated by long-term exposure to agonists is also available (Krobert et al., 2006). Actually, significantly increased 5-HT_7 receptor mRNA expression has been reported in raphe nuclei, hippocampus and

prefrontal cortex after treatment with the 5-HT_7 receptor agonist AS-19 (Pérez-García et al., 2006). Thus, if desensitization and/or down-regulation phenomena occur, as it has been reported following agonist exposure, they do not have noticeable consequences in the particular conditions of our study (e.g., they could require longer exposure to the agonist to become apparent) or are compensated by nerve injury-induced receptor up-regulation.

- **Pronociceptive effects of peripheral 5-HT_7 receptor activation and antinociceptive effects of spinal 5-HT_7 receptor activation**

In contrast to the antinociceptive (antiallodynic) effect found after systemic administration, a clear-cut pronociceptive (proallodynic) effect was found when the selective 5-HT_7 receptor agonist E-57431 was administered intraplantarly into the ipsilateral hind paw injected with a low subactive dose of capsaicin (*section 6.4*).

No effects were found when E-57431 was intraplantarly administered into the contralateral paw, suggesting that peripheral, local, mechanisms (i.e., activation of 5-HT_7 receptors on nerve endings) were responsible for its pronociceptive action (*section 6.4*). Interestingly, promotion of mechanical hypersensitivity did not occur when E-57431 was intraplantarly administered to rats receiving only vehicle (without capsaicin), indicating that a minimal sensitizing challenge with capsaicin was needed for the 5-HT_7 receptor agonist to exert its pronociceptive action. This suggests that recruitment of additional mechanisms other than activation of 5-HT_7 receptors is required to induce mechanical hypersensitivity at the periphery.

In fact, 5-HT released from platelets and mast cells at the periphery acts on different 5-HT receptor subtypes and in combination with other inflammatory mediators to excite afferent nerve fibres (Sommer, 2004). Activation of 5-HT_{2A} and 5-HT_3 receptor subtypes present on C-fibers has already been reported to contribute to 5-HT-mediated peripheral pronociceptive effects (Obata et al., 2000; Sommer, 2004). Our data allow the conclusion that 5-HT_7 receptors, in particular those expressed at the periphery by small and medium-sized DRG neurons (Meuser et al., 2002; Doly et al., 2005), also probably contribute to 5-HT-mediated pronociceptive effects.

Indeed, our results are supported by several convergent data in the literature: (1) intraplantar injection of the 5-HT_7 receptor antagonist SB-269970 reduced formalin-induced nociception

whereas intraplantar administration of non-selective 5-HT_7 receptor agonists such as 5-HT itself and 5-CT increased formalin-induced nociceptive behavior (Rocha-González et al., 2005); (2) intra-articular injection of the mixed 5-HT_{1A}/5-HT_7 receptor agonist 8-OH-DPAT induced c-Fos expression in the dorsal horn of the rat spinal cord and this effect was prevented by intra-articular administration of the non-selective 5-HT_7 receptor antagonist methiothepin (Meuser et al., 2002).

On the other hand, we found that intrathecal administration of the selective 5-HT_7 receptor agonist E-57431 inhibited mechanical hypersensitivity secondary to capsaicin injection and nerve injury-induced mechanical hypersensitivity (*section 6.4*). Thus, the antinociceptive effect mediated by central 5-HT_7 receptors seems to predominate over the pronociceptive effect at the periphery when selective 5-HT_7 receptor agonists are systemically administered.

In addition, we observed a significant increase of 5-HT_7 receptor immunoreactivity in laminae I–II and III–V of the ipsilateral dorsal horn of the spinal cord after sciatic nerve injury in mice (*section 6.3*). Increased levels of 5-HT_7 receptor messenger RNA have also been reported in rat DRG after bee venom- (Liu et al., 2005) and complete Freund's adjuvant-induced inflammation (Wu et al., 2001). This upregulation of 5-HT_7 receptors in the spinal cord after nerve injury suggests a "pain"-triggered regulation of receptor expression that may be relevant for the effectiveness of 5-HT_7 receptor agonists. Indeed, 5-HT_7 receptor upregulation might represent a compensatory, protective, spinal mechanism to reduce excessive nociception in neuropathic pain conditions.

Regarding the mechanism of action involved, we observed a co-localization of 5-HT_7 receptors on GABAergic cells (*section 6.3*). We reported by immunofluorescence that 5-HT_7 receptors co-localize with gamma-amino butyric acid (GABA) in neurons of the dorsal horn of the spinal cord in mice (*section 6.3*). Interestingly, agonists acting at spinal 5-HT_7 receptors cannot directly inhibit primary afferents or second-order nociceptive dorsal horn neurons because stimulation of the 5-HT_7 receptor has excitatory effects (Lovenberg et al., 1993; Chapin and Andrade, 2001). Therefore, an indirect action through activation of 5-HT_7 receptors localized on inhibitory interneurons is presumably required to inhibit nociceptive transmission (see figure 17).

In line with this, 5-HT_7 receptors in the spinal cord have been described located postsynaptically on local interneurons within the superficial laminae of the dorsal horn (Meuser et al., 2002; Doly et al., 2005). Actually, it has been reported that spinal GABAergic

interneurons, but not enkephalinergic interneurons, are involved in 5-HT_7 receptor-mediated antinociception (Bourgoin et al., 2008; Viguier et al., 2009). This is based on the finding that intrathecal pretreatment with the $GABA_A$ receptor antagonist bicuculline, but not the $GABA_B$ receptor antagonist phaclofen or the opioid receptor antagonist naloxone, prevented the antihyperalgesic effects exerted by 5-HT_7 receptor agonists in rats with constriction injury to the sciatic nerve (Bourgoin et al., 2008; Viguier et al., 2009).

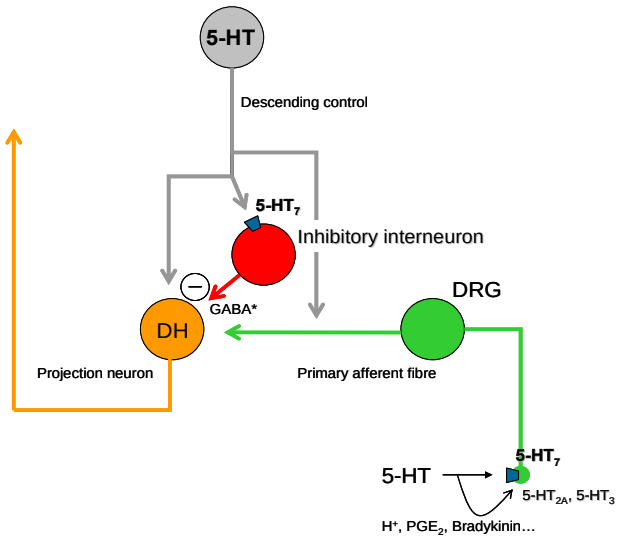

Fig 17. Significance of 5-HT_7 receptors and of their divergent neuronal localization to the modulation of nociceptive processing. Stimuli are exerted at terminals of primary afferent fibres (green line) where 5-HT_7 receptors are expressed. The activation of 5-HT_7 receptors by peripheral serotonin excites primary afferent fibres to transmite nociception to neurons expressed in the dorsal horn (DH). This nociceptive information is transmited to the brain (orange line). A descending inhibition pathway (grey and red line) is responsible to block pain transmission to cortical regions. In this process, activation of 5-HT_7 receptors expressed in inhibitory interneurones may release GABA to inhibit neurons in the dorsal horn. Different molecules other than serotonin (H^+, PGE2, bradykinin…) and 5-HT receptors other than 5-HT_7 receptors are also involved in pain transmission and modulation.

Recent data also suggest that, at the spinal level, 5-HT_7 receptor stimulation modulates pain signaling through Cl^- conductance-dependent mechanisms (Viguier et al., 2010). Accordingly, activation of spinal inhibitory GABAergic interneurons could underlie, or at least contribute to, the analgesic effects of 5-HT_7 receptor agonists. In contrast, recently published data from the department of Rocha-Gonzalez et al., suggest a pronociceptive role of spinal 5-HT_7 receptors in neuropathic rats. Spinal (0.3-30µg) administration of the selective 5-HT_7 receptor antagonist SB-269970 but not vehicle reduced in a dose-dependent manner established tactile allodynia (Amaya-Castellanos et al., 2011).

- ***In vivo* specific antinociceptive effects of 5-HT$_7$ receptor agonists in wild-type, but not in 5-HT$_7$ receptor knockout mice.**

We provided evidence that the formalin-induced nociceptive behavior in 5-HT$_7$ receptor knockout mice was not significantly different from wild-type mice as no significant differences in licking/biting time of the right hind paw injected with formalin 2.5% were found between both genotypes, in either the phase I or the phase II of the formalin test (*section 6.5*). However, the 5-HT$_7$ receptor agonists (AS-19, E-55888 and E-57431) inhibited the phase II of the formalin test in wild-type but not 5-HT$_7$ receptor knockout mice.

Altogether, these data suggest that basic mechanisms for transduction, transmission and perception of, as well as response to, nociceptive inputs are intact in mice lacking 5-HT$_7$ receptors. Adaptive changes in the central serotonergic system have been described to occur during development following the absence of a 5-HT receptor subtype, which may circumvent the loss-of-function of the deleted receptor (Compan, 2007). The constitutive deletion of the 5-HT$_7$ receptor may induce possible compensatory alterations, which could underlie their phenotypes, resulting in wildtype-like responses (Roberts et al., 2004; Sarkisyan and Hedlund, 2009; Semenova et al., 2008).

5-HT$_7$ receptor knockout mice provided us an additional tool to explore the function of this receptor subtype as well as a means for determining drug specificity at this target. Our data support the *in vivo* selectivity of assayed 5-HT$_7$ receptor agonists as they were devoid of activity on phase II formalin-induced nociceptive behavior in 5-HT$_7$ receptor knockout mice (*section 6.5*).

VIII. CONCLUSIONS

1.- 5-HT_7 receptors *per se* are not involved in the response to a noxius stimulus, but interact with the opiodergic system in nociceptive pain conditions. 5-HT_7 receptor knockout and wild-type mice show similar sensitivity to a noxious heat stimulus. Systemic 5-HT_7 receptor agonists and antagonists are ineffective in acute thermal nociceptive pain. However, 5-HT_7 receptor agonists enhance the analgesic effects exerted by morphine following acute thermal nociceptive stimulation.

2.- 5-HT_7 receptors are involved in pain conditions involving central sensitization. Systemic administration of 5-HT_7 receptor agonists and antagonists exert antinociceptive and pronociceptive effects, respectively, in capsaicin-induced neurogenic and neuropathic pain conditions. No tolerance to the antinociceptive effect is evidenced following repeated systemic administration of a 5-HT_7 receptor agonist.

3.- Activation of peripheral 5-HT_7 receptors promotes nociception whereas activation of spinal 5-HT_7 receptors exerts antinociceptive effects. Intraplantar injection of a 5-HT_7 receptor agonist results in pronociceptive effects in capsaicin-induced neurogenic pain conditions. However, intrathecal administration of a 5-HT_7 receptor agonist exerts antinociceptive effects in neurogenic and neuropathic pain conditions. These results support the hypothesis that spinal 5-HT_7 receptors are responsible for the antinociceptive effects of 5-HT_7 receptor agonists.

4.- 5-HT_7 receptors co-localize with GABAergic neurons in the dorsal horn of the spinal cord, suggesting that the activation of spinal inhibitory GABAergic interneurons could underlie or at least contribute to the analgesic effects of 5-HT_7 receptor agonists.

5.- Target-specific effects could be attributed to assayed 5-HT_7 receptor agonists based on both their *in vitro* binding selectivity profile and *in vivo* studies in 5-HT_7 receptor knockout mice.

6.- Selective 5-HT_7 receptor agonists could represent a new therapeutic approach for the treatment of neuropathic pain. However, no clinial studies have yet been performed to confirm the preclinical data obtained in animals.

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X. APPENDIX

Resum de la Tesi

El treball mostrat en aquesta Tesi ha format part del projecte "5-HT_7 i dolor neuropàtic" de l'empresa farmacèutica Esteve. Per tant, els objectius d'aquesta Tesi estan en línea amb l'objectiu del projecte 5-HT_7 d'Esteve focalitzat en el descobriment de compostos amb afinitat pel receptor 5-HT_7 pel tractament del dolor neuropàtic.

A partir de l'aproximació genètica amb l'ús de ratolins genoanul·lats pel receptor 5-HT_7 i d'eines farmacològiques com compostos amb afinitat pel receptor 5-HT_7, vàrem investigar a nivell preclínic el paper dels receptors 5-HT_7 en el dolor i l'interès terapèutic dels lligands del receptor 5-HT_7 en dolor. Entre els compostos utilitzats hi trobem el SB-258719 i el SB-269970 com antagonistes pel receptor 5-HT_7, i el AS-19, MSD-5a, E-55888 i el E-57431 com agonistes pel receptor 5-HT_7. E-55888 i E-57431 van ser descrits per primera vegada i es va estudiar el seu perfil d'afinitat, selectivitat i funcionalitat. Es van realitzar estudis de comportament *in vivo* en ratolí i rata sotmesos a unes condicions de dolor nociceptiu, inflamatori, neurogènic i neuropàtic.

Els nostres resultats van mostrar que els receptors 5-HT_7 per si mateixos no estaven implicats en la resposta a un estímul nociu, mentre que sí interaccionen amb el sistema opiodèrgic en condicions de dolor nociceptiu. Ratolins genoanul·lats pel receptor 5-HT_7 van mostrar la mateixa sensibilitat enfront un estímul tèrmic nociu. L'administració sistèmica de l'agonista del receptor 5-HT_7 E-55888 o l'antagonista del receptor 5-HT_7 SB-258719 no van mostrar efecte en el dolor agut nociceptiu. En canvi, vàrem observar que els efectes antinociceptius de la morfina per via oral obtinguts en resposta a l'estímul tèrmic nociu del *tail-flick*, eren potenciats amb l'administració sistèmica conjunta de l'agonista del receptor 5-HT_7 E-55888. Aquesta potenciació va ser revertida al mateix temps amb la coadministració de l'antagonista del receptor 5-HT_7 SB-258719.

També vàrem estudiar el paper dels receptors 5-HT_7 en condicions de dolor i sensibilització central. L'administració sistèmica d'agonistes selectius pel receptor 5-HT_7 inhibia la hipersensibilitat mecànica induïda per capsaicina, la hipersensibilitat mecànica i tèrmica induïda per la lesió del nervi ciàtic, i el dolor induït per la fase II del model de la formalina. Això suggeria la implicació dels receptors 5-HT_7 en condicions de sensibilització central. En canvi, es va observar una promoció de la hipersensibilitat mecànica amb els antagonistes del receptor 5-HT_7. Tant els efectes dels agonistes i antagonistes del receptor 5-HT_7 es van revertir amb la coadministració d'antagonistes i agonistes del receptor 5-HT_7, respectivament. És important senyalar que les dosis amb eficàcia analgèsica dels agonistes del receptor 5-HT_7

eren inferior a les dosis que produïen efectes adversos amb el test del *rota-rod*. A més, no es va observar tolerància de l'efecte analgèsic amb l'administració de dosis repetides de l'agonista del receptor 5-HT_7 E-57431.

L'efecte analgèsic obtingut amb l'administració sistèmica dels agonistes pel receptor 5-HT_7 semblava ser degut a l'activació de receptors 5-HT_7 localitzats a nivell espinal. Vàrem trobar que l'administració intratecal de l'agonista del receptor 5-HT_7 E-57431 inhibia la hipersensibilitat mecànica secundària a la injecció de capsaicina i la induïda per la lesió del nervi ciàtic. En canvi, es va observar un increment de la hipersensibilitat mecànica induïda per capsaicina amb la injecció local intraplantar de l'agonista del receptor 5-HT_7 E-57431. En resum, l'efecte antinociceptiu obtingut a través de l'activació dels receptors 5-HT_7 a nivell espinal sembla predominar respecte l'efecte pronociceptiu de la perifèria quan s'administra sistèmicament un agonista pel receptor 5-HT_7. En línea amb l'efecte antinociceptiu observat a nivell espinal, vàrem trobar un increment de la immunoreactivitat dels receptors 5-HT_7 de l'asta dorsal de la medul·la espinal en ratolins amb lesió del nervi ciàtic. Aquest increment en l'expressió del receptor 5-HT_7 en l'asta dorsal induït per la lesió del nervi podria representar un mecanisme espinal fisiològic, compensatori i protector rellevant pel control de dolor en condicions de dolor neuropàtic.

Vàrem observar una col·localització dels receptors 5-HT_7 en cèl·lules GABAèrgiques. En aquest sentit, l'activació dels receptors 5-HT_7 col·localitzats en interneurones inhibitòries de l'asta dorsal de la medul·la espinal podria ser el mecanisme d'acció implicat en els efectes antinociceptius observats amb els agonistes del receptor 5-HT_7.

Finalment, utilitzant ratolins genoanul·lats pel receptor 5-HT_7, vàrem demostrar que els agonistes pel receptor 5-HT_7 AS-19, E-55888 i E-57431 exercien efectes diana específics en el control de dolor. L'administració subcutània d'aquests agonistes pel receptor 5-HT_7 reduïren la nocicepció induïda per formalina de la fase II en ratolins salvatges però no en ratolins genoanul·lats, suggerint una especificitat dels efectes obtinguts *in vivo* a través del receptor 5-HT_7.

Aquest treball aporta un millor coneixement del paper del receptor 5-HT_7 en el control del dolor i suggereix un nou potencial ús terapèutic dels agonistes pel receptor 5-HT_7 com a fàrmacs prometedors pel tractament del dolor neuropàtic.

Introducció

El dolor es defineix com una experiència sensorial i emocional desagradable associada amb una lesió tissular actual o potencial. El maneig del dolor necessita considerar les característiques multidimensionals del dolor basades en la nocicepció, la percepció, el patiment i el comportament del dolor.

L'increment de la sensibilitat del dolor que es produeix en una lesió és degut als mediadors de la inflamació com les citoquines que són alliberades en el lloc de la lesió activant nociceptors que porten impulsos nociceptius del lloc de la lesió cap a l'asta dorsal de la medul·la espinal. Els nociceptors són extremadamente heterogenis, diferint en els neurotransmissors que contenen, els receptors i canals iònics que expressen, la velocitat de conducción, les propietats de resposta a estímuls nocius, i la seva capacitat de ser sensibilitzats durant la inflamació, lesió o malaltia.

El dolor es transmet a través de les vies ascendents des dels nociceptors de l'asta dorsal de la medul·la espinal al tàlam, sistema límbic i estructures corticals. Diferents àreas del sistema nerviós central estan relacionades amb el processament del dolor, com el tàlam, l'ínsula, el córtex sensorial secundari i el córtex cingulat anterior i parietal posterior. El dolor és un procés complex en el que estan implicats neurotransmissors i molècules que actuen tant a nivell perifèric com central, tals com la noradrenalina, serotonina i acetilcolina. Els impulsos nociceptius que arriben a la medul·la espinal són inhibits pel sistema inhibidor que actua com un component inhibidor endogen del dolor, mitjançant senyals provinents de regions supraespinals. També existeix el sistema facilitador descendent que és activat després de la lesió, contribuïnt a la hiperalgèsia secundària i als canvis plàstics relacionats amb el dolor crònic.

El dolor també produeix alteracions de llarga duració del sistema nerviós central, canvis plàstics que produeixen canvis en la sensibilitat dels nociceptors. Aquesta plasticitat neural resulta en un dolor persistent després d'haver corregit la patologia i l'extensió del dolor a altres àreas que les inicialment implicades en la patologia. El dolor neuropàtic és un forma de dolor crònic que és generat de manera persistent i que no té una funció beneficiosa per a l'individu afectat. El sistema noradrenèrgic, serotoninèrgic, gabèrgic i glutamatèrgic són els principals implicats en aquest tipus de dolor.

La majoria dels models animals de dolor crònic utilitzen la lesió mitjançant lligament, tall o pinçaments dels nervis i l'aplicació d'irritants químics amb la finalitat de lesionar o irritar els nervis perifèrics per tal de causar dolor. Entre els models animals més comuns de dolor utilitzats podem destacar els models de: dolor nociceptiu, dolor inflamatori, dolor neuropàtic i dolor idiopàtic. Els models de dolor neuropàtic induïts per la constricció, lligadura o transecció total o parcial dels nervis o dels ganglis de l'asta dorsal constitueixen els models animals més freqüentment utilitzats per estudiar el dolor neuropàtic. Els principals comportaments observats en aquests animals són la autonomia i/o la hipersensibilitat mecànica i tèrmica.

El neurotransmissor serotonina està implicat en moltes funcions tant en invertebrats com en vertebrats. En mamífers està present de manera abundant tant en el sistema nerviós central (SNC) com en la perifèria. El nom serotonina té els seus orígens com a component químic de la sang (sèrum) conegut per provocar contracció (tonus) dels vasos sanguinis. La serotonina està implicada en la etiologia de nombroses malalties com la depressió, ansietat, fòbia social, esquizofrènia, desordres obsessius compulsius i del pànic. També influeix parcialment en malalties com la migranya, la hipertensió, els trastorns de la gana, el vòmit i el síndrome del colon irritable.

Com a resultat dels estudis realitzats per tal de determinar la funció en la perifèria i el sistema nerviós central, el 1964 el compost fou identificat com a neurotransmissor (Rapport et al., 1948a, 1948b). Aquest descobriment va donar lloc a una nova etapa en el desenvolupament dels agents terapèutics amb l'objectiu de modificar les funcions de la serotonina.

El receptor 5-HT_7 és un dels subtipus de receptors de serotonina acoplat positivament a la proteïna G més recentment descobert, i menys caracteritzat (Hoyer et al., 1994, 2002). El primer clonatge del receptor 5-HT_7 fou publicat el 1993 per varis laboratoris independentment. El receptor ha estat clonat en els genomes humans, rata, ratolí, porc, cobai, conill i granota. Malgrat l'estructura primària (seqüència d'aminoàcids) del receptor 5-HT_7, aquest presenta un elevat grau d'homologia interespècie (95%), però mostra una baixa homologia amb la resta de receptors de serotonina (<50%). La longitud del receptor 5-HT_7 és de 445-479 aminoàcids amb 7 dominis transmembrana.

El gen del receptor 5-HT_7 humà està localitzat en el cromosoma 10 (10q21–q24) i té 2 introns en la regió codificadora (Gelernter et al., 1995; Erdmann et al., 1996; Heidmann et al., 1997). El *splicing* alternatiu del segon intró dóna lloc almenys a 4 isoformes del receptor 5-HT_7 que

difereixen principalment en l'extrem C-terminal intracel·lular, anomenat 5-HT_7(a), (b), (c) en rata i 5-HT_7(a), (b), (d) en humans. Recentment una cinquena isoforma ha estat identificada en rata, provisionalment 5-HT_7(e). La relativa abundància de les isoformes del receptor 5-HT_7 és diferent entre humans i rates, possiblement indicant propietats funcionals i rols fisiològics diferents per cada una d'aquestes isoformes. La isoforma 5-HT_7(a) és la més àmpliament distribuïda, mentre que la variant 5-HT_7(b) s'expressa generalment amb baixos nivells, en canvi és la principal isoforma transcrita en el múscul llis. Les isoformes espècie depenents 5-HT_7(c) i 5-HT_7(d) s'expressen en molt baixos nivells. A dia d'avui no s'han demostrat diferències farmacològiques, de transducció de senyals i distribució de teixits entre les diferents isoformes. En totes les espècies estudiades les diferents isoformes del receptor 5-HT_7 estan positivament acoplades a la adenilat ciclasa a través de la proteïna Gαs.

Estudis addicionals van revelar que l'estimulació del receptor 5-HT_7(a) no només provoca l'activació de l'adenilat ciclasa 5 (AC_5) a través de l'acoplament amb la proteïna Gαs, sinó també s'acopla a isoformes d'adenilat ciclasa sensibles a Ca^{2+}-calmodulina, AC_1 i AC_8, regulant l'AMPc intracel·lular mitjançant la mobilització del calci intracel·lular (Baker et al., 1998). També s'ha descrit en cultius primaris neuronals que el receptor 5-HT_7 activa la proteïna quinasa ERK activada per mitogen (Errico et al., 2001; D'Sa and Duman, 2002).

Variacions en la longitud de l'extrem C-terminal i en el número de llocs consens de fosforilació per les proteïnes quinases A i C (PKA i PKC) depenent d'AMPc, suggereixen la possibilitat que les variants de *splicing* puguin mostrar diferències en les propietats de desensibilització i tràfic de senyals (Heidmann et al., 1997). Les 3 isoformes de rata i les variants de *splicing* en humans 5-HT_7(a) i 5-HT_7(b) no tenen llocs consens de fosforilació potencial en la seqüència intracel·lular C-terminal. Mentre que les variants de *splicing* 5-HT_7(d) tenen 2 llocs consens de fosforilació potencial, un per la proteïna quinasa C i un altre per la casein quinasa II. A més, les variants 5-HT_7(b) i 5-HT_7(c) contenen una regió consens per proteïnes amb domini PDZ, suggerint la possibilitat que aquestes variants de *splicing* puguin acoplar-se a rutes alternatives de senyalització (Hamblin et al., 1998).

Recentment s'ha descobert que el receptor 5-HT_7(a) s'uneix post-transduccionalment de manera covalent a l'àcid palmític a través d'un pont tioéster i que pateix repetits cicles de palmitació/despalmitació. A més la presència d'agonistes fan que el receptor 5-HT_7(a) pateixi processos de palmitació de forma dinàmica i depenent d'agonista d'importància funcional (Kvachnina et al., 2009).

Un recent treball ha demostrat que el receptor 5-HT_7 és desensibilitzat després d'un llarg període d'activació sense produir-se una regulació descendent en el número de receptors 5-HT_7 presents a la membrana (Iceta et al., 2009). També s'ha descrit fenòmens de regulació ascendent en el nombre de receptor 5-HT_7 presents a la membrana en situacions patològiques (Pierce et al., 1996, 1997).

L'observació per diferents grups d'investigadors que concentracions creixents d'antagonistes del receptor 5-HT_7 (agonistes inversos) redueixen l'activitat basal de l'adenilat ciclasa en cèl·lules que expressen el receptor 5-HT_7, ha suggerit una activitat constitutiva dels receptors 5-HT_7 (Krobert and Levy, 2002). Això significa que inclús en absència d'un agonista, aquests receptors són capaços d'activar la proteïna G i mutacions en diferents dominis estructurals del receptor poden augmentar o reduir aquesta activitat independent d'agonista (Kvachnina et al., 2009).

S'han detectat elevats nivells del receptor 5-HT_7 al cervell, mentre que la seva presència és baixa en una varietat de teixits perifèrics: pulmó, ronyó, fetge, pàncrees, placenta, melsa, testicles, ovaris, retina, cor, artèries coronàries, pulmonars i uterines, vena capa superior, vena safena, varies regions del tracte gastro-intestinal incloent l'estòmac, el colon, el íleum (Krobert et al., 2001). Concretament, en el sistema nerviós central l'expressió del receptor 5-HT_7 és elevada en regions del còrtex, sèptum, cerebel, estriat, tàlam, hipotàlam, nuclis supraquiasmàtics, complex olfactori, gangli trigeminal, mesencèfal e hipocamp (Gustafson et al., 19960; Neumaier et al., 2001).

El desenvolupament de molècules selectives pel receptor 5-HT_7 fou necessari per tal de contribuir a un major coneixement del perfil farmacològic i del paper biològic d'aquest receptor en el sistema nerviós central i la perifèria. El primer antagonista del receptor 5-HT_7 identificat i àmpliament utilitzat fou el compost LY-215840 (Cohen, 1992), però presentava una baixa selectivitat. L'obtenció de molècules selectives pel receptor 5-HT_7 començà amb el desenvolupament del compost SB-258719 que s'unia al receptor 5-HT_7 amb una elevada afinitat (Ki = 31.6 nM) i de l'estructura anàloga SB-269970 (Ki = 1.2 nM) (Forbes et al., 1998; Lovell et al., 2000). Una altra molècula àmpliament utilitzada en diferents estudis fou el DR-4446 degut a la seva elevada afinitat pel receptor 5-HT_7 (Ki = 10 nM) i a la presència d'un grup metil accessible químicament convertint-lo en un bon candidat com a radiolligand i en estudis de tomografia d'emissió de positrons (Kikuchi et al., 2002a, b).

Respecte l´ús d'agonistes selectius pel receptor 5-HT_7, el més freqüentment utilitzat ha estat el AS-19 (Sanin et al., 2003), però estudis recents d'afinitat varen mostrar que el AS-19 tenia afinitat pel receptor 5-HT_{1A} i α_{2a} (Boker et al., 2009). També és important destacar el compost 8-OH-DPAT que era considerat inicialment com un agonista selectiu pel receptor 5-HT_{1A}, i quan es va descobrir que tenia afinitat pel receptor 5-HT_7 es va haver de reconsiderar moltes de les funcions assignades fins aquell moment al receptor 5-HT_{1A} (Ehlen et al., 2001; Sprouse et al., 2004).

Estudis farmacològics i/o amb ratolins genoanul·lats que no expressen el receptor 5-HT_7, han estat dissenyats per tal de constituir models animals de comportament capaços de reproduir almenys en part els desordres clínics en humans (Roberts et al., 2004; Guscott et al., 2005; Hedlund et al., 2005; Galici et al., 2008; Shelton et al., 2009; Liu et al., 2009). Interessants descobriments s'han fet implicant el receptor 5-HT_7 en diferents desordres relacionats amb la regulació dels ritmes circadians, la termoregulació, l'aprenentatge contextual i el processament de la memòria, la regulació neuroendocrina, el síndrome del colon irritable, el control de la micció, el sistema reproductiu, els desordres psiquiàtrics i la depressió. Malgrat tot, resultats discordants s'han obtingut en diferents estudis degut probablement a la manca de molècules realment selectives pel receptor 5-HT_7 utilitzades com eines farmacològiques. Aquestes discrepàncies les trobem principalment en estudis relacionats amb els desordres de l'ansietat, el dolor, l'esquizofrènia, els desordres psiquiàtrics i neurològics. En canvi, resultats consistents els trobem en els estudis referents als trastorns de la son i del camp de la depressió amb l'observació que la inhibició dels receptors 5-HT_7 potencia els efectes dels antidepressius utilitzats en la clínica.

La presència dels receptors 5-HT_7 en àrees supraespinals com el tàlam, en l'asta dorsal de la medul·la espinal, i en els ganglis de l'asta dorsal han suggerit la implicació del receptor 5-HT_7 en el dolor (Gustafson et al., 1996; Neumaier et al., 2001). En la medul·la espinal el receptor 5-HT_7 es troba localitzat principalment en la làmina superficial de l'asta dorsal, postsinàpticament en interneurones locals, i presinàpticament en fibres peptidèrgiques i en astrocits. La lesió dels teixits perifèrics provoca una alliberació de serotonina que actua en combinació amb altres mediadors de la inflamació per tal d'excitar i sensibilitzar les fibres nervioses aferents.

Malgrat l'ús de compostos no selectius i els escassos estudis realitzats per examinar el paper nociceptiu del receptor 5-HT_7 els resultats publicats suggereixen un implicació del receptor 5-

HT_7 en el dolor diferent en funció de la seva localització, segons sigui central o perifèrica. En aquest sentit, s'ha demostrat que l'activació dels receptors 5-HT_7 a nivell espinal juga un paper important en l'efecte analgèsic dels opioides. És probable que els receptors 5-HT_7 tinguin una acció pronociceptiva o antinociceptiva depenent de la localització del receptor, la qualitat i modalitat de l'estímul. A més, diferents regions de la perifèria i del sistema nerviós central han estat suggerides per la seva implicació en el dolor degut a la serotonina, i concretament a través del receptor 5-HT_7. Futurs estudis ajudaran a clarificar la relació entre els receptors 5-HT_7 i el dolor.

Hipòtesi

La serotonina ha estat reconeguda com un dels principals neurotransmissors que participen en la transmissió del dolor, el processament i el seu control. La presència de receptors 5-HT_7 en àreas del sistema nerviós central relacionades amb el dolor, en particular el tàlam, l'asta dorsal de la medul·la espinal, i els ganglis de l'arrel dorsal, suggerien un paper dels receptors 5-HT_7 en la nocicepció (Meuser et al., 2002; Doly et al., 2005). Malgrat la presència inicialment de dades que donaven suport al paper dels receptor 5-HT_7 en el dolor, els resultats eren escassos i s'havien obtingut principalment utilitzant lligands no selectius. En particular, també era remarcable l'absència de dades en dolor crònic (exemple, dolor neuropàtic).

Els únics dos estudis referents a la funció en la nocicepció dels receptors 5-HT_7 perifèrics, coincidien en afirmar el paper pronociceptiu dels receptors 5-HT_7 de la perifèria (Meuser et al., 2002; Rocha-González et al., 2005). En canvi, s'havien publicat resultats contradictoris respecte a la contribució a nivell espinal dels receptors 5-HT_7 en la nocicepció (Rocha-González et al., 2005; Dogrul and Seyrek, 2006). Àrees supraespinals, en particular el tàlam, havien estat també descrites per la seva implicació en la modulació de la nocicepció induïda per serotonina a través dels receptors 5-HT_7 (Díaz-Reval et al., 2004; Harte et al., 2005).

Per altra part, el receptor 5-HT_7 té un perfil d'unió de lligands molt similar al receptor 5-HT_{1A}. En aquest sentit s'havia proposat que alguns dels efectes antinociceptius atribuïts al receptor 5-HT_{1A} podrien ser deguts al receptor 5-HT_7 (Harte et al., 2005).

Basat en estudis previs i en línea amb els objectius del projecte 5-HT_7 d'Esteve focalitzat en el descobriment de fàrmacs amb afinitat 5-HT_7 pel tractament de dolor neuropàtic, en els

estudis aquí presents **vàrem examinar la possibilitat que agonistes o antagonistes selectius del receptor 5-HT$_7$ poguessin ser d'interès pel tractament del dolor.**

Objectius

Per tal d'investigar el paper dels receptors 5-HT$_7$ en el dolor i l'interès terapèutic de nous fàrmacs selectius del receptor 5-HT$_7$ pel tractament del dolor, els següents objectius van ser plantejats:

1.- Estudiar el paper dels receptors 5-HT$_7$ en condicions de dolor nociceptiu i la seva interacció amb el sistema opioide. Amb aquest propòsit, vam avaluar els efectes dels agonistes i antagonistes del receptor 5-HT$_7$ administrats sistèmicament per se o coadministrats amb morfina, en resposta a un estímul nociu tèrmic utilitzant el *tail-flick* en ratolí.

2.- Investigar el paper dels receptors 5-HT$_7$ en condicions de dolor i sensibilització central. Amb aquest propòsit, vam avaluar els efectes en la hipersensibilitat mecànica i la hiperalgèsia tèrmica dels agonistes i antagonistes del receptor 5-HT$_7$ administrats per via sistèmica, en models en ratolí de dolor neurogènic (test de la capsaicina), inflamatori (test de la formalina) i neuropàtic (lesió del nervi ciàtic).

3.- Explorar el lloc i mecanisme d'acció dels receptors 5-HT$_7$ implicat en el control del dolor. Amb aquest propòsit, vam administrar agonistes del receptor 5-HT$_7$ en la perifèria per via intraplantar en la pota de l'animal i en la medul·la espinal per via intratecal, en rates sotmeses a condicions de dolor neurogènic i neuropàtic. A més, vam investigar la immunoreactivitat dels receptors 5-HT$_7$ en la medul·la espinal i la col·localització amb neurones GABAèrgiques.

4.- Determinar l'especificitat *in vivo* dels agonistes del receptor 5-HT$_7$ pels receptors 5-HT$_7$. Amb aquest propòsit, vam avaluar els efectes dels agonistes del receptor 5-HT$_7$ administrats per via sistèmica en la fase II de la formalina de ratolins genoanul·lats del receptor 5-HT$_7$ i ratolins salvatges.

Resum dels mètodes

Aprofitant l'aproximació genètica (ratolins genoanul·lats pel receptor 5-HT_7) i farmacològica (lligands pel receptor 5-HT_7), vàrem examinar el paper dels receptors 5-HT_7 en el control de la nocicepció. Els experiments es van realitzar utilitzant ratolins o rates sotmeses a una lesió directa (lesió del nervi o amb un estímul tèrmic agut i nociu) o amb l'aplicació d'irritants (capsaicina o formalina) per lesionar o irritar un nervi perifèric i causar dolor.

El dolor nociceptiu es va mesurar amb animals naïve (aquells que no han estat subjectes a cap procediment previ) utilitzant un estímul tèrmic agut i nociu. El dolor neurogènic (injecció de capsaicina a la pota) i neuropàtic es va avaluar amb el *von Frey* automàtic o manual (hipersensibilitat mecànica) i el test plantar (hiperalgèsia tèrmica). El temps de llepat i mossegat va ser analitzat després de la injecció de formalina per avaluar la fase I (0-10 min) i la fase II (15-45 min).

Els fàrmacs es van administrar sistèmicament (oral, intraperitoneal o subcutani), perifèricament en la pota o intratecal en la medul·la espinal, i testat en diferents aproximacions experimentals. Dos nous agonistes pel receptor 5-HT_7, potents i eficients, E-55888 i E-57431 van ser desenvolupats per Esteve i descrits per primera vegada. També vàrem utilitzar altres lligands del receptor 5-HT_7 descrits (SB-258719 i SB-269970 com antagonistes pel receptor 5-HT_7; i AS-19 i MSD-5a com agonistes pel receptor 5-HT_7). Inicialment, el perfil d'afinitat, selectivitat i funcionalitat van ser avaluats pels fàrmacs testats *in vivo*. A més, per tal d'investigar els efectes específics de diana dels agonistes del receptor 5-HT_7 testats en dolor, els seus efectes varen ser examinats en el test de la formalina en ratolins genoanul·lats pel receptor 5-HT_7 i en ratolins salvatges.

L'anàlisi estadístic es va realitzar per testar diferències significatives entre grups utilitzant ANOVA, post-hoc i el *Student's t test*. Les dades es varen presentar com la resposta mitja en la corresponent unitat ± SEM o el percentatge mitjà de l'analgèsia calculat respecte al corresponent grup control. El nivell de significancia es va fixar en $p < 0.05$. L'anàlisi de les dades i la representació gràfica es va realitzar utilitzant el GraphPad Prism software (versió 4.0; GraphPad Software, Inc., USA).

Resultats

Potenciació de l'efecte analgèsic de la morfina degut a l'activació adjuvant dels receptors 5-HT_7

En aquest estudi vàrem observar que l'activació dels receptors 5-HT_7 potenciava els efectes antinociceptius de la morfina enfront un estímul tèrmic nociu utilitzant el test del *tail-flick*.

Malgrat l'agonista selectiu del receptor 5-HT_7 E-55888 no tenia efectes per si sol en el *tail-flick*, vàrem descobrir que era capaç de potenciar els efectes antinociceptius de la morfina en el *tail-flick*. A més, vam comprovar que la potenciació observada era deguda a efectes farmacodinàmics i no farmacocinètics ja que no vam observar diferències en la concentració de morfina i del seu metabòlit M3G, tant en plasma com en cervell quan la morfina era coadministrada amb l'agonista selectiu del receptor 5-HT_7 E-55888. Els resultats d'aquest treball ens van permetre demostrar que els agonistes selectius pel receptor 5-HT_7 podrien ser d'interès terapèutic en l'alleugeriment del dolor com a adjuvants d'altres analgèsics com els opioides.

L'activació del receptor 5-HT_7 inhibeix la hipersensibilitat mecànica secundària a la sensibilització per capsaicina en ratolins.

Aquest treball tenia com objectiu estudiar el paper potencial del receptor 5-HT_7 en la nocicepció secundària a la injecció intraplantar de la capsaicina en els ratolins. La capsaicina provoca una hipersensibilitat de les neurones de l'asta dorsal (sensibilització central). Amb aquest propòsit, vàrem avaluar l'efecte en la hipersensibilitat mecànica induïda per la capsaicina de diferents lligands selectius (agonistes del receptor 5-HT_7: AS-19, MSD-5a, E-55888; antagonistes del receptor 5-HT_7: SB-258719, SB-269970; l'agonista del receptor 5-HT_{1A}: F-13640; i l'antagonista del receptor 5-HT_{1A}: WAY-100635). Es va avaluar també el perfil *in vitro* d'afinitat i d'eficàcia intrínseca en estimular el receptor 5-HT_7, dels agonistes del receptor 5-HT_7 utilitzats. E-55888 va resultar ser un agonista complet en canvi el AS-19 i el MSD-5a es van comportar com agonistes parcials, amb un efecte màxim corresponent al 77% i 61%, respectivament. Els nostres resultats *in vivo* van mostrar que l'administració sistèmica d'agonistes del receptor 5-HT_7 produïa un efecte antinociceptiu dosi-depenent que era revertit pels antagonistes del receptor 5-HT_7, però no per un antagonista 5-HT_{1A}. L'ordre d'eficàcia *in vivo* (E-55888>AS-19>MSD-5a) va resultar correspondre a les seves eficàcies *in*

vitro com agonistes del receptor 5-HT_7. Contràriament als agonistes, l'administració dels antagonistes del receptor 5-HT_7 promovia la hipersensibilitat mecànica, reforçant la implicació del receptor 5-HT_7 en el control de la hipersensibilitat mecànica induïda per capsaicina. Els resultats del present treball ens van evidenciar el paper inhibidor de la serotonina en el control de la nocicepció a través de l'activació dels receptors 5-HT_7, i ens van suggerir l'interès terapèutic dels agonistes del receptor 5-HT_7 en el camp de l'analgèsia.

L'activació farmacològica dels receptors 5-HT_7 redueix la hipersensibilitat mecànica i tèrmica induïda per la lesió del nervi

Amb els resultats del present estudi vàrem demostrar la participació del receptor 5-HT_7 en la hipersensibilitat mecànica i tèrmica induïda per la lesió del nervi ciàtic dels ratolins. L'activació dels receptors 5-HT_7 a través de l'administració sistèmica de l'agonista del receptor 5-HT_7, AS-19 (1 i 10 mg/kg) va produir una reducció de la hipersensibilitat mecànica i tèrmica la qual vàrem revertir amb la coadministració de l'antagonista selectiu del receptor 5-HT_7, SB-258719. També interessant és que el bloqueig dels receptors 5-HT_7 amb SB-258719 (2.5 i 10 mg/kg) va promoure la hipersensibilitat mecànica (però no tèrmica) en ratolins amb lesió del nervi ciàtic i en ratolins *sham* (operats sense lesionar el nervi). El tractament crònic de dos vegades al dia durant 11 dies de l'agonista selectiu del receptor 5-HT_7, E-57431 (10 mg/kg) va demostrar mantenir l'eficàcia del tractament després de repetides administracions sistèmiques, no desenvolupant tolerància de l'efecte antial·lodínic i antihiperalgèsic. El receptor 5-HT_7 va resultar col·localitzar amb cèl·lules GABAèrgiques en l'asta dorsal de la medul·la espinal, suggerint que l'activació d'interneurones GABAèrgiques inhibidores a nivell espinal podrien contribuir a l'efecte analgèsic dels agonistes del receptor 5-HT_7. A més, vam detectar per immunohistoquímica un increment significatiu dels receptors 5-HT_7 en l'asta dorsal ipsilateral de la medul·la espinal després de la lesió del nervi, suggerint una regulació de l'expressió del receptor 5-HT_7 induïda pel dolor. Els resultats obtinguts en el present treball ens van permetre evidenciar el potencial interès terapèutic dels agonistes selectius del receptor 5-HT_7 en el tractament del dolor neuropàtic, i suggerir que l'activació del sistema GABAèrgic sembla ser el responsable dels efectes antinociceptius resultant de l'activació del receptor 5-HT_7.

Paper dels receptor 5-HT$_7$ a nivell perifèric i espinal en la modulació del dolor en condicions de sensibilització.

L'objectiu del present treball va ser estudiar la contribució dels receptors 5-HT$_7$ presents a nivell perifèric i central responsables de l'efecte antinociceptiu observat amb l'administració sistèmica dels agonistes selectius del receptor 5-HT$_7$. Amb aquest propòsit, vàrem mesurar la hipersensibilitat mecànica secundària a la capsaicina després d'administrar l'agonista del receptor 5-HT$_7$ E-57431 per la via oral (nivell sistèmic), via intratecal (nivell espinal) i intraplantar (nivell perifèric) en les rates.

Vàrem observar una inhibició dosi-depenent de la hipersensibilitat mecànica induïda per capsaicina després d'administrar per la via oral l'agonista selectiu del receptor 5-HT$_7$ E-57431 (1,25 - 10 mg/kg). L'administració intratecal de 100 µg de E-57431 va resultar també inhibir la hipersensibilitat mecànica induïda per capsaicina. Contràriament a l'activació espinal, vàrem administrar per la via intraplantar l'agonista del receptor 5-HT$_7$ E-57431 (0.01, 0.1 i 1µg) resultant en la promoció de la hipersensibilitat mecànica induïda per capsaicina, reforçant el paper pronociceptiu dels receptors 5-HT$_7$ en la perifèria. L'agonista E-57431 el vàrem també administrar per via sistèmica e intratecal en un model de lesió del nervi ciàtic en rates per tal de confirmar els resultats obtinguts en el model de la capsaicina. L'administració intraperitoneal de 10 mg/kg e intratecal de 100 µg va resultar també inhibir la hipersensibilitat mecància induïda per la lesió del nervi ciàtic en rates. Els resultats del present estudi ens van permetre demostrar utilitzant un model de dolor neuropàtic (lligadura del nervi ciàtic) i un model de dolor neurogènic (sensibilització per capsaicina), que l'efecte antinociceptiu observat amb l'administració sistèmica d'un agonista selectiu del receptor 5-HT$_7$ es deu possiblement a l'activació dels receptors 5-HT$_7$ a nivell espinal, contrarrestant el paper pronociceptiu dels receptors 5-HT$_7$ en la perifèria.

Estudi de la selectivitat dels agonistes pel receptor 5-HT$_7$ utilitzant tests de dolor i termoregulació en ratolins genoanul·lats i salvatges pel receptor 5-HT$_7$.

Basat en resultats preclínics, els agonistes pel receptor 5-HT$_7$ representen un potencial terapèutic pel tractament del dolor en condicions de sensibilització central. L'objectiu d'aquest estudi fou investigar el efectes diana específics dels agonistes pel receptor 5-HT$_7$ en el model de la formalina en ratolins genoanul·lats pel receptor 5-HT$_7$ i salvatges.

Aquest estudi va mostrar que els ratolins genoanul·lats pel receptor 5-HT_7 i salvatges mostraven la mateixa sensibilitat a estímuls tèrmics nocius i químics com la formalina. L'administració subcutània dels agonistes pel receptor 5-HT_7 AS-19 (10 mg/kg), E-55888 (20 mg/kg) i E-57431 (10 mg/kg) reduïren la resposta nociceptiva de la fase II de la formalina en el ratolins salvatges però no en els ratolins genoanul·lats pel receptor 5-HT_7.

Aquestes dades suggereixen que l'efecte antinociceptiu observat en el test de la formalina dels agonistes pel receptor 5-HT_7 AS-19, E-55888 i E-57431 es deu possiblement a l'activació específica dels receptor 5-HT_7 i confirma el potencial terapèutic dels receptors 5-HT_7 pel tractament de dolor.

Discussió

L'activació dels receptors 5-HT_7 a nivell espinal sembla ser responsable dels efectes antinociceptius observats amb l'administració sistèmica d'agonistes selectius del receptor 5-HT_7 en condicions de dolor neuropàtic. El paper antinociceptiu dels receptors 5-HT_7 de la medul·la espinal contrarestaria així els efectes pronociceptius deguts a l'activació dels receptors 5-HT_7 presents en la perifèria. Així doncs, en base als resultats publicats en aquesta Tesi, si un equilibri existeix entre l'acció pronociceptiva i antinociceptiva depenent de la localització del receptor i la natura de l'estímul nociceptiu, l'efecte global de l'administració sistèmica d'agonistes selectius del receptor 5-HT_7 sembla ser clarament analgèsic.

Vam demostrar que el llindar nociceptiu era el mateix entre els dos genotips en diferents tests de dolor tèrmic nociceptiu, com el *tail-flick*, el *tail-immersion* i el *hot plate*. Aquests resultats concorden amb un estudi publicat on tampoc es van trobar diferències en el llindar nociceptiu dels ratolins genoanul·lats del receptor 5-HT_7 utilitzant el test del *tail-flick* (Roberts et al., 2004). A més, vàrem demostrar que l'administració sistèmica de lligands del receptor 5-HT_7 (l'agonista del receptor 5-HT_7 E-55888 o l'antagonista del receptor 5-HT_7 SB-258719) no tenien ningún efecte en el *tail-flick*.

En canvi, quan vàrem coadministrar per la via sistèmica l'agonista del receptor 5-HT_7 E-55888 juntament amb la morfina, vàrem observar que l'activació dels receptors 5-HT_7 potenciava els efectes antinociceptius de la morfina en el *tail-flick*. Vàrem demostrar que aquesta potenciació era deguda a una interacció farmacodinàmica i no farmacocinètica, al no trobar diferències en els nivells de morfina i del seu metabòlit M3G en plasma i cervell, amb

la coadministració de l'agonista del receptor 5-HT_7 E-55888 i la morfina. A més, aquesta potenciació dels efectes analgèsics de la morfina van ser revertits amb l'administració conjunta de l'antagonista selectiu del receptor 5-HT_7 SB-258719. Estudis publicats demostren també que l'activació dels receptors 5-HT_7 a nivell espinal juguen un paper clau en els efectes antinociceptius del opiacis, probablement a través del sistema inhibidor descendent (Dogrul et al., 2009; Yanarates et al., 2010).

Per altra part, vàrem administrar sistèmicament diferents agonistes selectius pel receptor 5-HT_7 (AS-19, MSD-5a, E-55888 i E-57431), resultant en una inhibició dosi-depenent de la hipersensibilitat mecànica induïda per la capsaicina, la hipersensibilitat tèrmica i mecànica induïda per la lesió del nervi ciàtic i la fase II del model de formalina. A més l'administració sistèmica d'agonistes selectius del receptor 5-HT_7 de manera repetida en els animals lesionats no induïa tolerància de l'efecte antinociceptiu, i les dosis analgèsiques no produïen afectació de la coordinació motora.

A diferència de l'efecte antinociceptiu dels agonistes del receptor 5-HT_7, vàrem observar que l'administració sistèmica d'antagonistes del receptor 5-HT_7 (SB-258719 i SB-269970) produïa un efecte pronociceptiu en els ratolins sensibilitzats amb baixes dosis subactives de capsaicina. En canvi, aquests mateixos antagonistes no produïen cap canvi en animals que havien rebut vehicle enlloc de la capsaicina. Semblava ser que una mínima sensibilització era necessària per permetre als antagonistes exercir un efecte pronociceptiu. Això ens va suggerir que la injecció intraplantar de dosis subactives de capsaicina (però no de vehicle) produeix una activació endògena dels receptors 5-HT_7, i que per tant els efectes pronociceptius observats amb els antagonistes és fruit de contrarestar aquesta activitat endògena dels receptors. Per altra banda, vàrem demostrar que els efectes antinociceptius dels agonistes del receptor 5-HT_7 i els efectes pronociceptius dels antagonistes del receptor 5-HT_7 podien ser bloquejats per antagonistes i agonistes del receptor 5-HT_7, respectivament. Els nostres resultats indicaren que el receptor 5-HT_7 està implicat definitivament en el control de la hipersensibilitat mecànica secundària a un estímul sensibilitzant.

Vàrem observar resultats similars utilitzant el model de dolor neuropàtic induït per la lligadura parcial del nervi ciàtic en ratolins. Els resultats ens van demostrar que l'administració sistèmica de l'antagonista del receptor 5-HT_7 SB-258719 promovia la hipersensibilitat mecànica, però no la hipersensibilitat tèrmica. Les diferències observades entre la hipersensibilitat mecànica i tèrmica podrien ser degudes a diferències en el to

serotoninèrgic endogen i/o en la modulació dels receptors 5-HT_7 en les vies sensorials/nociceptives depenent de la natura (mecànic vs. tèrmic) i de la intensitat (alodínia vs hiperalgèsia) de l'estímul. A més, l'antagonista SB-258719 va resultar promoure no només la hipersensibilitat mecànica en ratolins amb lligadura parcial del nervi ciàtic sinó també en els animals *sham* (animals control sotmesos a l'operació però sense lesionar el nervi).

A més, vàrem demostrar injectant dosis subactives de capsaicina a la superfície plantar de les rates, que l'administració local intraplantar de l'agonista selectiu del receptor 5-HT_7 E-57431 produïa un efecte pronociceptiu dosi-depenent. Aquests resultats en línea amb estudis previs publicats, suggerien un paper pronociceptiu dels receptors 5-HT_7 en la perifèria en el context de la lesió tissular i la inflamació. L'increment de la hipersensibilitat mecànica degut a l'acció perifèrica de l'agonista del receptor 5-HT_7 E-57431 requeria una mínima sensibilització resultant de la injecció d'una dosi subactiva de capsaicina.

Per altra part, vàrem administrar l'agonista del receptor 5-HT_7 E-57431 per la via intratecal en el model de la capsaicina i de la lligadura del nervi ciàtic en rates. El resultat va ser que l'activació dels receptors 5-HT_7 a nivell espinal inhibia la hipersensibilitat mecànica induïda per la capsaicina i per la lligadura del nervi ciàtic. En aquest sentit, sembla ser que els efectes antinociceptius debuts a l'activació dels receptors 5-HT_7 a nivell central predominin respecte els efectes pronociceptius observats a nivell perifèric, quan els agonistas selectius pel receptor 5-HT_7 són administrats sistèmicament.

També, vam detectar nivells incrementats del nombre de receptors 5-HT_7 a la medul·la espinal, concretament a la làmina I-II i III-IV de l'asta dorsal del costat ipsilateral després d'onze dies de l'operació. Aquesta regulació ascendent en el nombre de receptors 5-HT_7 expressats com a conseqüència de la lesió ens va suggerir que aquests canvis en la regulació de l'expressió dels receptors 5-HT_7 podria tenir conseqüències rellevants en l'eficàcia dels agonistes del receptor 5-HT_7 i en els mecanismes fisiològics, compensadors i protectors que tenen lloc a nivell espinal i que són essencials en el control del dolor neuropàtic.

Els efectes antinociceptius dels agonistes del receptor 5-HT_7 que vàrem observar en els experiments anteriorment descrits, no podien ser deguts directament a la inhibició de fibres primàries aferents o de neurones de segon ordre de l'asta dorsal ja que el receptor 5-HT_7 està acoplat positivament a l'adenilat ciclasa. En aquest sentit, vam demostrar per estudis de immunoreactivitat que els receptors 5-HT_7 col·localitzaven amb neurones GABAèrgiques de l'asta dorsal de la medul·la espinal, la qual cosa podria explicar els efectes antinociceptius

observats amb els agonistes del receptor 5-HT_7 a través de l'activació de receptors 5-HT_7 expressats en interneurones inhibitòries GABAèrgiques.

Vam demostrar que el llindar nociceptiu del ratolins genoanul·lats del receptor 5-HT_7 no era significativament diferent que el dels ratolins salvatges en condicions de sensibilització central. El temps de llepat i mossegat de la pota dreta posterior injectada amb formalina 2,5% era igual en els dos genotips en la fase I i II del test de la formalina, suggerint que els mecanismes bàsics de transducció, transmissió i percepció dels senyals sensorials i nociceptius estaven intactes en els ratolins genoanul·lats. En canvi, l'administració subcutània dels agonistes pel receptor 5-HT_7 AS-19 (10 mg/kg), E-55888 (20 mg/kg) i E-57431 (10 mg/kg) reduïren la resposta nociceptiva de la fase II de la formalina en el ratolins salvatges però no en el ratolins genoanul·lats pel receptor 5-HT_7.

Aquest estudi amb ratolins genoanul·lats pel receptor 5-HT_7 va permetre determinar l'especificitat *in vivo* al receptor dels agonistes del receptor 5-HT_7 AS-19, E-55888 i E-57431. A més, aquestes dades suggereixen que l'efecte antinociceptiu observat en el test de la formalina dels agonistes pel receptor 5-HT_7 AS-19, E-55888 i E-57431 es deu a l'activació específica dels receptors 5-HT_7.

Conclusions

1. Els receptors 5-HT_7 no estan implicats per si mateixos en la resposta a un estímul nociu, mentre que interaccionen amb el sistema opioide en condicions de dolor nociceptiu. Els ratolins genoanul·lats del receptor 5-HT_7 i salvatges mostren un sensibilitat similar enfront a un estímul tèrmic nociu. L'administració sistèmica d'agonistes i antagonistes del receptor 5-HT_7 resulta ineficaç en el dolor nociceptiu enfront un estímul tèrmic agut. Malgrat això, en aquestes condicions de dolor, els agonistes del receptor 5-HT_7 potencien el efectes analgèsics de la morfina.
2. Els receptors 5-HT_7 estan implicats en condicions de dolor i sensibilització central. L'administració sistèmica d'agonistes i antagonistes del receptor 5-HT_7 exerceixen efectes antinociceptius i pronociceptius, respectivament, en condicions dolor neurogènic induït per capsaicina i de dolor neuropàtic. A més, no s'observa una tolerància de l'efecte antinociceptiu amb l'administració sistèmica repetida d'un agonista del receptor 5-HT_7.

3. L'activació dels receptors 5-HT_7 en la perifèria promou el dolor, mentre que l'activació dels receptors 5-HT_7 a nivell espinal produeix efectes antinociceptius. La injecció intraplantar d'un agonista del receptor 5-HT_7 resulta en efectes pronociceptius en condicions de dolor neurogènic induït per la capsaicina. En canvi, l'administració intratecal d'un agonista del receptor 5-HT_7 produeix efectes antinociceptius en condicions de dolor neurogènic i neuropàtic. Aquests resultats donen suport a la hipòtesi que els receptors 5-HT_7 localitzats a nivell espinal són els responsables dels efectes antinociceptius dels agonistes del receptor 5-HT_7.

4. Els receptors 5-HT_7 colocalitzen en neurones GABAèrgiques de l'asta dorsal de la medul·la espinal, la qual cosa suggereix que l'activació d'interneurones inhibitòries GABAèrgiques a nivell espinal podria explicar o almenys contribuir en els efectes analgèsics dels agonistes del receptor 5-HT_7.

5. Efectes diana específics podrien ser atribuïts als avaluats agonistes del receptor 5-HT_7 basat en el perfil de selectivitat *in vitro* i en els estudis *in vivo* en ratolins genoanul·lats del receptor 5-HT_7.

6. Agonistes selectius del receptor 5-HT_7 podrien representar una nova aproximació terapèutica pel tractament del dolor neuropàtic. Malgrat això, no s'han realitzat estudis clínics que confirmin els resultats preclínics obtinguts amb animals.

Informe del director

Durant el seu doctorat sota la nostra direcció, l'Àlex Brenchat Barberà ha contribuït en l'elaboració de 5 manuscrits. L'Àlex és el primer autor en els 5 articles, cosa que demostra la seva gran capacitat per a produir treball científic d'alta qualitat. A continuació resumiré la contribució de l'Àlex i el factor d'impacte dels cinc manuscrits presentats en aquesta Tesi doctoral. Els manuscrits es presenten de forma cronològica:

- **[Brenchat et al., 2009]** Pain 2009;141:239-247 [Factor d'impacte = 5.371]

Alex Brenchat, Luz Romero, Mónica García, Marta Pujol, Javier Burgueño, Antoni Torrens, Michel Hamon, José Manuel Baeyens, Helmut Buschmann, Daniel Zamanillo, José Miguel Vela.

5-HT$_7$ receptor activation inhibits mechanical hypersensitivity secondary to capsaicin sensitization in mice.

Aquest treball tenia com objectiu estudiar el paper del receptor 5-HT$_7$ en la nocicepció secundària a l'estímul sensibilitzant de la capsaicina. Per aquest propòsit, es va avaluar la hipersensibilitat mecànica induïda per capsaicina després d'administrar diferents agonistes i antagonistes selectius del receptor 5-HT$_7$ en els ratolins.

L'Àlex ha dut a terme tots els experiments del manuscrit, excepte els referents a la taula 1 i figura 1. A més l'Àlex ha contribuït en l'elaboració d'aquest manuscrit.

- **[Brenchat et al., 2010]** Pain 2010;149:483-494 [Factor d'impacte = 5.371]

Alex Brenchat, Xavier Nadal, Luz Romero, Sergio Ovalle, Asunción Muro, Ricard Sánchez-Arroyos, Enrique Portillo-Salido, Marta Pujol, Ana Montero, Xavier Codony, Javier Burgueño, Daniel Zamanillo, Michel Hamon, Rafael Maldonado, José Miguel Vela

Pharmacological activation of 5-HT$_7$ receptors reduces nerve injury-induced mechanical and thermal hypersensitivity.

Aquest treball tenia com objectiu estudiar l'interès terapèutic del receptor 5-HT$_7$ en el tractament del dolor crònic, concretament el dolor neuropàtic. En el present estudi, diferents agonistes i antagonistes selectius del receptor 5-HT$_7$ foren avaluats per estudiar el paper del receptor 5-HT$_7$ en la hipersensibilitat mecànica i tèrmica induïda per la lligadura del nervi ciàtic en els ratolins.

L'Àlex ha dut a terme tots els experiments del manuscrit, excepte els referents a la taula 1, els de la figura 7 i 8. L'Àlex ha contribuït en l'elaboració d'aquest manuscrit.

- **[Brenchat et al., 2011]** Journal of Pharmacological Sciences 2011;116:388-391 [Factor d'impacte = 2.260]

Alex Brenchat, Miriam Ejarque, Daniel Zamanillo, José Miguel Vela, Luz Romero

Potentiation of morphine analgesia by adyuvant activation of 5-HT$_7$ receptors.

Aquest treball tenia com objectiu estudiar la potenciació de la morfina amb l'activació dels receptors 5-HT$_7$. L'administració de l'agonista selectiu del receptor 5-HT$_7$ E-55888 va demostrar que els efectes antinociceptius de la morfina podien ser potenciats amb l'activació dels receptors 5-HT$_7$.

L'Àlex ha realitzat els experiments referents a la figura 1, 2 i 3. En l'elaboració del manuscrit l'Àlex també hi ha contribuït.

- **[Brenchat et al., 2012]** European Journal of Pain 2012 ;16 :72-81 [Factor d'impacte = 3.371]

Alex Brenchat, Daniel Zamanillo, José Miguel Vela, Luz Romero

Role of peripheral versus spinal 5-HT$_7$ receptors in the modulation of pain under sensitizing conditions

Aquest treball tenia com objectiu estudiar el lloc d'acció dels agonistes selectius del receptor 5-HT$_7$ responsables dels efectes antinociceptius. En el present estudi, es va examinar l'efecte resultant d'activar els receptors 5-HT$_7$ a nivell espinal i perifèric en la hipersensibilitat mecànica induïda per la capsaicina o la lligadura del nervi ciàtic de les rates.

L'Àlex ha dut a terme tots els experiments del manuscrit. A més l'Àlex ha contribuït en l'elaboració d'aquest manuscrit.

- **[Brenchat et al.]** Advances in Pharmacological Sciences (enviat)

Alex Brenchat, Maria Rocasalbas, Daniel Zamanillo, José Miguel Vela, Luz Romero

Assessment of 5-HT$_7$ receptor agonists selectivity using nociceptive and thermoregulation tests in knockout versus wild-type mice

Aquest treball tenia com objectiu estudiar els efectes antinociceptius dels agonistes selectius pel receptor 5-HT$_7$ en el model de la formalina. L'ús de ratolins genoanul·lats que no expressen el receptor 5-HT$_7$ permetia confirmar que els efectes antinociceptius observats amb els agonistes selectius del receptor 5-HT$_7$ eren realment deguts a l'activació del receptor 5-HT$_7$.

L'Àlex ha realitzat conjuntament amb LR i MR els experiments referents a la figura 2 i 3. En l'elaboració del manuscrit l'Àlex també hi està contribuïnt.

Barcelona, a de de 2012

Jose Miguel Vela Hernández — Director de Tesi

Luz Romero Alonso — Codirectora de Tesi